AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology

The Trk Potassium Transporter Is Required for RsmB-Mediated Activation of Virulence in the Phytopathogen *Pectobacterium wasabiae*

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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 an *N*-acyl homoserine lactone-based quorum sensing system and through 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 noncoding RNA that is essential for the 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* and *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 for 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 the 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.

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 cabbages. 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 the expression and activity of the global posttranscriptional 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 an *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 noncoding RNA that binds RsmA (6). This binding of RsmA by RsmB inhibits the 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 system 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 references 8 and 9), the chemical identity of the molecule(s) responsible for their activation remains unknown. Accumulation of intermediates of the Krebs cycle have been shown to stimulate the Gac/Rsm systems in *Pseudomonas fluorescens* and *Vibrio fischeri* (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

Received 28 July 2015 **Accepted** 14 October 2015

Accepted manuscript posted online 19 October 2015

Citation Valente RS, Xavier KB. 2016. The Trk potassium transporter is required for RsmB-mediated activation of virulence in the phytopathogen *Pectobacterium wasabiae*. J Bacteriol 198:248–255. doi:10.1128/JB.00569-15.

Editor: G. A. O'Toole

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00569-15.

the expression of *csrB* (a functional *rsmB* homologue) in *E. coli* or *Salmonella enterica* subsp. *enterica* serovar Typhimurium at low pH, but the stimuli that activate the system at neutral pH have not been identified (12, 13).

To identify the physiological stimuli involved in the activation of the Gac/Rsm system and to understand how this system regulates virulence in the well-characterized *P. wasabiae* strain SCC3193, we performed a genetic screen to identify mutants affected in the expression of *rsmB*, 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, we investigated the importance of potassium and the Trk system to 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 of the strains and plasmids used in this study are listed in Table S1 in the supplemental material. *P. wasabiae* strains are derived from the wild-type (WT) strain SCC3193 (14). Strains were grown at 30°C with aeration in Luria-Bertani (LB) broth, M9 minimal medium (MM), or minimal potassium medium (15) with 0.4% (wt/vol) glycerol. When specified, the medium was supplemented with 0.4% 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: 100 mg liter^{-1} ampicillin (Amp), 100 mg liter^{-1} streptomycin (Str), 50 mg liter^{-1} kanamycin (Kan), 50 mg liter^{-1} spectinomycin (Spec), and 25 mg liter^{-1} chloramphenicol (Cm). To assess bacterial growth, the optical density at 600 nm (OD_{600}) was determined by measuring absorbance at 600 nm in the Bioscreen C reader system (multiplate reader; Oy Growth Curves Ab Ltd.).

Genetic and molecular techniques. Primers used in this study are listed in Table S2 in the supplemental material. The plasmid carrying the *rsmB* promoter fused to the green fluorescence protein (GFP) (P_{rsmB}::*gfp*) was constructed using a modified version of the promoterless::*gfp* vector pCMW1 (16). First, the chloramphenicol resistance gene (*cm*) was amplified by PCR from pKD3 (17) using P0276-pKD3/4(BamHI) and P0277-pKD3/4(BamHI) primers and introduced into pCMW1, yielding PRSV59. Next a 392-bp fragment containing the promoter region of *rsmB* was amplified by PCR using the P0528-rsmB(SphI) and P0529-rsmB-(SalI) primers and was ligated into PRSV59, yielding pRSV206. Deletion mutants were constructed by chromosomal gene replacement with an antibiotic marker using the λ Red recombinase system (17, 18). The DNA region of *gacA*, including approximately 500 bp upstream and 500 bp downstream of the gene, was amplified by PCR and cloned into pUC18 (19) using SalI and SacI. This construct, containing the *gacA* gene and its flanking regions, was divergently amplified by PCR using primers to introduce an XhoI restriction site in the 5′ and 3′ regions of the gene. The streptomycin resistance gene (*str*) was amplified from pKNG101 (20) with primers containing the XhoI restriction site. The amplified fragment containing the gene for streptomycin resistance was digested with XhoI and was introduced into the XhoI-digested PCR fragment. The final construct contained the antibiotic resistance marker flanked by the upstream and downstream regions of *gacA*. The 500-bp–*str*–500-bp fragment was amplified by PCR, and approximately 3 ng of DNA was electroporated into a strain expressing the λ Red recombinase system from pKD46 to allow recombination (17). To construct the *trkA*$^+$ and *trkH*$^+$ complementation plasmids, a 400-bp–*trkA*–400-bp fragment and a 400-bp *trkH* fragment were amplified from SCC3193 and were cloned into pOM18 using SalI and SacI or PstI and XbaI, respectively. pOM18 was constructed by amplifying the multiple cloning site of pUC18 and by cloning it into the BglII site created from the divergently amplified pOM1 (21). PCRs for cloning purposes were performed using the proofreading Herculase II polymerase (Agilent). Dream *Taq* polymerase (Fermentas) was used for all other PCRs. Digestions were done using FastDigest enzymes (Fermentas), and ligations were 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 SCC3193 mutants was constructed by transposon mutagenesis using the EZ-Tn*5* <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 multiwell plates at 30°C in MM supplemented with Cm and Kan and were diluted 1:100 into fresh medium in black multiwell plates. After 24 h of growth, GFP expression was assessed using a multilabel counter (Victor^3; PerkinElmer). We identified 29 mutants with <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 Taxonomy ID: 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 and were then inoculated into fresh medium in multiwell plates at a starting OD_{600} of 0.02. Aliquots were collected at an OD_{600} of 0.3 or 0.4, as indicated in the figure legends. Cells were diluted 1:100 into phosphate-buffered saline (PBS), and the fluorescence intensity of GFP per cell was assessed immediately in the flow cytometer (LSRFortessa; BD). Results were analyzed with Flowing Software v2.5.1. A minimum of 5,000 GFP-positive single cells were acquired per sample and were analyzed for their *rsmB* expression. *rsmB* expression of the mutants with the P_{rsmB}::*gfp* fusion is reported as the median GFP expression of the GFP-positive single cells in arbitrary units (a.u.).

Pel activity assay. Overnight cultures of bacterial strains were diluted to an OD_{600} of 0.02 in fresh LB supplemented with PGA in multiwell plates. Bacteria were subcultured to an OD_{600} of 0.4, at which time cell-free supernatants were harvested by centrifugation. Extracellular Pel activity was measured using the previously described TBA colorimetric method (22). Briefly, supernatants were incubated for 3 h with the substrate mixture at 37°C. The reaction was stopped by acidification, TBA (Sigma T5500) was added, and the reaction mixture was boiled for 1 h. The pink coloration was measured at 548 nm using a multilabel counter (Victor^3; Perkin-Elmer). The absorbance values obtained at 548 nm were divided by the OD600 of the corresponding cultures.

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

Statistical analysis. Data were analyzed using Graphpad Prism 6 software and R program v3.0.2. The Mann-Whitney test was performed to

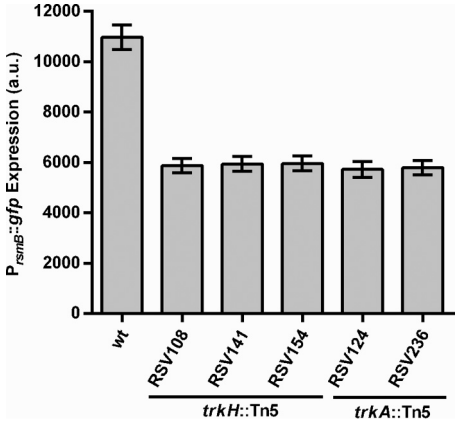

FIG 1 Trk mutants have low *rsmB* expression. Expression of P_{rsmB}::*gfp* promoter fusion from pRSV206 in *P. wasabiae* WT and RSV108 (*trkH*::Tn*5*), RSV141 (*trkH*::Tn*5*), RSV154 (*trkH*::Tn*5*), RSV124 (*trkA*::Tn*5*), or RSV236 (*trkA*::Tn*5*) mutant strains was measured by fluorescence flow cytometry of cells grown for 24 h in MM. Error bars represent standard deviations (SD); $n = 3$.

determine statistical significance, and *P* values were adjusted using the Holm-Bonferroni correction for multiple comparisons. An adjusted *P* value of <0.05 was used as the cutoff for statistical significance.

RESULTS

Mutants with mutations 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 *P. wasabiae* SCC3193 transposon mutants for changes in Pel activity. We obtained 58 mutants with reduced Pel activity compared to that of the WT strain. *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 <75% *rsmB* expression in comparison to the WT strain (see Table S3 in the supplemental material). 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 2-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, whose product is annotated as the potassium uptake protein TrkH. This protein 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 the two 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, whose product is annotated as the potassium uptake protein TrkA. This protein 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.

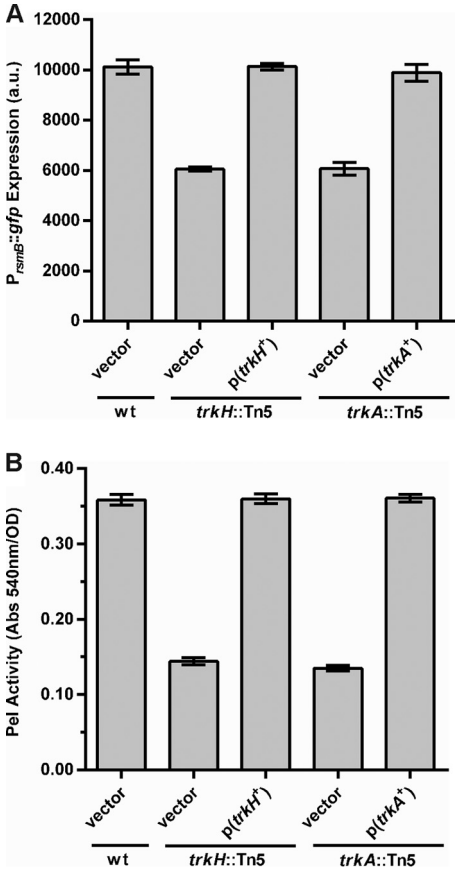

FIG 2 Complementation of the *trkH* and *trkA* mutants. (A) Expression of a P_{rsmB}::*gfp* promoter fusion was measured in WT bacteria harboring the control vector (pOM18), the *trkH*::Tn*5* mutant carrying either the pOM18 empty vector or the vector expressing *trkH*, p(*trkH*$^+$), and the *trkA*::Tn*5* mutant carrying either the pOM18 empty vector or the vector expressing *trkA*, p(*trkA*$^+$). Fluorescence was measured in cells collected from cultures grown to an OD_{600} of 0.4 in MM. (B) Pel activity was measured in cell-free supernatants from cultures of the bacterial strains indicated above grown in LB supplemented with PGA at an OD_{600} of 0.4. Error bars represent standard errors of the means (SEM); $n = 6$.

We tested the *trkH*::Tn*5* and *trkA*::Tn*5* mutants for complementation in *trans* with the *trkH* or *trkA* gene, respectively, for *rsmB* expression and Pel activity (Fig. 2). Due to the growth defect for both *trkH* and *trkA* mutant strains (see Fig. S1 in the supplemental material), 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 it remained low in mutants carrying the empty vector (Fig. 2A). When tested for their ability to produce PCWDEs, these mutants had a >2-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 the consequent effect upon the downstream induction of PCWDEs observed were a consequence of the disruption of *trkH* and *trkA* by the transposon insertion, and they suggested that the Trk system could affect regulation of virulence in *P.*

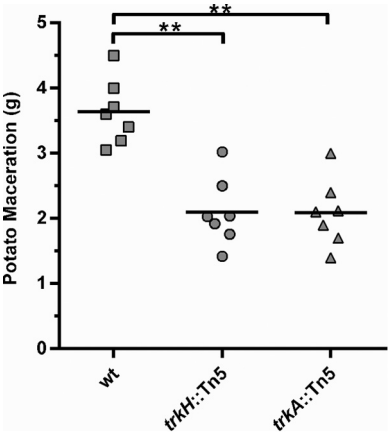

FIG 3 Mutants in the Trk system are impaired in virulence. The virulence of WT bacteria and *trkH*::Tn*5* and *trkA*::Tn*5* mutants was measured by quantification of the mass of potato tuber maceration induced by these bacteria 48 h after inoculation of the potato tubers. Potatoes were inoculated with approximately 3×10^5 cells of the respective strain grown overnight in LB. **, $P < 0.01$; $n = 7$. This is a representative experiment from three independent experiments.

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

Previous studies of the Gac/Rsm system in pectobacteria and other bacterial species have established GacA as the key response regulator responsible for activating the expression of *rsmB*. To verify whether potassium-dependent regulation of RsmB occurs via this two-component system, we measured *rsmB* expression in mutants lacking *gacA* and determined the effect of potassium in the absence of this response regulator. In line with current literature, disruption of *gacA* resulted in reduced expression of *rsmB* compared to that of WT bacteria (Fig. 4A). Nonetheless, *rsmB* expression in the *gacA* mutant can still be induced with intermediate levels of potassium, which is similar to what was observed with WT bacteria. Furthermore, deletion of *gacA* in a *trkA* mutant background (*gacA trkA*::Tn*5* 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 than in potatoes infected with bacteria prepared in buffer supplemented with 4.5 mM potassium or no potassium (squares in Fig. 5). As for the mutants, the 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 the virulence of the WT bacteria occurred when potatoes were inoculated with cells prepared in 4.5 mM potassium compared to the virulence in those prepared without the addition of potassium. However, bacterial inocula were grown in LB (a medium that contains approximately 8 mM potassium [15]) prior to infections, 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 and to determine if extracellular potassium at the site of infection is essential

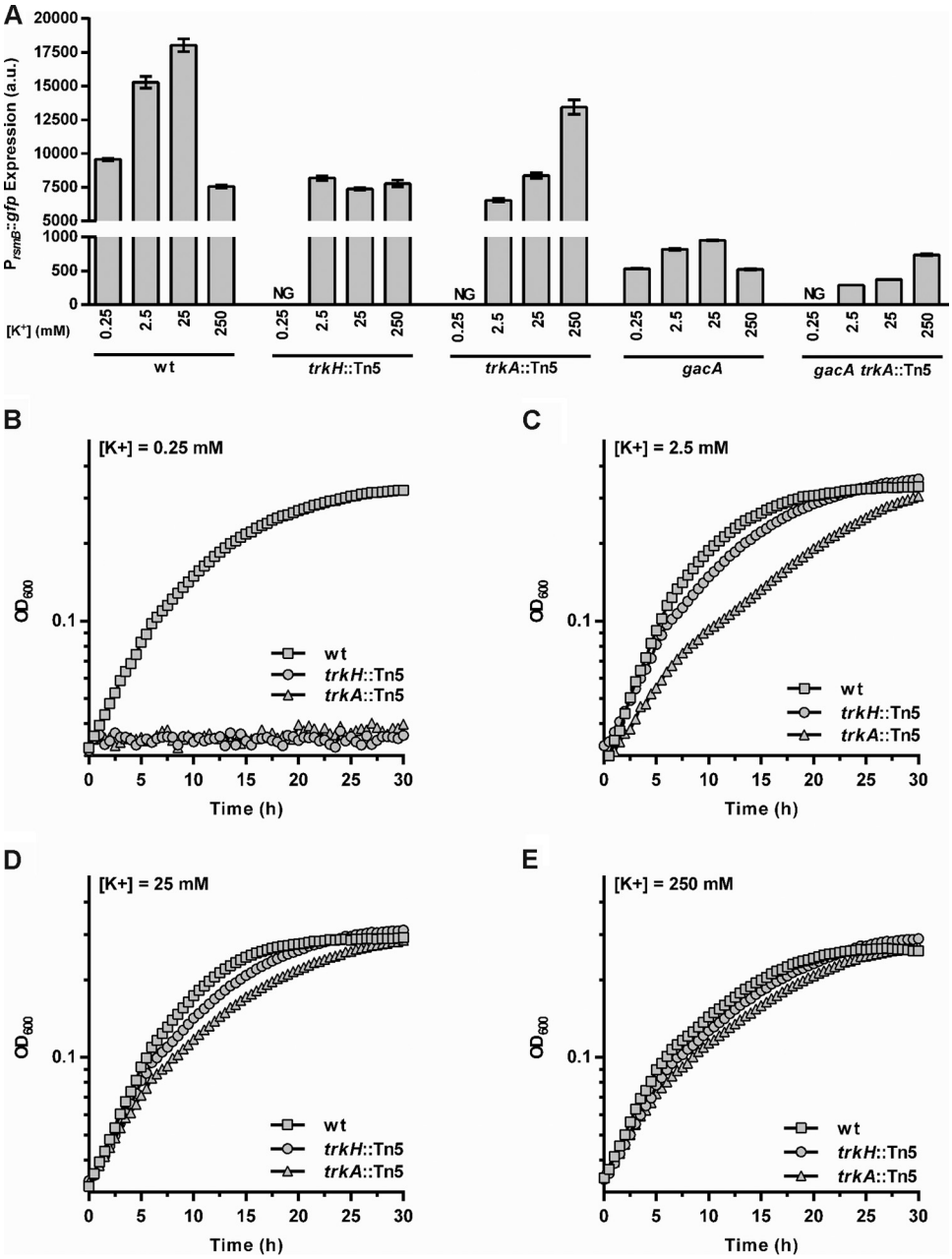

FIG 4 Effect of extracellular potassium on *rsmB* expression and growth in *P. wasabiae*. (A) The expression of the *rsmB* promoter fusion (P_{rsmB}::*gfp*) was measured by flow cytometry of cultures of WT bacteria and *trkA*::Tn*5*, *trkH*::Tn*5*, *gacA*, *gacA trkA*::Tn*5* mutants grown to an OD_{600} of 0.3 in minimal potassium medium supplemented with a final potassium concentration of 0.25 mM, 2.5 mM, 25 mM, or 250 mM. NG, no growth. Error bars represent SEM; $n = 6$. (B to E) The OD_{600} was measured throughout growth for WT bacteria and *trkH*::Tn*5* and *trkA*::Tn*5* mutants in minimal potassium medium supplemented with 0.25, 2.5, 25, or 250 mM KCl (for growth rates, see Table S4 in the supplemental material).

to induce virulence, WT *P. wasabiae* was grown under noninducing conditions in minimal potassium medium supplemented with low potassium (0.25 mM) and was then resuspended 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 noninducing conditions. Again, high concentrations of potassium (250 mM) inhibited the induction of virulence; the mass of macerated tissue was as low under 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-medi-

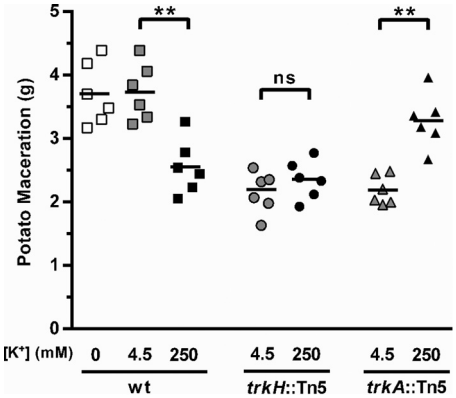

FIG 5 Regulation of virulence by extracellular potassium concentration. The virulence of WT bacteria and *trkH*::Tn*5* and *trkA*::Tn*5* mutants was measured by quantification of the mass of macerated tissue 48 h after inoculation of potato tubers. Cells were cultured overnight in LB (approximately 8 mM potassium) and were harvested and resuspended in potassium-free PBS (WT only) or PBS supplemented with a final concentration of either 4.5 mM or 250 mM potassium. Potatoes were inoculated with approximately 3×10^5 cells of the respective strain at the different potassium concentrations. Error bars represent SEM; $n = 6$; **, $P < 0.01$; ns, not significant. This is a representative experiment from three independent experiments.

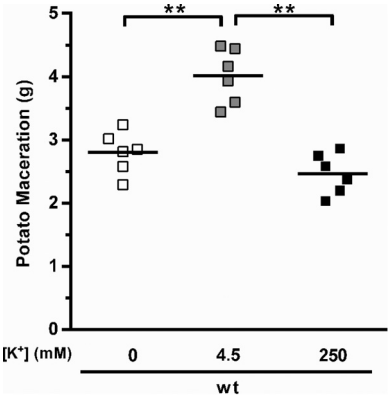

FIG 6 Induction of virulence by extracellular potassium. Virulence of WT bacteria was measured by determining the mass of damaged tissue 48 h after the infection of potato tubers. Potatoes were inoculated with approximately 3×10^5 cells of the respective strain grown overnight in minimal potassium medium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

ated repression of PCWDE 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 the 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 the expression of *rsmB* and virulence. We showed that intermediate concentrations of potassium (2.5 to 25 mM) were required to induce RsmB, but high potassium concentrations (250 mM) inhibited the 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, which had the transposon inserted in the third gene of the annotated *sapABCDF* operon (RSV238) (see Table S3 in the supplemental material). This operon, in particular *sapD*, has been implicated in potassium transport via the Trk system in *E. coli*, as *sapD* mutants 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 similarly to the *E. coli* Trk system. However, although 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 system responsible for potassium uptake at concentrations lower than 5 mM (reviewed in reference 28), but in *P. wasabiae* the *trk* mutants had a strong growth defect at a 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*, may lack a high-affinity potassium transporter and may rely solely on Trk at low potassium concentrations.

Our results showed that the Trk system regulated *rsmB* expression and provided strong support for the hypothesis that extracellular levels of potassium are important for virulence in *P. wasabiae*. Accordingly, we observed that extracellular potassium was required to fully induce maceration of plant tissue in WT bacteria 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 bacteria at a concentration of 250 mM potassium is unlikely to be a consequence of growth inhibition because, at this potassium concentration *in vitro*, WT bacteria still grow better than the *trkA* mutant. In addition, despite its growth defect, the *trkA* mutant can cause almost as much tissue maceration with 250 mM potassium as the maximal levels observed with the WT strain (Fig. 4E and 5; see also Table S4 in the supplemental material). We also determined that the supplementation of a high concentration of potassium had no effect on the viability of the inoculum applied to the potato tubers in any of strains tested as shown in Fig. S2 in the supplemental material.

The mechanism by which the extracellular levels of potassium regulate *rsmB* expression via the Trk system is still unclear. As *rsmB* transcription is activated by the GacS/GacA system, we investigated whether the observed effects of potassium were also linked with or dependent upon the function of this two-component system. Although disruption of the *gacA* gene decreased the extent to which *rsmB* expression was induced, surprisingly potassium-dependent and Trk-dependent regulation was still observed in a *gacA* mutant. Two additional regulators in *Pectobacterium* spp., KdgR and RsmC, have been shown to repress RsmB expression (7, 29). However, disruption of these regulators had no effect on potassium-dependent regulation of *rsmB* expression (data not shown). These results provide evidence for additional players in the regulation of RsmB. The identity of such regulators could

come from a genetic approach to isolate mutants that no longer respond to changes in extracellular levels of potassium. For example, it is possible that an additional two-component system is involved in such regulation. In fact, some transport systems have been associated with the activation of two-component systems (reviewed in reference 30). In *E. coli*, the DcuS/DcuR two-component system, which is activated by C_4-carboxylates in the periplasm, is also regulated by the DcuB antiporter, which takes up C_4-carboxylates. It has been proposed that, in the absence of C_4-carboxylates, protein-protein interactions between DcuB and DcuS repress the autophosphorylation activity of the DcuS sensor kinase. Upon transport of these compounds, this repression is absent presumably because DcuB releases DcuS, which can then be activated by the periplasmic levels of C_4-dicarboxylates (31). A similar interaction might happen between the Trk transport proteins and an unknown two-component system to regulate *rsmB*. Alternatively, it is possible that the observed regulation of *rsmB* is not responding to potassium flux through the Trk system but that it is instead sensitive to changes in intracellular potassium concentrations, as has been shown for the induction of biofilm formation in *Bacillus subtilis* as a response to potassium leakage (32). In this case, it was proposed that in the presence of natural compounds that cause potassium leakage, the membrane kinase KinC responds to transient decreases in cytoplasmic potassium concentrations, activating a phosphorylation cascade that results in induction of exopolysaccharide production and biofilm formation.

The Trk system is widespread among bacteria, and it is the potassium transporter found in the largest number of species. This transport system is crucial for many of the intracellular functions of potassium, such as the maintenance of cell turgor pressure, the regulation of intracellular pH, adaptation to osmotic stress, and the function of many cytoplasmic enzymes that require potassium (reviewed in reference 28), but importantly it has also been implicated in the regulation of virulence in diverse bacteria. Trk mutants of *Vibrio vulnificus*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, and *Francisella tularensis* are impaired in virulence; however, the molecular mechanisms involved in such regulation have not been identified (15, 33, 34). As both *V. vulnificus* and *S. enterica* also use homologues of the Rsm system (Csr) to regulate virulence, it is conceivable that Trk regulation of virulence in these organisms might also take place through the regulatory RNAs of the Csr system. Therefore, it is possible that the mechanisms identified here are conserved among the other pathogens that have the Gac/Rsm system. As the Gac/Rsm system has been shown to modulate carbon fluxes (10, 11), we propose that for bacteria that use the Trk transporter to regulate components of the Gac/Rsm pathway cells, we might benefit from coupling the information obtained from perceiving changes in potassium concentrations with the information on the metabolic state of the cell to modulate functions that go beyond maintaining the physiological functions of potassium to control activities that contribute to host colonization. For instance, when short-chain fatty acids accumulate during fermentation, bacterial cells typically use potassium transporters to manipulate intracellular potassium levels to cope with changes in cytoplasmic pH and to control turgor pressure (reviewed in reference 35). It is interesting that the Gac/Rsm homologue system in *E. coli* has been shown to respond under certain conditions to these weak organic acids (12), and thus, it is tempting to speculate that the link between the regulation of potassium transport and 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 to 100 μM range, contrary to the 100 mM concentration found in eukaryotic cells (36). Hence, upon arrival of the bacteria 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 continue to cause 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 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*.

ACKNOWLEDGMENTS

We thank Joana Amaro for technical assistance and Jessica Thompson and Pol Nadal for helpful discussions. We are very grateful to Andres Mäe (Institute of Molecular and Cell Biology) for sharing protocols and strains and to Tapio Palva (University of Helsinki) for providing strains used in this study.

FUNDING INFORMATION

Howard Hughes Medical Institute (HHMI) provided funding to Rita S. Valente and Karina B. Xavier under grant number HHMI 55007436. Science and Technology Foundation, Ministry of Education and Science (Fundação para a Ciência e a Tecnologia) provided funding to Rita S. Valente under grant number SFRH/BD/33570/2008. Science and Technology Foundation, Ministry of Education and Science (Fundação para a Ciência e a Tecnologia) provided funding to Karina B. Xavier under grant number PTDC/BIA-BCM/101585/2008.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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