The Trk potassium transporter is required for RsmB-mediated activation of virulence in the phytopathogen *Pectobacterium wasabiae*

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Running Head: Potassium regulates RsmB-mediated virulence activation

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Abstract

Pectobacterium wasabiae (previously known as *Erwinia carotovora*) is an important plant pathogen that regulates the production of plant cell wall-degrading enzymes through both an *N*-acyl-homoserine lactone-based quorum sensing system and the GacS/GacA two component system (also known as ExpS/ExpA). At high cell density, activation of GacS/GacA induces the expression of RsmB, a non-coding RNA which is in turn essential for activation of virulence in this bacterium. A genetic screen to identify regulators of RsmB revealed that mutants defective in components of a putative Trk potassium transporter (*trkH*, *trkA*) had decreased *rsmB* expression. Further analysis of these mutants showed that changes in potassium concentration influenced *rsmB* expression and consequent tissue damage in potato tubers, and that this regulation required an intact Trk system. Regulation of *rsmB* expression by potassium via the Trk system occurred even in the absence of the GacS/GacA system, demonstrating that these systems act independently and are both required for full activation of RsmB and the downstream induction of virulence in potato infection assays. Overall, our results identified potassium as an essential environmental factor regulating the Rsm system, and the consequent induction of virulence, in the plant pathogen *P. wasabiae*.

Importance

Crop losses from bacterial diseases caused by pectolytic bacteria are a major problem in agriculture. By studying the regulatory pathways involved in controlling expression of plant cell wall-degrading enzymes in *Pectobacterium wasabiae* we showed that the Trk potassium transport system plays an important role in the regulation of these pathways. The data presented further identify potassium as an important environmental factor in the regulation of virulence in this plant pathogen. We showed that a reduction in virulence can be achieved by increasing the extracellular concentration of potassium. Therefore, this work highlights how elucidation of the mechanisms involved in regulating virulence can lead to the identification of environmental factors that can influence the outcome of infection.

Introduction

Pectobacterium spp. are Gram-negative, rod-shaped bacteria belonging to the Enterobacteriaceae family. They cause soft-rot disease in several plants, including potatoes, carrots and cabbage. Damage to plant tissues is caused by the action of a mixture of cellulases, proteases, pectate lyases (Pel), pectin lyases and polygalacturonases secreted by these bacteria. The enzymes degrade plant cell wall components, releasing nutrients that fuel bacterial growth. Production of these plant cell wall-degrading enzymes (PCWDEs), is carefully coordinated by a complex multipartite regulatory system that integrates internal and external information to ensure that virulence is only switched on when conditions are optimal (1).

In *Pectobacterium wasabiae* [previously *Erwinia carotovora* (2)], production of PCWDEs is regulated mainly through two signal transduction systems. These systems coordinately control expression and activity of the global post-transcriptional regulator, RsmA, which represses the expression of PCWDEs by binding to their mRNAs. Transcription of *rsmA* is regulated by ExpI/ExpR, a typical quorum sensing system that relies on *N*-acyl homoserine lactone (AHL) autoinducer (3–5). The second sensory pathway regulating RsmA, and therefore virulence, is the GacS/GacA two-component system (also known as ExpS/ExpA). The response regulator, GacA, is the major transcriptional activator of RsmB, a non-coding RNA which binds RsmA (6). This binding of RsmA by RsmB inhibits RsmA-mediated repression of PCWDEs, ultimately promoting the expression of these virulence factors (7). Therefore, activation of RsmB expression is essential to induce virulence in *P. wasabiae*. Homologues of the Gac/Rsm system exist in many Gram-negative bacteria, including the Gac/Rsm system in *Pseudomonas* spp. and the BarA/UvrY/Csr in *Escherichia coli*. Though these systems regulate a wide range of important physiological functions in bacteria, including primary and secondary metabolism, biofilm formation, motility and virulence [reviewed in (8, 9)], the chemical identity of the molecule(s) responsible for its activation remains unknown. Accumulation of intermediates of the Krebs Cycle have been shown to stimulate the Gac/Rsm in *Pseudomonas fluorescens* and *Vibrio fisheri* (10, 11), but the physiological conditions

that lead to the accumulation of these metabolites with the consequent activation of the Gac/Rsm system are still poorly understood. Additionally, short-chain fatty acids, such as acetate, formate, propionate and butyrate, have been shown to influence the expression of *csrB* (a functional *rsmB* homologue) in *E. coli* or *Salmonela enterica enterica* serovar Typhimurium at low pH, but the stimuli that activate the system at neutral pH have not been identified (12, 13).

To identify physiological stimuli involved in the activation of the Gac/Rsm system and understand how this system regulates virulence in the well-characterized *P. wasabiae* strain SCC3193, we performed a genetic screen to identify mutants affected in *rsmB* expression, the main target of the Gac/Rsm system in this bacterium. The screen revealed 5 mutants defective in genes coding for components of a putative homologue of the *E. coli* Trk potassium uptake system (10). Therefore, here we investigated the importance of potassium and the Trk system for the regulation of RsmB. Our results demonstrated that extracellular potassium is a critical environmental factor influencing virulence in *Pectobacterium* spp.

Materials and Methods

Bacterial strains, plasmids and media. All strains and plasmids used in this study are listed in Table S1. *P. wasabiae* strains are derived from the wild-type (wt) strain SCC3193 (14). Strains were grown at 30ºC with aeration in Luria-Bertani (LB) broth, M9 Minimal Media (MM) or Minimal Potassium Media (15) with 0.4% (w/v) Glycerol. When specified, media were supplemented with 0.4% of polygalacturonic acid (PGA; Sigma P3850) to induce the expression of PCWDEs. Where mentioned, KCl was added at various final concentrations. Antibiotics were used at the following concentrations: ampicillin (Amp) 100 mgL^{-1}; streptomycin (Str) 100 mgL^{-1}; kanamycin (Kan) 50 mgL^{-1}; spectinomycin (Spec) 50 mgL^{-1} and chloramphenicol (Cm) 25 mgL^{-1}. To assess bacterial growth the optical density (OD_{600}) was determined by measuring absorbance at 600 nm in Bioscreen C Reader System (Multi-plate reader Oy Growth Curves Ab Ltd).

104 **Genetic and molecular techniques.** Primers used in this study are listed in Table S2. The plasmid
105 encoding the *rsmB* promoter fused to green fluorescence protein (GFP) (P_{rsmB}::*gfp*) was constructed
106 using a modified version of the promoterless::*gfp* vector pCMW1 (16). First the chloramphenicol
107 resistance gene (*cm*) was amplified by PCR from pKD3 (17) using P0276-pKD3/4(BamHI) and
108 P0277-pKD3/4(BamHI) primers and introduced into pCMW1, yielding PRSV59. Next a 392 bp
109 fragment containing the promoter region of *rsmB* was amplified by PCR using P0528-rsmB(SphI)
110 and P0529-rsmB(SalI) primers and ligated into PRSV59, yielding pRSV206. Deletion mutants were
111 constructed by chromosomal gene replacement with an antibiotic marker using the λ Red
112 recombinase system (17, 18). The DNA region of *gacA*, including approximately 500 bp upstream
113 and 500 bp downstream of the gene, was amplified by PCR and cloned into pUC18 (19) using SalI
114 and SacI. This construct, containing the *gacA* gene and its flanking regions, was divergently
115 amplified by PCR using primers to introduce an XhoI restriction site in the 5' and 3' regions of the
116 gene. The antibiotic resistance gene (*str*) was amplified from pKNG101 (20) with primers
117 containing the XhoI restriction site. The amplified fragment containing str^R was digested with XhoI
118 and introduced into the XhoI digested PCR fragment. The final construct contained the antibiotic
119 resistance marker flanked by the upstream and downstream regions of *gacA*. The 500bp-*str*-500bp
120 fragment was amplified by PCR and approximately 3 ng of DNA were electroporated into a strain
121 expressing the λ Red recombinase system from pKD46, to allow recombination (17). To construct
122 the $trkA^+$ and $trkH^+$ complementation plasmids, a 400bp-*trkA*-400bp fragment and a 400bp-*trkH*
123 fragment were amplified from SCC3193 and cloned into pOM18 using SalI and SacI or PstI and
124 XbaI, respectively. pOM18 was constructed by amplifying the multiple cloning site of pUC18 and
125 cloning it into the BglII site created from the divergently amplified pOM1 (21). PCR reactions for
126 cloning purposes were performed using the proof reading Herculase II Polymerase (Agilent).
127 Dream Taq Polymerase (Fermentas) was used for all other PCR reactions. Digestions were done
128 using Fast Digest Enzymes (Fermentas) and ligations performed with T4 DNA ligase (New

England Biolabs). All cloning steps were performed in *E. coli* DH5α. All mutants and constructs were confirmed by sequencing at the Instituto Gulbenkian de Ciência sequencing facility.

Isolation of transposon insertion mutants with low *rsmB* expression. A library of 15,126 mutants in SCC3193 was constructed by transposon mutagenesis using the EZ-Tn5™ <R6Kγori/KAN-2>Tnp Transposome™ kit, according to the manufacturer's instructions (Epicentre). Mutants were tested in 96-well plates for Pel activity levels using the thiobarbituric acid (TBA) method (22). The pRSV206 plasmid, which contained the P_{rsmB}::*gfp* fusion, was introduced into mutants with low Pel activity by electroporation. To measure *rsmB* expression levels, these strains were grown in multi-well plates at 30°C in MM supplemented with Cm and Kan and diluted 1:100 into fresh medium in black multi-well plates. After 24 h of growth, GFP expression was assessed using a multi-label counter (Victor^3, PerkinElmer). We identified 29 mutants with less than 75% of *rsmB* expression level shown by wt bacteria. The transposon insertion site of these mutants was amplified by a two-step arbitrary PCR using the transposon specific primers, P0058-Kan_SP1 and P0057-Kan_SP2, and the arbitrary primers, P0052-Arb1K, P0053-Arb2k and P0054-Arb6K (23, 24). The insertion site was identified by DNA sequencing coupled with BLAST analysis against the *Pectobacterium* SCC3193 complete genome sequence (NCBI taxid:1166016).

Analysis of expression of P_{rsmB}::*gfp*. Analysis of *rsmB* expression was performed by flow cytometry. Mutant and wt strains of *P. wasabiae* SCC3193 containing the *rsmB* reporter fusion were grown overnight in MM supplemented with Cm and Kan then inoculated into fresh medium in multi-well plates at a starting OD_{600} of 0.02. Aliquots were collected at OD_{600} = 0.3 or 0.4, as indicated in the figures legends. Cells were diluted 1:100 into Phosphate Buffered Saline (PBS) and fluorescence intensity of GFP per cell was assessed immediately in the flow cytometer (LSR Fortessa, BD). Results were analyzed with Flowing Software v2.5.1. A minimum of 5,000 GFP-positive single cells were acquired per sample and analyzed for their *rsmB* expression. *rsmB*

154 expression of the mutants with the P_{rsmB}::*gfp* fusion is reported as the median GFP expression of the
155 GFP-positive single cells in arbitrary units (a.u.).

156 **Pel activity assay.** Overnight cultures of bacterial strains were diluted to an OD_{600} of 0.02 in fresh
157 LB supplemented with PGA in multi-well plates. Bacteria were sub-cultured to an OD_{600} of 0.4, at
158 which time cell-free supernatants were harvested by centrifugation. Extracellular Pel activity was
159 measured using the previously described TBA colorimetric method (22). Briefly, supernatants were
160 incubated for 3 h with the substrate mixture at 37º C. The reaction was stopped by acidification,
161 TBA (Sigma T5500) was added, and the reaction mixture boiled for 1 h. The pink coloration was
162 measured at 548 nm using a multi-label counter (Victor^3, Perkin-Elmer) and normalized to the
163 OD_{600} of the culture.

164 ***P. wasabiae* virulence assay.** Virulence was determined using a modified protocol to assess
165 maceration of potato tubers (25). Potatoes were washed and surface sterilized by soaking for 10 min
166 in 10% bleach followed by 10 min in 70% ethanol. To prepare bacteria for inoculation, overnight
167 cultures were washed twice in PBS, which contained a total of 4.5 mM of potassium, or in PBS
168 with various concentrations of KCl, as indicated. 30 µL of cells at an OD_{600} of 0.05 were inoculated
169 into previously punctured potatoes and incubated at 28ºC with relative humidity above 90% for 48
170 h. After incubation, potatoes were sliced and macerated tissue was collected and weighed. To
171 quantify the inoculum, serial dilutions of this bacterial suspension were performed in PBS, plated
172 and bacterial growth was quantified by the number of colony forming units (cfu) present in 30 µl.

173 **Statistical analysis.** Data were analyzed using Graphpad Prism6 software and R program version
174 3.0.2. The Mann-Whitney test was performed to determine statistical significance and P-values
175 were adjusted using the Holm-Bonferroni correction for multiple comparisons. An adjusted P-value
176 <0.05 was used as the cut-off for statistical significance. ^{ns}Not significant; *P-value <0.05; **P-
177 value <0.01; ***P-value <0.001.

178

179

Results

Mutants in the Trk potassium uptake system are impaired in *rsmB* expression and production of PCWDEs

To identify regulators of the Gac/Rsm system we generated and screened a library of 15,126 transposon mutants in *P. wasabiae* SCC3193 for changes in Pel activity. We obtained 58 mutants with reduced Pel activity compared to wt. *rsmB* expression levels were then tested in these mutants following the introduction of a plasmid-encoded *rsmB* promoter-GFP fusion construct (pRSV206 – P_{rsmB}::*gfp*) into the mutants. Of these, 29 independent mutants showed less than 75% of *rsmB* expression in comparison to wt (Table S3). From those 29 mutants, five were found to have transposon insertions in genes annotated as components of a putative Trk system, which is involved in potassium uptake in *E. coli* (26). As shown in Fig. 1, all five mutants had an approximately two-fold reduction in the expression of the *rsmB*::*gfp* fusion compared to wt levels. Three of those mutants (RSV108, RVS141 and RSV154) had transposon insertions in the W5S_4400 gene, annotated as the potassium uptake protein, TrkH. The protein encoded by this gene has 89% sequence identity with one of the potassium uptake channel proteins (TrkH) from *E. coli*. This bacterium has two redundant Trk channel proteins, TrkH and TrkG: disruption of both proteins is required for the abolishment of potassium uptake by the Trk system. In contrast, *P. wasabiae,* has only one putative Trk channel protein, similar to most other bacteria containing the Trk transporter. The other two mutants (RSV124 and RSV236) had transposon insertions in the W5S_4132 gene, annotated as the potassium uptake protein, TrkA. The protein encoded by this gene shares 85% sequence identity with the TrkA protein from *E. coli*, a regulator of the Trk potassium uptake system. In *E. coli*, disruption of *trkA* results in a lower rate of potassium uptake by the Trk system (26). To investigate the role of the Trk system in the regulation of RsmB, one mutant with a transposon insertion in the *trkH* gene (RSV141) and another with an insertion in the *trkA* gene (RSV236) were selected for further characterization.

We tested the *trkH*::Tn5 and *trkA*::Tn5 mutants for complementation in *trans* with the *trkH* or *trkA* genes, respectively, both for *rsmB* expression and Pel activity (Fig. 2). Due to the growth defect for both *trkH* and *trkA* mutant strains (Fig. S1), cells were collected and analyzed at the same cell density (OD_{600} = 0.4). P_{rsmB}::*gfp* expression was restored to wt levels in *trkH* and *trkA* mutants when the respective genes were expressed under the control of their own promoter, but remained low in mutants carrying the empty vector (Fig. 2A). When tested for their ability to produce PCWDEs, these mutants had a more than two-fold decrease in extracellular Pel activity which was restored to wt levels upon expression of either *trkH* or *trkA* in *trans* (Fig. 2B). These data showed that the reduction in *rsmB* expression, and consequent effect upon the downstream induction of PCWDEs observed, were a consequence of the disruption of *trkH* and *trkA* by the transposon insertion, and suggested that the Trk system could affect regulation of virulence in *P. wasabiae*. We therefore investigated the ability of the selected *trk* mutants to cause tissue damage in potatoes. As shown in Fig. 3, the mutant strains were impaired in virulence, showing an approximately 40% reduction in the mass of macerated potato tuber tissue compared to that in tubers infected with wt bacteria.

Extracellular potassium levels influence *rsmB* expression

The requirement for a functional Trk potassium uptake system for full activation of *rsmB* expression indicated that induction of virulence might in fact be regulated by potassium. Therefore, we analyzed *rsmB* expression levels in wt *P. wasabiae* grown in different concentrations of potassium. Bacteria grown in 0.25, 2.5 and 25 mM potassium induced *rsmB* expression by responding positively to increasing concentrations of potassium. However, in cells grown in 250 mM potassium, induction was as low as that observed in cells with 0.25 mM potassium (Fig. 4A).

To verify that this regulation was dependent upon the Trk system, we analyzed *rsmB* expression in the *trkA* and *trkH* mutants cultured in the same range of potassium concentrations. As neither mutant grew in the lowest concentration tested (0.25 mM, Fig. 4B), it was not possible to

231 determine the level of *rsmB* expression in these mutants under these conditions. Importantly, this
232 lack of growth shows that the Trk system is required for the growth of *P. wasabiae* in low
233 concentrations of potassium, supporting the predicted function of these genes in potassium uptake.
234 Furthermore, as shown in Fig. 4A, the two mutants yielded distinct phenotypes with regards to
235 potassium-dependent regulation of *rsmB* expression, in agreement with the putative functions
236 assigned by similarity to the Trk system in *E. coli*. In the *trkH* mutant, where based on knowledge
237 from *E. coli*, we would expect Trk-dependent potassium uptake to be absent, *rsmB* expression was
238 low when it was grown in the potassium concentrations tested. This result showed that no
239 potassium-dependent regulation of *rsmB* expression was observed upon disruption of the putative
240 Trk potassium channel protein. In contrast, *trkA* mutants retained some ability to induce *rsmB*
241 expression in response to changes in potassium availability, though 100-fold higher concentrations
242 (250 mM) were needed to reach the level of activation seen in the wt cultured with 2.5 mM
243 potassium. This suggests that Trk-dependent potassium uptake is less efficient in *P. wasabiae trkA*
244 mutants than in wt, similar to what was reported for *E. coli* (26). Together these phenotypes support
245 the prediction that these genes are part of a functional homologue of the Trk potassium transport
246 system in *P. wasabiae*, and these results show that this system is involved in the potassium
247 mediated regulation of RsmB.

248 Previous studies of the Gac/Rsm system in both pectobacteria and other bacterial species,
249 have established GacA as the key response regulator responsible for activating expression of *rsmB*.
250 To verify whether potassium-dependent regulation of RsmB occurs via this two component system,
251 we measured *rsmB* expression in mutants lacking *gacA* and determined the effect of potassium in
252 the absence of this response regulator. In line with current literature, disruption of *gacA* resulted in
253 reduced expression of *rsmB* compared to that of wt bacteria (Fig. 4A). Nonetheless, *rsmB*
254 expression in the *gacA* mutant could still be induced with intermediate levels of potassium, similar
255 to that observed in wt bacteria. Furthermore, deletion of *gacA* in a *trkA* mutant background (*gacA*
256 *trkA*::Tn5 double mutant) resulted in the same potassium-dependent activation of *rsmB* expression

observed for the *trkA* single mutant. Therefore, potassium-dependent regulation of RsmB is not mediated by the GacS/GacA signal transduction pathway.

Overall, our results demonstrate that *rsmB* expression is regulated by extracellular levels of potassium and that this regulation requires the Trk system, but is independent of the GacS/GacA system.

Virulence in *P. wasabiae* is regulated by the extracellular concentration of potassium

Next we tested whether the potassium-dependent effect on *rsmB* expression observed in the *in vitro* studies described above had consequences for virulence *in vivo,* using the maceration of potato tubers. We tested whether supplementation of the inoculum with different concentrations of potassium affected the outcome of infection. In agreement with the *in vitro* results for *rsmB* expression, high concentrations of extracellular potassium (250 mM) had an inhibitory effect on the virulence of wt bacteria: less tissue maceration was observed in potatoes inoculated with cells resuspended in buffer with 250 mM potassium compared to that of potatoes infected with bacteria prepared in buffer supplemented with 4.5 mM potassium or no potassium (squares in Fig. 5). As for the mutants, virulence of the *trkH* mutant was low at all potassium concentrations tested, while that of the *trkA* mutant was also low at low potassium concentrations, though supplementation of high concentrations of potassium (250 mM) resulted in an increase in virulence to near wt levels (Fig. 5). The results for the *trkH* and *trkA* mutant strains are in full agreement with their respective phenotypes for RsmB induction obtained *in vitro* (Fig. 4A).

Nonetheless, no increase in virulence of the wt bacteria occurred when potatoes were inoculated with cells prepared in 4.5 mM potassium compared to those prepared without the addition of potassium. However, bacterial inocula were grown in LB prior to infections, a medium which contains approximately 8 mM potassium (15), and because our data showed that concentrations higher than 2.5 mM were sufficient to induce *rsmB* expression in wt bacteria *in vitro* (Fig 4), we reasoned that such induction might sustain *rsmB* expression, and therefore virulence,

during infection. To address this possibility, as well as to determine if extracellular potassium at the site of infection could be essential to induce virulence, wt *P. wasabiae* was grown under non-inducing conditions in Minimal Potassium Medium supplemented with low potassium (0.25 mM) and then re-suspended in buffer with different potassium concentrations before inoculation into potatoes. The *trkH* and *trkA* mutant strains were not tested under these conditions due to their lack of growth in low concentrations of potassium (Fig. 4B). As shown in Fig. 6, wt bacteria grown in 0.25 mM caused some tissue maceration even when no potassium was added to the inoculum, but, importantly, an increase in virulence was observed with the addition of 4.5 mM potassium (Fig. 6). These results demonstrate that the addition of potassium at the time of infection induced virulence in cells that were grown under non-inducing conditions. Again, high concentrations of potassium (250 mM) inhibited the induction of virulence: the mass of macerated tissue was as low in these conditions as when no potassium was added to the inoculum (Fig. 6).

Discussion

The small RNA, RsmB, has a major role in the signaling network controlling virulence in *P. wasabiae* by preventing RsmA-mediated repression of PCWDEs expression. Consequently, activation of *rsmB* transcription is essential for the production of these enzymes, and thus unraveling the signals and mechanisms involved in this regulation is a key step in understanding the environmental factors influencing virulence in *Pectobacterium* spp.

In a screen for regulators of RsmB, we isolated five mutants disrupted in genes involved in the putative Trk potassium uptake system (*trkH* and *trkA*). We demonstrated that these mutants presented a reduced *rsmB* expression and that the Trk system is important for production of PCWDEs. These results led us to investigate the role of potassium in expression of *rsmB* and virulence. We showed that intermediate concentrations of potassium (2.5 – 25 mM) were required to induce RsmB, but high potassium concentrations (250 mM) inhibited expression of this regulatory RNA. The conclusion that potassium and Trk participate in RsmB regulation is further

supported by the identification of another mutant isolated in our screen, that had the transposon inserted in the third gene of the annotated *sapABCDF* operon (RSV238, Table S3). This operon, in particular SapD, has been implicated in potassium transport via the Trk system in *E. coli,* as mutants in *sapD* present no potassium uptake by the Trk system (27). It is thus possible that a transposon insertion in *sapC* also affects the Trk system.

Our analysis of the *trk* mutants showed that in *P. wasabiae* the Trk potassium system functions in a similar way to the *E. coli* Trk system. However, althought in *E. coli* the Trk system seems to be important mainly at intermediate concentrations of potassium, in *P. wasabiae* this system appeared to be relevant in a broader range of potassium concentrations. In *E. coli* an inducible high affinity potassium system, the Kdp system, is the major responsible for potassium uptake at concentrations lower than 5mM [reviewed in (28)], but, in *P. wasabiae* the *trk* mutants had a strong growth defect at low potassium concentration (2.5 mM) and did not even grow at 0.25 mM. These results showed that the Trk system is important at low potassium concentrations. This, together with the fact that we could not find any *kdp*-like gene in the genome of *P. wasabiae* indicates that *P. wasabiae*, in contrast to *E. coli*, might lack a high affinity potassium transporter and relies solely on Trk at low potassium concentrations.

Our results showing that the Trk system regulated *rsmB* expression provided strong support for the hypothesis that extracellular levels of potassium were important for virulence in *P. wasabiae*. Accordingly, we observed that extracellular potassium was required to fully induce maceration of plant tissue in wt and that this induction required an intact Trk system. Moreover, we observed that virulence was inhibited at high extracellular concentrations of potassium (250 mM). Importantly, this inhibition of virulence in wt at a concentration of 250 mM potassium is unlikely to be a consequence of growth inhibition since *in vitro*, at this potassium concentration, wt still grows better than the *trkA* mutant. In addition, the *trkA* mutant despite its growth defect, with 250 mM potassium can cause almost as much tissue maceration as the maximal levels observed with the wt (Fig. 4E, Fig. 5 and Table S4). We also determined that supplementation of high concentration of

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335 potassium had no effect on the viability of the inoculum applied to the potato tubers in any of
336 strains tested, as shown in Fig. S2.

337 The mechanism by which the extracellular levels of potassium regulate *rsmB* expression via
338 the Trk system is still unclear. As *rsmB* transcription is activated by the GacS/GacA system, we
339 investigated whether the observed effects of potassium were also linked with or dependent upon the
340 function of this two component system. Though disruption of the *gacA* gene decreased the extent to
341 which *rsmB* expression was induced, surprisingly potassium- and Trk-dependent regulation was
342 still observed in a *gacA* mutant. Two additional regulators in *Pectobacterium* spp., KdgR and
343 RsmC, have been shown to repress RsmB expression (7, 29). However, again disruption of these
344 regulators had no effect in potassium-dependent regulation of *rsmB* expression (data not shown).
345 These results provide evidence for additional players in this regulation of RsmB. The identity of
346 such regulators could come from a genetic approach to isolate mutants that no longer respond to
347 changes in extracellular levels of potassium. For example, it is possible that an additional two-
348 component system is involved in such regulation. In fact, some transport systems have been
349 associated with the activation of two-component systems [reviewed in (30)]. In *E. coli*, the
350 DcuS/DcuR two-component system which is activated by C4-carboxylates in the periplasm is also
351 regulated by the DcuB antiporter that uptakes C4-carboxylates. It has been proposed that in the
352 absence of C4-carboxylates, protein-protein interactions between DcuB and DcuS repress the
353 autophosphorilation activity of the DcuS sensor kinase. Upon transport of these compounds, this
354 repression is absent presumably because DcuB releases DcuS which can then be activated by the
355 perisplamic levels of C4-dicarboxylates (31). A similar interaction might be happening between the
356 Trk transport proteins and an unknown two-component system to regulate *rsmB*. Alternatively, it is
357 possible that the observed regulation of *rsmB* is not responding to potassium flux through the Trk
358 system but instead it is sensitive to changes in intracellular potassium concentrations, as it has been
359 shown for the induction of biofilm formation in *Bacillus subtilis* as a response to potassium leakage
360 (32). In this case it was proposed that in the presence of natural compounds that cause potassium

361 leakage, the membrane kinase KinC responds to transient decreases in cytoplasmic potassium
362 concentrations, activating a phosphorylation cascade that results in induction of exopolysaccharides
363 production and biofilm formation.

364 The Trk system is widespread among bacteria and it is the potassium transporter most
365 commonly found in the largest number of species. This transport system is crucial for many of the
366 intracellular functions of potassium, such as maintenance of cell turgor pressure, regulation of
367 intracellular pH, adaptation to osmotic stress and function of many cytoplasmic enzymes that
368 require potassium [reviewed in (28)], but importantly it has also been implicated in regulation of
369 virulence in diverse bacteria. Trk mutants are impaired in virulence in *Vibrio vulnificus*, *Salmonella*
370 *enterica enterica* serovar Typhimurium and *Francisella tularensis*, however the molecular
371 mechanisms involved in such regulation have not been identified (15, 33, 34). As both *V. vulnificus*
372 and *S. enterica* also use homologues of the Rsm system (Csr) to regulate virulence, it is conceivable
373 that Trk regulation of virulence in these organisms might also take place through the regulatory
374 RNAs of the Csr system. Therefore, it is possible that the mechanisms identified here are conserved
375 among the other pathogens that have the Gac/Rsm system. As the Gac/Rsm system has been shown
376 to modulate carbon fluxes (10, 11) we propose that in bacteria that use the Trk transporter to
377 regulate components of the Gac/Rsm pathway cells might benefit from coupling the information
378 obtained from perceiving changes in potassium concentrations with the information on the
379 metabolic state of the cell, to modulate functions that go beyond maintaining the physiological
380 functions of potassium to control activities that contribute to host colonization. For instance, when
381 short-chain fatty acids accumulate during fermentation, bacterial cells typically use potassium
382 transporters to manipulate intracellular potassium levels, to cope with changes in cytoplasmic pH
383 and to control turgor pressure [reviewed in (35)]. It is interesting that the Gac/Rsm homologue
384 system in *E. coli* has been shown to respond under certain conditions to these weak organic acids
385 (12) and thus, it is tempting to speculate that the link between the regulation of potassium transport
386 with the regulation of the Gac/Rsm system might be related to the need to adapt to the presence of

short-chain fatty acids. The benefit of regulating virulence in response to changes in potassium concentrations might also be related to the environmental changes typically experienced by pathogens during host invasion. For example, plant pathogens, such as *P. wasabiae*, are typically found in the soil where potassium concentrations are in the 10-100 µM range, contrary to the 100 mM concentration found in eukaryotic cells (36). Hence, upon arrival to a wounded host, the local increased potassium concentration might provide a cue for the activation of the production of PCWDEs. The action of these enzymes will further disrupt the host cells with the consequent leakage of intracellular potassium, and the bacteria will have to adapt to the increasing potassium levels which can ultimately reach the levels found inside the eukaryotic plant cells.

Crop losses resulting from bacterial diseases remain significant agricultural and economic concerns. Understanding the regulatory networks responsible for the activation of virulence genes will help to define and improve control strategies that target this problem. The results presented here identify potassium as an important environmental factor in the regulation of virulence in the plant pathogen *P. wasabiae* and show that a reduction in wt virulence can be achieved by increasing the extracellular concentration of potassium (Fig. 5 and Fig. 6). Although additional work is required to fully characterize the molecular mechanisms behind the regulation of virulence by potassium via RsmB, our study highlights how potassium levels in the soil could affect the outcome of virulence in the plant pathogen *P. wasabiae*.

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Legends

Fig. 1. Trk mutants have low *rsmB* expression. (A) Expression of P_{rsmB}::*gfp* promoter fusion from pRSV206 in *P. wasabiae* wt, RSV108 (*trkH*::Tn5), RSV141 (*trkH*::Tn5), RSV154 (*trkH*::Tn5), RSV124 (*trkA*::Tn5) or RSV236 (*trkA*::Tn5) mutant strains was measured by fluorescence flow cytometry of cells grown for 24 h in MM. Error bars represent SD, n = 3.

514 **Fig. 2. Complementation of the *trkH* and *trkA* mutants.** (A) Expression of a P_{rsmB}::*gfp* promoter
515 fusion was measured in wt harboring the control vector (pOM18), the *trkH*::Tn5 mutant carrying
516 either the pOM18 empty vector or the vector expressing *trkH* (p(*trkH*$^+$)), and the *trkA*::Tn5 mutant
517 carrying either the pOM18 empty vector or the vector expressing *trkA* (p(*trkA*$^+$)). Fluorescence was
518 measured in cells collected from cultures grown to OD_{600} = 0.4 in MM. (B) Pel activity was
519 measured in cell-free supernatants from cultures of the bacterial strains indicated above grown in
520 LB supplemented with PGA at OD_{600} = 0.4. Error bars represent SEM, n = 6.
521
522 **Fig. 3. Mutants in the Trk system are impaired in virulence.** Virulence of wt (squares),
523 *trkH*::Tn5 (circles) and *trkA*::Tn5 (triangles) was measured by quantification of the mass of potato
524 tuber maceration induced by these bacteria 48 h after inoculation of the potato tubers. Potatoes were
525 inoculated with approximately 3×10^5 cells of the respective strain grown overnight in LB. n = 7,
526 **P-value < 0.01. This is a representative experiment from three independent experiments.
527
528 **Fig. 4. Effect of extracellular potassium on *rsmB* expression and growth in *P. wasabiae*.** (A)
529 The expression of the *rsmB* promoter fusion (P_{rsmB}::*gfp*) was measured by flow cytometry of
530 cultures of wt, *trkA*::Tn5 and *trkH*::Tn5, *gacA* and *gacA trkA*::Tn5 grown to OD_{600} = 0.3 in Minimal
531 Potassium Media supplemented with a final potassium concentration of 0.25 mM, 2.5 mM, 25 mM
532 or 250 mM. NG stands for no growth. Error bars represent SEM, n = 6. (B – D) OD_{600} was
533 measured throughout growth for wt (squares), *trkH*::Tn5 (circles), *trkA*::Tn5 (triangles) in Minimal
534 Potassium Media supplemented with (B) 0.25 mM, (C) 2.5 mM, (D) 25 mM or (E) 250 mM KCl
535 (growth rates in Table S4).
536
537 **Fig. 5. Regulation of virulence by extracellular potassium concentration.** Virulence of wt
538 (squares), *trkH*::Tn5 (circles) and *trkA*::Tn5 (triangles) was measured by quantification of the mass
539 of macerated tissue 48 h after inoculation of potato tubers. Cells cultured overnight in LB

(approximately of 8 mM potassium) were harvested and re-suspended in potassium-free PBS (wt only, white squares), or PBS supplemented with a final concentration of either 4.5 mM (grey) or 250 mM (black) potassium. Potatoes were inoculated with approximately 3×10^5 cells of the respective strain at the different potassium concentrations. Error bars represent SEM, n = 6, **P-value < 0.01, ^{ns}Not significant. This is a representative experiment from three independent experiments.

Fig. 6. Induction of virulence by extracellular potassium. Virulence of wt bacteria was measured by determining the mass of damaged tissue 48 h after infection of potato tubers. Potatoes were inoculated with approximately 3×10^5 cells of the respective strain grown overnight in Minimal Potassium Media supplemented with a final potassium concentration of 0.25 mM and re-suspended in potassium-free PBS (0 mM, white), in PBS (4.5 mM of potassium, grey) or in PBS supplemented with potassium to a final concentration of 250 mM (black). Error bars represent SEM, n = 6, **P-value < 0.01. This is a representative experiment from two independent experiments.