Pectobacterium carotovorum is a ubiquitous soft rot pathogen that uses global virulence regulators to coordinate pathogenesis in response to undefined environmental conditions. We characterize an operon in *P. carotovorum* required for gluconate metabolism and virulence. The operon contains four genes that are highly conserved among proteobacteria (initially annotated *ygbJKLM*), one of which was misassigned as a type III secreted effecter, (*ygbK* originally known as *hopAN1*). A mutant with a deletion-insertion within this operon is unable to metabolize gluconate, a precursor for the pentose phosphate pathway. The mutant exhibits attenuated growth on the leaves of its host of isolation, potato, and those of *Arabidopsis thaliana*. Notably, the mutant hypermacerates potato tubers and is deficient in motility. Global virulence regulators that are responsive to cell wall pectin breakdown products and other undefined environmental signals, KdgR and FlhD, respectively, are misregulated in the mutant. The alteration of virulence mediated via changes in transcription of known global virulence regulators in our *ygbJ-M* operon mutant suggests a role for host-derived catabolic intermediates in *P. carotovorum* pathogenesis. Thus, we rename this operon in *P. carotovorum* vguABCD for virulence and gluconate metabolism.
quent progress through the stages of infection (Whitehead et al. 2002; Lazdunski et al. 2004; Yang et al. 2008; Li et al. 2009). Although KdgR is responsive to pectin-breakdown products, other virulence regulators that feed into the Rsm system are responsive to as yet unknown environmental cues. The FlhDC hexomer, another key virulence regulator, acts downstream of environmental signals and upstream of the Rsm system (Cui et al. 2008). Considered the master regulator of motility, FlhDC interacts genetically with multiple virulence regulators, including the two-component system, GacA/S, and a LysR-like transcriptional regulator, HexA (Harris et al. 1998; Mukherjee et al. 2000). FlhDC and HexA are responsive to unknown extracellular environmental factors, and HexA, in turn, regulates rsmB expression. The sensor kinase GacS is thought to detect cell density, growth phase, and other undetermined environmental factors, while its cognate response regulator, GacA, is itself regulated by medium composition and growth phase (Kitten et al. 1998; de Souza et al. 2003; Cui et al. 2008). GacA regulates toxins, PCWDE, and rsmB downstream of GacS (Chatterjee et al. 2003). In addition to regulating HexA and GacA/S, FlhDC in Escherichia coli is responsible for regulating a shift to aerobic respiration and genes involved in the Entner-Doudoroff pathway, an alternative glycolysis pathway (Pruss et al. 2003; Leatham et al. 2005). Thus, the activity of FlhDC not only provides a clear regulatory link between virulence and motility (Pruss et al. 2006) but between metabolism and environmental conditions as well.

We characterize a P. carotovorum operon required for gluconate metabolism that modulates the expression of key virulence regulators and affects virulence in plants. Both and associates (2002) employed an in vivo expression technology screen in P. syringae for T3SS virulence determinants and identified a gene they designated ipx53. Gene ipx53 was considered a potential type III effector for a short time and assigned the name HopAN1. Unlike other T3SS effectors, this gene is highly conserved across enterobacteria and other bacterial plant pathogens (Miriagou et al. 2005; Grant et al. 2006; Zienkiewicz et al. 2007). The hopAN1 homolog of strain WPP14 exists in an operon of four genes encoding, in order: an oxidoreductase, hopAN1, a class II aldolase, and an isomerase (Glasner et al. 2008). The operon is highly conserved in enterobacteria and the four genes were named ygbJKL and M in E. coli, which are temporary names for genes of unknown function (Rudd 1998). Here, we find that the operon is required for gluconate and virulence and, thus, name the P. carotovorum operon vguABCD for virulence and gluconate.

**RESULTS**

The vguABCD operon is widely distributed in proteobacteria.

The structure of the vguABCD operon in P. carotovorum (Fig. 1A) is generally conserved in enterobacteria. A similar operon also exists in more distantly related plant-pathogenic bacteria, although one or two of the open reading frames (ORF) are missing in some species. The conservation of the operon structure in select proteobacteria is illustrated in Supplementary Figure S1, which shows a Bayesian inference of phylogeny tree (Huelsenbeck and Ronquist 2001). The operon is conserved in non-enterobacteria plant pathogens such as Ralstonia picketti and Pseudomonas syringae B728a. In many species, the chromosome regions are flanked by other genes predicted to be involved in sugar metabolism, such as epimerases and decarboxylases. The conserved genomic structure of this operon suggests that its products could be involved in a metabolic function.

The vgu operon mutant has significantly attenuated growth on potato and Arabidopsis leaves.

We constructed a deletion-insertion mutation that replaces vguB with a chloramphenicol cassette. This has a polar effect and disrupts the expression of both the up- and downstream overlapping genes in the operon as determined by reverse-transcription polymerase chain reaction (RT-PCR). This mutation does not affect expression of the next gene downstream, gntP, which encodes gluconate permease (data not shown). The mutation was confirmed by PCR and resequencing of the entire vgu operon. The vgu operon mutant had the same growth rate as the
wild type in 2x yeast extract tryptone (YT), tuber-extract, hrp-repressing, and swimming minimal media (Supplementary Fig. S2), and was as tolerant to oxidative stress as the wild type using a hydrogen peroxide tolerance assay (data not shown). To determine whether the vgu operon is involved in virulence, we compared growth of the wild type to that of the vgu operon mutant in its native host, potato (Fig. 1B), as well as a model host, Arabidopsis thaliana (Fig. 1C). In both potato and A. thaliana leaves, P. carotovorum subsp. carotovorum WPP14 grew to 10^9 CFU/ml by 3 days postinfection. In potato leaves, the vgu operon mutant grew three logs less than strain WPP14 by 3 days postinfection, illustrating a severe attenuation in virulence. Similarly, in A. thaliana leaves, the vgu operon mutant grew two logs less than WPP14 by 3 days postinfiltration on leaves of both hosts. The vgu operon mutant failed to produce maceration symptoms typically seen during Pectobacterium carotovorum infection on leaves of both hosts. These begin as a rotting lesion spreading from the site of infiltration over time. Both attenuation of growth and lack of maceration mutant phenotypes were rescued with the vgu operon cloned with its native promoter in the entry clone pCR2.1 and mated into the vgu operon mutant strain (Fig. 1).

The vgu operon mutant hypermacerates potato tubers compared with the wild type.

To determine whether the vgu operon mutant was also defective in tuber maceration, we compared wild-type maceration to that of the vgu operon mutant. Each tuber was infected with three strains: P. carotovorum subsp. carotovorum WPP14, the vgu operon mutant, and the complemented vgu operon mutant, as well as a negative control for maceration, 10 mM MgCl_2 buffer. The wild type and the vgu operon mutant carrying the complementation clone both macerated just over 1 g of potato tuber in 5 days, while the vgu operon mutant macerated over 3 g of potato tuber (Fig. 2). The concentration of bacteria was approximately 1 x 10^9 CFU per 100 mg of macerated tuber tissue for both the mutant and the wild type. This indicated that the vgu operon mutant is hypermacerating, suggesting a misregulation of the expression of PCWDE that characterize soft rotting diseases. This hypervirulence in the tuber was unexpected, given the attenuated virulence phenotypes on leaves reported above.

The vgu operon mutant is deficient in swimming motility.

Previous studies of P. carotovorum and other phytopathogens have demonstrated coregulation of motility modes during pathogenesis. Therefore, we compared the vgu operon mutant with the wild type on twitching, swarming, and swimming motility agar plates. The vgu operon mutant was able to swim and twitch like wild-type strain WPP14; however, the vgu operon mutant exhibited significant reduction in swimming plates. On swimming plates, the wild type and the vgu mutant carrying the complementation clone swam almost twice the diameter of the vgu operon mutant (Fig. 3). This indicates that the vgu operon mutant is also unable to properly swim or perform chemotaxis.

The vgu operon is neither HrpL-regulated nor upregulated in leaves.

vguB has homology to the Pseudomonas syringae gene originally annotated as a type III effector called hopA11 (Lindeberg et al. 2005). We determined whether HrpL, the alternative sigma factor responsible for T3SS regulation in Pectobacterium carotovorum (Chatterjee et al. 2002), regulates vguB transcription in strain WPP14. Cultures of WPP14 containing the native hrpL ORF cloned under the control of an arabinose-inducible promoter in pCF430 (Chang et al. 2005) were grown with or without 200 mM arabinose in hrpL-repressing media. Quantitative (q)RT-PCR was used to assess the relative expression of vgu and a known HrpL-dependent T3SS effector, dspE (Fig. 4A). In the presence of overexpressed hrpL, expression of the type III effector gene dspE increased over 200-fold. However, vgu expression was not significantly altered by hrpL overexpression. By contrast to the Pseudomonas syringae hopA11 gene, vgu expression is also not induced in infected plants. We infiltrated A. thaliana leaves with wild-type strain WPP14 cultures with approximately 4 x 10^6 CFU/ml. RNA was extracted 2.5 h postinfection. Simultaneously, a WPP14 culture was grown in hrp-repressing minimal media to the same concentration as the infiltrated culture, and RNA was extracted. qRT-PCR performed using RNA from infected leaves or culture indicated that hrpL transcription increased approximately sevenfold in leaves compared with expression in the hrp-repressing minimal media (Fig. 4B). The expression of vguB, however, was not altered in leaves compared with minimal media and exhibited low-level expression in both conditions.

The vgu operon mutant exhibits altered expression of key virulence regulators in leaf infections and in the presence of tuber extract.

Our data indicated that virulence of the vgu operon mutant was attenuated in the leaf but overactive in the tuber. We used qRT-PCR to assess the expression level of known virulence

![Fig. 2. The vgu operon mutant hypermacerated infected potato tubers. Potato tubers were inoculated with either 10 mM MgCl_2, Pectobacterium carotovorum subsp. carotovorum WPP14, the vgu operon mutant, or this mutant carrying pCR2.1 vgu operon. In all, 10^9 CFU were injected into a 1.5-mm-deep hole and tubers were incubated at 28°C for 5 days. The experiment had 10 internal replicates and was repeated twice; a representative experiment is shown, and error bars are two times standard error.](image)

![Fig. 3. The vgu operon mutant was defective in swimming motility. Swimming motility plates were inoculated with 10^9 CFU into the center of the plate, each with 50 ml of motility agar and appropriate antibiotics. Plates were grown for 18 h at room temperature. The experiment had four internal replicates and was repeated three times; a representative experiment is shown, and error bars are two times standard error.](image)
regulators in both conditions. In leaves, the vgu operon mutant exhibited higher mRNA levels of kdgR and the T3SS helper protein hrpN than the wild type (Fig. 5A). In the presence of tuber extract, flhD (Cui et al. 2008) and the LysR-like transcription regulator, hexA (Harris et al. 1998), were severely downregulated in the vgu mutant compared with the wild type (Fig. 5B). Though weakly expressed relative to the flh housekeeping gene control, rsmB was also downregulated eightfold in the vgu operon mutant compared with the complementation strain in the presence of tuber extract (Fig. 5B). Following leaf infection, flhD and rsmB were expressed at very low levels in both the vgu operon mutant and the wild type (Fig. 5A).

The vgu operon is required for gluconate metabolism.

To address whether the vgu operon is involved in sugar metabolism, as BLAST analysis suggests, we used a phenotyparray plate to assess the metabolic phenotype of our vgu mutant in relation to the wild type. Wild-type strain WPP14 was capable of using all carbon sources tested, with the exception of D-gluconate (carbon sources tested are listed in Supplementary Table S2). The ability to grow on gluconate was rescued by the expression of the operon in the complementation strain. In Pectobacterium carotovorum, gluconate is a precursor sugar for the pentose phosphate pathway (Truesdell et al. 1991) and the first enzyme in the vgu operon has similarity to the first enzyme in the pentose phosphate pathway, 6-phosphogluconate dehydrogenase. However, the mutant was able to grow as well as the wild type on carbon sources that are sugar intermediates of the pentose phosphate pathway, xylulose and ribulose. This indicates that these pentose sugars can be metabolized through multiple pathways or that the defect in the vgu mutant may be limited to early steps in the pentose phosphate pathway.

The vgu operon is required for the accumulation of pentose sugars.

We compared the metabolite profiles of wild-type P. carotovorum subsp. carotovorum WPP14 and the vgu mutant grown in tuber extract media by gas chromatography (GC) coupled to mass spectrometry (MS). Cultures were grown in tuber extract media and metabolites were extracted during a cold chloroform extraction protocol established for GC-MS metabolite profiling by Ruijter and Visser (1996). We found six differential peaks in the chromatograph of the wild type and the vgu operon mutant (Table 1). One of the peaks was present in the

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**Fig. 4.** Expression of the vgu operon is independent of type III secretion system regulation. A, Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) data showing relative expression of vgu and dspE expressed in Pectobacterium carotovorum subsp. carotovorum WPP14 carrying pCF430 with hrpL-WPP14. Cultures were grown in hrpL-repressing minimal media and hrpL expression was induced with 200 mM arabinose. B, qRT-PCR data showing relative expression of vgu and hrpL expressed in WPP14. Strains were grown either in hrpL-repressing minimal media overnight or infiltrated into Arabidopsis thaliana leaves at 10⁶ CFU/ml with RNA extraction 2.5 h postinoculation. Expression is relative to the flh housekeeping gene. Each experiment was repeated at least twice and includes three technical replicates; error bars are two times standard error. Lowercase letters represent significantly different groups determined using an analysis of variance and a Tukey test. P values for expression differences between growth conditions are as follows: A, vguB, P = 0.2211 and dspE, P = 1.552 e⁻³; B, vguB, P = 0.386 and hrpL, P = 7.207 e⁻³.

**Fig. 5.** The vgu operon is required for normal expression of key virulence regulators and determinants in Pectobacterium carotovorum subsp. carotovorum WPP14, the vgu operon mutant, or the vgu operon mutant carrying pCR2.1-vgu operon, from leaf infection conditions after 2.5 h and in the presence of tuber extract after 3 h of growth. Expression is relative to the flh housekeeping gene. Each experiment was repeated at least twice and includes three technical replicates; error bars are two times standard error. Lowercase letters represent significantly different groups determined using an analysis of variance and a Tukey test. P values for expression differences between growth conditions are as follows: A, vguB, P = 0.0264; rsmB, P = 3.75e⁻³; hexA, P = 0.7054; kdgR, P = 0.0205; hrpN, P = 0.0005; and in the tuber: flhD, P = 3.228e⁻³; rsmB, P = 0.000634; hexA, P = 3.735e⁻³; kdgR, P = 0.1180; hrpN, P = 0.0769.
mutant but absent in the wild type. The mass spectrograph of that peak matched that of chloramphenicol, the antibiotic selection that is used for the mutant, but not the wild type, and the only known difference in the media composition between the two strains. The remaining five chromatograph peaks were all present in the wild type but absent in the mutant. One peak had a mass spectrograph that resembled that of 2-phenyl-2,3-dihydrobenzothiophene. It is unclear how this derivatized metabolite relates to the vgu operon mutant’s metabolic deficiency. The remaining four spectrographs represent silylated sugar derivatives, including D-glucuronic acid, ribose, erythrose, and sedoheptulose. Erythrose, ribose, and sedoheptulose are intermediates involved in the pentose-phosphate shunt. D-glucuronic acid is the precursor sugar for this pathway, suggesting that upstream catabolism is also altered in the vgu operon mutant. It should be noted that the P. carotovorum genome encodes two glucuronate transporters, one of which is encoded just downstream of the vgu operon. Because the vgu mutation does not alter expression of the downstream transporter (data not shown), we anticipate that the reduced glucuronate levels are due to the lack of the vgu operon-encoded functions. As a control, pure forms of these sugars (Sigma-Aldrich, St. Louis) were derivatized and analyzed with GC-MS to confirm the identity of the metabolite ion profiles that differed between the mutant and the wild type.

**DISCUSSION**

We demonstrate that the vgu operon is involved in glucuronic metabolism and is required for proper expression of virulence via KdgR, FlhD, HexA, and the rsm system. A mutant lacking the vgu operon has attenuated virulence in leaves, hypermacerates potato tubers, and is deficient in motility. The inability of the mutant to grow on glucuronic and its lack of intermediate pentose sugars from glucuronic metabolism suggests that the vgu operon encodes enzymes for glucuronic metabolism. In *P. carotovorum*, glucose can be oxidized to 2,5-diketogluconate, reduced to glucuronic, and subsequently metabolized through the pentose phosphate pathway as it is in related species (Truesdell et al. 1991). The predicted functions of the vgu operon-encoded proteins suggest that they operate in a pathway similar to the pentose phosphate pathway. Thus, we provide evidence suggesting that glucuronic catabolism affects virulence determinants by modulating known regulators.

**Glucuronic metabolism in *P. carotovorum***

In environments with high glucose concentrations, many bacteria oxidize glucose extracellularly to glucuronic or 2-keto-glucuronic through membrane-bound dehydrogenases. When glucose is depleted in the environment, the oxidized glucuronic can be transported into the cell and phosphorylated in an ATP-dependent manner, forming 6-phosphogluconate or 2-keto-6-phosphogluconate, respectively. These phosphorylated, intracellular sugars can then be used for the Entner-Doudoroff pathway, an alternative to the Embden-Meyerhof-Parnas glycolysis pathway, or the pentose phosphate shunt (Eisenberg and Dobrogosz 1967; Truesdell et al. 1991; Murray and Conway 2005).

The genome of *P. carotovorum* encodes genes for ketoaldonic acid metabolism and may oxidize extracellular glucose to 2,5-diketogluconate, which can be reduced to glucuronic in two separate pathways (Truesdell et al. 1991). However, once in the cell, 6-phosphogluconate may solely be metabolized through the pentose phosphate pathway, because enzymes for the ED pathway were not found in significant levels in a related species (Truesdell et al. 1991). Generally, if 6-phosphogluconate were to be metabolized through the pentose phosphate shunt, it would first be converted to ribulose 5-phosphate by the enzyme 6-phosphogluconate dehydrogenase, which is then converted to ribose 5-phosphate. The first gene in the vgu operon has high similarity to 6-phosphogluconate dehydrogenase. Consistent with this prediction, preliminary GC-MS data indicates that ribose does not accumulate in the vgu operon mutant.

The ribose 5-phosphate resulting from the first step of the pathway is further rearranged by transaldolases and isomerases to 6-glucose-phosphate and glyceraldehyde 3-phosphate. Although vguB, the second gene in the operon, does not have similarity to any other protein with a known function, the last two operon members encode a putative aldolase and isomerase. It should be noted that the *P. carotovorum* genome contains genes predicted to encode all other enzymes typically involved in the pentose phosphate pathway. However, these other enzymes are dispersed around the genome and many are neither within an operon nor clustered with other metabolic genes. Our observation that the vgu operon mutant is incapable of metabolizing glucuronic suggests that these other predicted enzymes either are not expressed in the conditions we tested or are involved in different pathways. Using GC-MS, we compared the metabolite profiles of the wild type and the operon mutant and found the mutant deficient in accumulation of pentose sugar intermediates of the pentose phosphate pathway. Therefore, based on our evidence that the vgu operon is required for glucuronic metabolism and the operon’s homology to enzymes in the pathway, we predict that the vgu operon is involved in a pathway similar to the pentose phosphate pathway.

The pentose phosphate pathway is also a main method for generating reducing power via the production of NADPH, which can be used to prevent oxidative stress. Plant hosts produce reactive oxygen species as a defense against invasion (Dong 2004). However, in oxidative stress assays using hydrogen peroxide, the vgu operon mutant was not more sensitive than the wild type to oxidative stress. Additionally, the vgu operon mutant did not produce different pH conditions in macerated tuber tissue or in medium, suggesting that the virulence phenotypes of the vgu operon mutant are not due to stress conditions in the host.

**Table 1.** Gas chromatography–mass spectrometry metabolic profiles show the vgu mutant to be depleted of sugars that are the immediate precursor and the products of the pentose phosphate pathway

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>WTb</th>
<th>vgu</th>
<th>Timec</th>
<th>Spectrogram peaksd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconic acid</td>
<td>+</td>
<td>–</td>
<td>16.91</td>
<td>73-387-299-315-357</td>
</tr>
<tr>
<td>2-Phenyl-2,3-dihydrobenzo[b]thiophene</td>
<td>+</td>
<td>–</td>
<td>17.59</td>
<td>212-211-134-135-178</td>
</tr>
<tr>
<td>Erythrose</td>
<td>+</td>
<td>–</td>
<td>18.6</td>
<td>73-315-357-299-217</td>
</tr>
<tr>
<td>D-ribose</td>
<td>+</td>
<td>–</td>
<td>20.15</td>
<td>73-315-217-299-316</td>
</tr>
<tr>
<td>O,O bis chloramphenicol</td>
<td>–</td>
<td>+</td>
<td>20.2</td>
<td>73-225-208-75-224</td>
</tr>
<tr>
<td>Sedoheptulose</td>
<td>+</td>
<td>–</td>
<td>20.53</td>
<td>204-73-191-217-205</td>
</tr>
</tbody>
</table>

a Differential presence (+) or absence (−) of metabolite.
b Wild type.
c Time metabolite peak was detected in the chromatograph.
d Top five signature peaks of the mass spectrogram for that metabolite peak.
Glucanate metabolism affects the expression of key regulators.

Previous studies identified a complex network of transcriptional and post-transcriptional virulence regulators (Barnard and Salmond 2007; Barnard et al. 2007; Mole et al. 2007) that regulate virulence determinants such as the T2SS and macerating enzymes, the T3SS, motility, quorum sensing, and antibiotic production. We evaluated the expression of these known key regulators in the vgu operon mutant to better understand the virulence phenotypes.

In leaves, the vgu operon mutant overexpresses kdgR, a transcriptional repressor known to regulate hrpN and genes encoding macerating enzymes (Liu et al. 1999). KdgR activity is responsive to pectin breakdown products and KdgR releases from target promoters after binding to catabolic intermediates of pectin degradation (Pouyssegur and Stoeber 1974; Nasser et al. 1991; Liu et al. 1999). Therefore, in the presence of pectin and its catabolic intermediates, such as polygalacturonate (PGA), the KdgR repressor activity is blocked and KdgR targets, such as genes encoding macerating enzymes and hrpN, are expressed.

Glucanate and, presumably, the catabolic intermediates of glucanate inhibit the production of macerating enzymes in the presence of KdgR inducers PGA and galacturonate (Nasser et al. 1991). Accordingly, in our vgu operon mutant, we observed hypermaceration in tubers and an increase in hrpN expression. The absence of glucanate metabolism in this mutant may allow for macerating enzymes and hrpN to be expressed unabated. HrpN is a T3SS-associated helper protein, known to induce plant defense responses. Overexpression of hrpN in P. carotovorum has been shown to induce a defense response of infected tobacco leaves (Cui et al. 1996), and purified HrpN protein from P. carotovorum can induce hypersensitive responses indicative of induced host defenses when applied to A. thaliana leaves (Kariola et al. 2003). Therefore, the induced defense responses caused by elevated expression of hrpN in the vgu operon mutant may be sufficient to explain its loss of virulence on leaves. A model for this is shown in Figure 6A, in which KdgR repressor activity is blocked by pectin breakdown products but sustained with the addition of glucanate. From our data and the previously published data by Nasser and associates (1991), we hypothesize that the catabolic pathways for pectin and glucanate are antagonistic. The increase that we see in kdgR expression in the vgu operon mutant is presumably due to autoregulation of the repressor; however, there is no data on the regulation of kdgR.

In tuber extract media, the vgu operon mutant shows no relative changes in kdgR or hrpN expression. However, the vgu operon mutant does exhibit downregulation of flhD, hexA, and rsmB in tuber extract compared with the wild type. These genes are weakly expressed in both the wild type and the mutant during leaf infection. The FlhDC complex was first identified as the master regulator of motility (Smith and Hoover 2009) in E. coli, and regulates the switch from aerobic respiration to anaerobic respiration (Pruss et al. 2003). FlhDC interacts with HexA, a LysR-like transcription factor, as well as with the Rsm system (Chatterjee et al. 2009). HexA directly binds the promoters of genes encoding extracellular enzymes. The loss of HexA was previously reported to cause a hypermacerating phenotype (Harris et al. 1998). This explains how our vgu operon mutant, which has relatively weak expression of hexA, hypermacerates the tuber. Likewise, downregulation of flhD, the master regulator of the flagellar system, in the vgu operon mutant can explain the deficiency in motility that we observed on motility plates. Previous studies, however, have indicated that FlhD and HexA activity are antagonistic (Chatterjee et al. 2009). This begs an explanation for how both could be downregulated simultaneously in our vgu operon mutant. The answer may lie in unresolved environmental factors that can putatively influence the expression of both flhD and hexA. For example, flhD is regulated by FliT, which is responsive to unknown environmental factors (Chatterjee et al. 2009). Similarly, hexA expression is also mediated by unknown environmental factors through an unknown mechanism (Chatterjee et al. 2009). We propose a model (Fig. 6B) for the influence of the vgu operon and inferred glucanate catabolic intermediates on these key regulators during tuber infection that correlates the observed phenotypes, expression data, and published regulatory network information (Barnard et al. 2007; Cui et al. 2008).

Although it is surprising that the vgu operon mutant is attenuated for virulence in the leaf while being hypervirulent in the tuber, the two in planta environments are very different. The tuber environment is anaerobic and contains a high level of starch. Thus, it is a glucose-rich environment. The apoplastic spaces of the leaf are aerobic and nutrient-poor (Rico and Preston 2008). Nutrient availability and other environmental factors have dramatic effects on virulence. For instance, hrpN is regulated by the alternative sigma factor HrpL, in addition to KdgR. HrpL is activated by two-component system, HrpX/Y, that is responsive to unknown exogenous metabolites. In tubers, HrpL is not upregulated by HrpX/Y and, thus, does not activate hrpN in either the wild-type WPP14 or the vgu operon mutant.

Conclusion and outlook.

The life history of P. carotovorum, encompassing saprophytic environmental microbe to opportunistic phytopathogen, demands the ability to sense environmental shifts and to respond accordingly. Despite existing data on the network of regulators that control expression of virulence in P. carotovorum, it is still unclear what environmental signals are monitored to determine virulence expression. Here, we identified an operon that encodes enzymes necessary for glucanate metabolism that acts as a cue for virulence regulation. Our model for how the actions of

**Fig. 6.** Hypothetical model of the vgu operon’s role in virulence. KdgR blocks transcription of macerating enzymes and hrpN in the absence of pectin breakdown products. In the presence of only pectin, KdgR repressor activity is blocked and its target genes, such as hrpN, can be expressed. In the vgu mutant, which does not metabolize glucanate, we see overexpression of the KdgR target gene, hrpN. In the presence of glucanate, KdgR is not blocked by the presence of pectin and KdgR target genes are not expressed. hrpN is also under the control of HrpL, which is responsive to the two-component signal transduction system, HrpX/Y. Glucanate metabolism also regulates FlhDC and HexA through unknown mechanisms. FlhDC is known to regulate motility and HexA regulates rsmB and plant cell-wall-degrading enzymes.
these enzymes affect the expression of virulence regulators favors the straightforward hypothesis that catabolic intermediates are, themselves, cues. However, competing hypotheses include more interplay or antagonism between metabolic pathways and differences in metabolic fluxes that can influence virulence expression. There is evidence to support these hypotheses, including the role of FlhDC in E. coli in the switch from aerobic to anaerobic respiration (Pruss et al. 2003; Leatham et al. 2005) and the role of glucanate to interfere with KDG binding to KdgR. However, more work is needed to elucidate the role of nutrient availability and subsequent metabolic network fluxes on virulence expression and the mechanisms by which these cues act.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains were maintained in lysogeny broth (LB) agar or 2× YT broth, containing appropriate antibiotics (Sambrook and Russell 2001). The minimal media for hrpL repression was described by Chang and associates (2005). Tuber extract media was made by autoclaving 200 g of chopped Yukon gold potatoes with 200 ml of water. After autoclaving, the water-potato slurry was centrifuged at 12,000 × g for 30 min. The supernatant was diluted 1:5 in M9 minimal media lacking a carbon source. When required, antibiotics and drugs were supplemented at the following concentrations: ampicillin (Amp), 100 µg/ml; chloramphenicol (Cm), 30 µg/ml; gentamicin, 25 µg/ml; kanamycin (Kan), 30 µg/ml; rifampicin, 100 µg/ml; spectinomycin, 50 µg/ml; and tetracycline, 5 µg/ml. Media was solidified with 1.5% (wt/vol) Bacto agar (BD Biosciences, San Jose, CA, U.S.A.). Cultures were grown at 28°C and, if in liquid, shaken at 250 rpm.

DNA manipulations.

Standard procedures were used for plasmid and chromosomal DNA isolation, electroporation, restriction endonuclease digestions, ligations, gel electrophoresis, and triparental mating. Enzymes were obtained from either New England Biolabs (Beverly, MA, U.S.A.) or Invitrogen (Carlsbad, CA, U.S.A.). Nucleotide sequences were determined by the University of North Carolina–Chapel Hill Genome Analysis Facility and sequences were analyzed using BLAST (Altschul et al. 1990).

Construction of the P. carotovorum vgu operon mutant.

To replace the vgu operon, we used splicing overlap extension (SOE) PCR (Horton et al. 1989) to create a construct with a Cm cassette flanked by 1 kb of sequence surrounding vguB on each side. The vguB reading frame overlaps the downstream and upstream genes, vguA and vguC, respectively, by 4 bp each (Fig. 1). Briefly, a 1-kb region upstream of vguB was amplified using SOE primers containing a SalI site for the insertion of a Cm cassette on the 3′ end, and a 1-kb downstream region was amplified similarly with the SalI site on the 5′ end with SOE primers (Supplementary Table S1, primers 1 and 2). These amplicons were then fused together in a two-step SOE with SOE primers (Supplementary Table S1, primers 1 and 2).

The four-gene operon and a 500-bp region encompassing the operon promoter region were amplified with pfx polymerase (Invitrogen) with primers 5 and 6. Gel electrophoresis with a 1% (wt/vol) agarose (Invitrogen ultrapure agarose) was used to view and isolate the resulting amplicon. Gel extraction buffers and protocols were supplied from Qiagen Gel extraction kit (Qiagen, Basel, Switzerland). The isolated amplicon was then incubated in the presence of Taq polymerase (Invitrogen) and a PCR reaction mixture without primers for 5 min at 70°C in order to add overhanging As on the blunt amplicon. The amplicon was then cloned into pCR2.1 TA cloning vector (Invitrogen TA cloning kit) according to the manufacturer’s protocol. The resulting plasmid was then conjugated into the P. carotovorum vgu operon mutant via triparental mating with E. coli helper strain pRK2013. The resulting complementation strain was isolated on Cm and Kan plates and verified by PCR from vector-borne M13 primers (Invitrogen).

A. thaliana and potato leaf infection assay.

Cultures were grown overnight in 2× YT media, washed twice in 10 mM MgCl₂, and resuspended at 10⁶ CFU/ml in 10 mM MgCl₂. Cultures were hand infiltrated with a needleless 1-ml syringe into the leaves of either 4- to 5-week-old Yukon gold potatoes or 4- to 5-week-old A. thaliana. At subsequent time points, infected leaves were cored with a 6-mm-diameter cork borer and the tissue ground in the presence of 10 mM MgCl₂. The CFU per milliliter of present bacteria was quantified by titration and plating on LB plates with the appropriate antibiotics.

Tuber maceration assay.

Tubers were injected with 10 µl of bacteria resuspended in MgCl₂ at a concentration of 10⁷ CFU/ml. Bacteria were inoculated into 15-mm holes in the tuber, made with a pipette tip. The infected tubers were then placed in a plastic bag, which was sealed and kept at 28°C for 5 days. After 5 days, the soft, macerated tissue surrounding each injection site was carefully scooped out using a metal spatula and weighed. Each experiment contained 10 internal replicates.

Motility assays.

Motility media recipes were described by Rashid and Kornberg (2000). Swimming motility media contained tryptone (10 g/liter), NaCl (5 g/liter), and Bacto-Agar (BD) at 0.3% wt/vol. Swimming motility media contained nutrient broth (8 g/liter) and Bacto-Agar at 0.5% wt/vol. Twitching motility media contained tryptone (10 g/liter), yeast extract (5 g/liter), NaCl (10 g/liter), and Bacto-Agar at 1% wt/vol. Each motility plate contained 50 ml of motility media with appropriate antibiotics. Plates were inoculated with 10⁶ CFU of bacteria and incubated for 18 h at room temperature, at which point the diameter of the colony was measured.

RNA extraction.

From leaves. A. thaliana or potato leaves were infiltrated with wild-type WPP14 cultures at an optical density at 600 nm (OD₆₀₀) of 1.0 (approximately 4 × 10⁸ CFU/ml) using a needleless syringe. At 2.5 h postinfection, 10 leaves were col-
lected, ground in the presence of Qiagen Protect Bacteria, and homogenized over a Qashredder column before RNA was extracted using the RNeasy kit (Qiagen).

**From tuber extract media.** Cultures were grown in rich media overnight, centrifuged, and washed in 10 mM MgCl₂. Bacteria were resuspended at an OD600 of 0.5 (2 x 10⁸ CFU/ml) and grown for 3 h with shaking at 28°C. Bacteria were then harvested in the presence of Protect Bacteria (Qiagen) and RNA was extracted with the RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. However, the protocol was modified to include an RNA precipitation step with LiCl after lysis but before the ethanol precipitation and RNA being bound to the column. This step removed excessive sugars present in the tuber extract media.

**qRT-PCR.**

RNA was reverse transcribed into cDNA using the Ambion RETROscript kit. Briefly, 2 µg of RNA was primed with random decamers and reverse transcribed with the MMLV-RT enzyme. cDNA was then diluted and relative quantities of specific transcripts were determined using SYBR Green RT-PCR reagents. Fluorescence of double-stranded DNA was measured by a DNA Engine, Opticon 2 continuous fluorescence detector (MJ Research, San Francisco) and values were analyzed using Opticon Monitor 3 software (MJ Research). Relative gene expression was determined by normalizing to a control gene for qRT-PCR analysis by (Takle et al. 2007). Significance of differentially expressed genes was determined by an analysis of variance with a Tukey test using Jump v8 software (SAS Institute Inc., Cary, NC, U.S.A.).

**Hydrogen peroxide tolerance assay.**

Overnight cultures were centrifuged, washed with 200 mM MgCl₂, and resuspended at 2 x 10⁸ CFU/ml. Cultures were then incubated for 30 min in hydrogen peroxide at concentrations of 0.1, 1, 10, or 100 mM. Each culture at a given concentration of hydrogen peroxide was done in triplicate. Cultures were then titrated and plated.

**Carbon source utilization assays.**

Biolog GN2 plates, which contain 95 discrete carbon sources, were inoculated with P. carotovorum WPP14, the vgu operon mutant, and the complemented vgu operon mutant. Briefly, cells were grown in LB media at 28°C for 24 h, washed twice in 10 mM MgCl₂, and resuspended in 10 mM MgCl₂ to a final concentration at OD600 of 0.4. Aliquots of 150 µl were added to each well. The plates were sealed and allowed to incubate statically at room temperature for 24 h. Each plate contained a negative control that lacked a carbon source to ensure that metabolic activity of stored carbon reserves was not being measured. In response to respiration, tetrazolium in each well was reduced to produce a distinctive purple color indicating the oxidation of the available carbon source. Carbon utilization was additionally tested by growth in M9 salts and 100 mM of a specific carbon source. Bacteria were grown in liquid 2× YT culture overnight, washed in M9 salts two times, and resuspended at an OD600 of 0.04 in the M9 salts with a carbon source. The culture was then incubated for 18 to 20 h at 28°C, while shaking, and the OD600 was measured again.

**GC-MS.**

Preparation and methods for GC-MS analysis was as described (Koek et al. 2006). Cultures of the wild type and the vgu operon mutant were grown from single colonies in LB media overnight. The next day, overnight cultures were used to inoculate 100 ml of potato extract media and grown to an OD600 of 0.4 in approximately 4 h with shaking at 250 rpm at 28°C. Cellular metabolism was immediately quenched by the addition of 160 ml of methanol quenching buffer at −45°C to 40 ml of culture. Intracellular metabolites were extracted with chloroform as described by Ruijter and Visser (1996). At −45°C, chloroform was added to the methanol-water mixture to break the cell walls and denature enzymes. The water-methanol phase was then lyophilized and derivatized with 10 µl of a 56 mg/ml ethoxylamine hydrochloride solution in pyridine and 20 µl of pyridine. The samples were then incubated at 40°C for 90 min. The samples were then silylated for 50 min at 40°C with 70 µl of N-methyl-N-trimethylsilyl trifluoroacetamide. The derivatized extracts were prepared for GC-MS analysis by the addition of ethyl acetate as a carrier and filtering in the presence of acetyl nitrile to ensure the absence of metal ions. Samples were analyzed with an Agilent 6850 gas chromatograph coupled to an Agilent 5973 mass selective detector. The 5-µl aliquots of extract were injected into a capillary column (30 m × .25 mm i.d., 0.25-mm film thickness) at 250°C. The initial temperature of the gas chromatograph was 50°C and held for 3 min before ramping to 250°C at 10°C/min. Helium was used as a carrier gas. Detection was achieved using MS detection in electron impact mode and full scan monitoring mode (m/z 10 to 550). Data was analyzed using ChemStation (Enhanced Software, Mumbai, India).

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**LITERATURE CITED**


