Ralstonia solanacearum Depends on Catabolism of Myo-Inositol, Sucrose, and Trehalose for Virulence in an Infection Stage–Dependent Manner

Corri D. Hamilton,¹ Olivia R. Steidl,¹ April M. MacIntyre,^{1,2} Connor G. Hendrich,^{1,2} and Caitilyn Allen^{1,†}

¹ Department of Plant Pathology University of Wisconsin-Madison, 1630 Linden Drive, Madison, WI 53706, U.S.A.

² Microbiology Doctoral Training Program, University of Wisconsin-Madison, 1630 Linden Drive, Madison, WI 53706, U.S.A.

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The soilborne pathogen *Ralstonia solanacearum* causes a lethal bacterial wilt disease of tomato and many other crops by infecting host roots, then colonizing the water-transporting xylem vessels. Tomato xylem sap is nutritionally limiting but it does contain some carbon sources, including sucrose, trehalose, and myo-inositol. Transcriptomic analyses revealed that *R. solanacearum* expresses distinct catabolic pathways at low cell density (LCD) and high cell density (HCD). To investigate the links between bacterial catabolism, infection stage, and virulence, we measured in planta fitness of bacterial mutants lacking specific carbon catabolic pathways expressed at either LCD or HCD. We hypothesized that early in disease, during root infection, the bacterium depends on carbon sources catabolized at LCD, while HCD carbon sources are only required later in disease during stem colonization. A *R. solanacearum* $\Delta iolG$ mutant unable to use the LCD-catabolized nutrient myo-inositol was defective in tomato root colonization, but after it reached the stem this strain colonized and caused symptoms as well as wild type. In contrast, *R. solanacearum* mutants unable to use the HCD-catabolized nutrients sucrose ($\Delta scrA$), trehalose ($\Delta treA$), or both ($\Delta scrA/treA$), infected roots as well as wild-type *R. solanacearum* but were defective in colonization and competitive fitness in midstems and had reduced virulence. Further, xylem sap from tomato plants colonized by $\Delta scrA$, $\Delta treA$, or $\Delta scrA/treA$ *R. solanacearum* mutants contained twice as much sucrose as sap from plants colonized by wild-type *R. solanacearum*. Together, these findings suggest that quorum sensing specifically adapts *R. solanacearum* metabolism for success in the different nutritional environments of plant roots and xylem sap.

Keywords: bacterial catabolism, bacterial wilt disease, metabolomics, metabolic trade-offs, myo-inositol, sucrose, trehalose, xylem sap

Microbial success hinges on the ability to reduce the organic compounds available in a specific habitat. Microbes occupying niches with diverse but scarce nutrients need a broad set of catabolic pathways, but operating many such pathways imposes a cost (Dekel and Alon 2005; Fatima and Senthil-Kumar 2015; Gudelj et al. 2007; Pfeiffer and Bonhoeffer 2004; Polz and Cordero et al. 2016; Siebrecht et al. 2003). Sensing and signaling mechanisms help microbes make informed choices about when to express a given pathway to balance the energy cost of synthesizing transporters and enzymes with the energy gained from consuming the corresponding carbon sources (Wadhams and Armitage 2004).

The plant products myo-inositol, sucrose, and trehalose are potential energy sources for plant-associated microbes. Myo-inositol, a sugar alcohol, is often found in high quantities in planta. It is common in root exudates, including those of tomato; these exudates are the first plant nutrient interface for soil-borne microbes (Kravchenko et al. 2003; Wang and Bergeson 1974; Wei et al. 2015). Myo-inositol plays a vital role as the core of many signaling molecules and membrane and cell-wall structural components, and it is the most abundant carbon source in root nodules of the *Bradyrhizobium diazoefficiens*–pea mutualism (Sköt and Egsgaard 1984; Vélchez et al. 2020). Sucrose is the major product of photosynthesis. It is essential for plant growth and development, and plants continuously shuttle sucrose from source to sink (Pollock and Farrar 1996; Salanoubat and Belliard 1989; Sonnewald and Willmitzer 1992). The disaccharide trehalose, much less abundant than sucrose, plays key roles in plant metabolic regulation and stress tolerance (Lunn et al. 2014; Paul et al. 2008). Trehalose is a major contributor to tolerance of drought and temperature stress and plants use trehalose 6-phosphate signaling to balance their sucrose-to-starch storage ratio (Kolbe et al. 2005).

Ralstonia solanacearum is part of a heterogeneous species complex of plant pathogenic β -proteobacteria that cause bacterial wilt diseases (Allen et al. 2005; Elphinstone 2005; Genin and Denny 2012; Prior et al. 2016). Bacterial wilt affects over 100 economic crops, including tomato, potato, banana, tobacco, peanut, ginger, cloves, and eggplant, reducing yields for subsistence and commercial growers worldwide (Allen et al. 2005).

R. solanacearum cycles between two very different nutritional environments, rhizosphere soil and host xylem sap. The rhizosphere teems with diverse microbes and carbon sources (Yang and Crowley 2000). *R. solanacearum* is well adapted to this

[†]Corresponding author: C. Allen; callen@wisc.edu

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complex environment, with more than 50 catabolic pathways to use a wide variety of resources, although pathway repertoires vary somewhat within the species complex (Álvarez et al. 2010; Jacobs et al. 2012; Zuluaga et al. 2013). *R. solanacearum* is found at relatively low titers in bulk soil, reaching only 10⁴ CFU per gram in the most heavily infested fields (Allen et al. 2005; Hayward 1991).

Soil-dwelling *R. solanacearum* cells use chemotaxis to locate and infect tomato roots, moving to the developing vascular bundles and spreading systemically through the water transporting xylem (Caldwell et al. 2017; McGarvey et al. 1999). Xylem vessels are dead and xylem sap is described as a nutrient-poor solution of water and minerals (Evert and Eichhorn 2006). Nonetheless, *R. solanacearum* rapidly grows to cell densities >10⁹ CFU per milliliter of xylem sap, eventually forming a bio-film matrix that can fill entire vessels and obstruct water flow (Denny 2006; Yao and Allen 2007). These high bacterial populations coincide with the onset of symptoms, which include stunting, leaf wilting, vascular discoloration, and death.

Little is understood about how *R. solanacearum* grows to such high cell densities in xylem sap. Xylem sap from healthy tomato plants contains enough sugars, amino acids, and organic acids to support only limited bacterial growth in vitro (Coplin et al. 1974; Dixon and Pegg 1972; Evert and Eichhorn 2006; Fatima and Senthil-Kumar 2015; Wang and Bergeson 1974; White et al. 1981). Interestingly, xylem sap from *R. solanacearum*-infected plants is enriched in certain metabolites, including carbon sources that can support *R. solanacearum* growth, such as sucrose and trehalose (Lowe-Power et al. 2018a). This metabolomic finding, together with the observation that *R. solanacearum* grows better in sap harvested from infected plants than in sap from healthy plants, suggests that the pathogen somehow increases available nutrients in xylem vessels.

When *R. solanacearum* grows to high cell densities (HCD) in culture or in the confined environment of the xylem vessel, the PhcA quorum-sensing (QS) system upregulates expression of secreted bacterial wilt virulence factors, like extracellular polymeric substances (EPS) and plant cell wall-degrading enzymes. Studies with a $\Delta phcA$ mutant, which is genetically locked in a low cell density (LCD) state, revealed that this regulator also represses expression of dozens of transporters and catabolic pathways at HCD (Khokhani et al. 2017; Peyraud et al. 2016). In this way, *R. solanacearum* has evolved a metabolic trade-off between maximizing growth by using all available nutrients and maximizing pathogenic fitness by producing costly secreted virulence factors (Polz and Cordero 2016). In the context of the bacterial wilt disease cycle, this suggests that *R. solanacearum* uses a broad set of nutrients when it grows in the rhizosphere and during early root infection, but once the bacterium grows to large population sizes in xylem vessels, it shifts to catabolizing a much narrower set of compounds. These observations led us to hypothesize that *R. solanacearum* is adapted to use specific carbon sources at different points in its life history.

Here, we elucidate the relationships between *R. solanacearum* catabolism, infection stage, and ability to colonize and cause disease on tomato. We found that bacterial mutants lacking the ability to consume myo-inositol, sucrose, and trehalose had reduced fitness in an infection stage-specific manner. In particular, a LCD-induced gene encoding a myo-inositol-catabolizing enzyme was required only for infection, root endosphere colonization, and competitive fitness in roots very early in wilt disease development. In contrast, the HCD-induced genes encoding degradation of sucrose and trehalose were required for colonization and competitive fitness in the stem and for full virulence. Although sucrose is an important carbon source for *R. solanacearum* in the xylem, we found that the much less abundant sugar trehalose is not an important energy source for the bacterium in planta. However, trehalose degradation does contribute to *R. solanacearum* virulence by another mechanism.

RESULTS

The catabolic genes *iolG*, *scrA*, and *treA* are expressed during tomato infection and are regulated by the PhcA QS system.

R. solanacearum GMI1000 can grow on myo-inositol, sucrose, and trehalose as sole carbon sources, and all three metabolites are present in tomato xylem sap and root exudates (Lowe-Power et al. 2018a; Wang and Bergeson 1974; Wei et al. 2015). Infecting tomato plants with *R. solanacearum* increases levels in xylem sap of sucrose and myo-inositol less than two-fold, but trehalose concentrations are 19-fold higher in *R. solanacearum*-infected sap than in sap from healthy plants (Lowe-Power et al. 2018a). To determine the roles of these three carbon sources in the *R. solanacearum* life cycle, we mutated *iolG*, which encodes an oxidoreductase that is the first step in catabolism of myo-inositol, *scrA*, which encodes a sucrose-specific transport protein, and *treA*, which encodes an alpha-trehalase (Kanehisa and Goto 2000). As summarized in Table 1, during growth in tomato plants, *R. solanacearum* GMI1000 expresses *iolG*, *scrA*, and *treA* at the moderately high absolute expression levels of 10.15, 11.08, and 12.53, respectively (Jacobs et al. 2012). All three genes are regulated by the cell density-responsive PhcA QS system. While *iolG* is up-regulated in a *phcA* mutant, which is genetically locked in a LCD state, *treA* and *scrA* are both down-regulated in the $\Delta phcA$ strain (Khokhani et al. 2017) (Table 1).

To determine the roles of these three carbon sources across the *R. solanacearum* infection process, we created *R. solanacearum* mutants, $\Delta iolG$, $\Delta scrA$, and $\Delta treA$, lacking the three targeted catabolism pathways, respectively, and confirmed their phenotypes by measuring mutant growth on glucose, myo-inositol, sucrose, and trehalose as sole carbon sources (Supplementary Table S1). The $\Delta iolG$, $\Delta scrA$, $\Delta treA$, and $\Delta scrA/treA$ double mutant all grew as well as wild-type strain GMI1000 on glucose. As expected, $\Delta iolG$, $\Delta scrA$, $\Delta treA$, and $\Delta scrA/treA$ differed from wild type only

Table 1. *Ralstonia solanacearum* catabolic genes and metabolites of interest

Carbon source	Fold change (infected/healthy) ^a	Gene	Absolute expression ^b	In planta LogFC ^c	Gene annotation ^d	Locus tag
Myo-inositol	1.95	<i>iolG</i>	10.15	2.08	Oxidoreductase 2-dehydrogenase signal peptide protein	RSc1246
Sucrose	1.29	<i>scrA</i>	11.08	-0.54	Sucrose-specific transmembrane import protein	RSc1285
Trehalose	19.39	<i>treA</i>	12.53	-2.92	Alpha-trehalase signal peptide protein	RSp 0277

^a Values represent the relative metabolite level fold change in ex vivo xylem sap from wilt-susceptible tomato cultivar Bonny Best plants that were either healthy or infected with *R. solanacearum* (Lowe-Power et al. 2018a).

^b In planta gene absolute expression values were based on four biological replicates, normalized using the Robust Multichip Algorithm (Jacobs et al. 2012).

^c In planta gene expression logFC (fold change) values in a *R. solanacearum* $\Delta phcA$ mutant strain relative to wild-type strain GMI1000 (Denny and Hayward 2001; Khokhani et al. 2017).

^d Gene annotations based on NCBI reference sequences NC_003295.1 and NC_003296.1.

in growth on their respective targeted carbon source or sources. Complementing each mutation with a copy of the wild-type gene restored strain growth on the corresponding carbon source (Supplementary Table S1). These functional experiments confirmed the gene annotations of *iolG*, *scrA*, and *treA* and showed that the mutants could not use the corresponding sugars.

Myo-inositol catabolism is required for colonization and competitive fitness early in tomato root infection.

By 24 h after they reach a host root surface, *R. solanacearum* cells are multiplying in root cortical tissue and beginning to colonize root vascular bundles, but many have not yet reached the PhcA QS threshold of 1×10^7 CFU/ml; this timepoint thus represents early disease (Caldwell et al. 2017; McGarvey et al. 1999). By 72 h postinoculation (hpi), many vascular elements are densely colonized and most *R. solanacearum* populations have transitioned into the HCD gene expression pattern, corresponding to the later stage of disease. Because *R. solanacearum* upregulates the myo-inositol pathway at LCDs, we hypothesized that consuming this sugar increases bacterial success at the earliest step in the infection process. We tested this by placing wild type, $\Delta phcA$, $\Delta scrA$, $\Delta treA$, or $\Delta iolG$ cells onto the root surface of tomato seedlings, allowing infection to proceed for 24 or 72 h and then quantifying *R. solanacearum* population sizes inside surface-sterilized roots in the root endosphere (Fig. 1). As expected, very early in root infection the LCD-locked $\Delta phcA$ mutant was more successful than wild type but by 72 hpi, $\Delta phcA$ populations in the root endosphere were smaller than those of the wild-type strain. Because it can use a wider range of nutrients and does not synthesize metabolically costly virulence factors, the $\Delta phcA$ mutant grows faster in the earliest stages of plant infection (Khokhani et al. 2017; Peyraud et al. 2016). In contrast, subpopulations of wild-type cells experience intermittent HCDs and associated QS-mediated regulation that slows their growth as they fill xylem vessels and cortical tissue. However, the $\Delta phcA$ growth advantage in roots is short-lived, likely because virulence factor production is critical for success

in planta. The $\Delta scrA$, $\Delta treA$, and the $\Delta scrA/treA$ double mutant reached population sizes similar to those of wild type, suggesting that *R. solanacearum* success during the first 24 h of root infection does not depend on access to sucrose or trehalose. However, at 24 hpi the *iolG* mutant was 92-fold reduced in root colonization compared with wild type, indicating that myo-inositol consumption is important early in the root colonization process. In contrast, mutants unable to use either or both sucrose and trehalose reached smaller population sizes in seedling roots 72 hpi (Fig. 1).

To further quantify the contribution of specific sugars to *R. solanacearum* success early in root infection, we coinoculated roots with wild type and mutant strains to determine if the catabolic mutants were reduced in competitive fitness. Tomato seedling roots were surface-inoculated, as described above, with a 1:1 mixture of wild-type and either $\Delta scrA$, $\Delta treA$, or $\Delta iolG$ and the population sizes of each strain inside root endospheres were quantified at 24 and 72 hpi (Table 2). The $\Delta iolG$ mutant was out-competed by the wild-type strain by 87-fold in roots 24 hpi. At 72 hpi, there were no differences among strains in competitive fitness (Table 2). Together, these results indicate that the ability to use myo-inositol helps *R. solanacearum* multiply very early in plant infection while consuming sucrose and trehalose increases bacterial success only after the pathogen has reached high densities later in root colonization.

Sucrose catabolism is required for colonization and competitive fitness in tomato stems.

After *R. solanacearum* cells colonize root xylem vessels they spread rapidly up into the host stem. At this point in bacterial wilt disease development, most pathogen cells are in subpopulations that have surpassed the PhcA QS threshold of 1×10^7 CFU/ml (Genin and Denny 2012). Xylem sap metabolomics and in-plant transcriptomics suggest that trehalose and sucrose could be key carbon sources for *R. solanacearum* cells multiplying in tomato stems. To directly measure the contribution of specific xylem carbon sources to bacterial

Fig. 1. A *Ralstonia solanacearum* (*Rs*) mutant unable to catabolize myo-inositol colonized the root endosphere poorly at 24 h postinoculation (hpi), while mutants unable to use sucrose or trehalose showed defects only at 72 hpi. The roots of 4-day-old unwounded tomato seedlings were inoculated with 2,000 cells of *R. solanacearum* $\Delta phcA$ (yellow symbols), wild-type GM11000 (black), $\Delta iolG$ (green), $\Delta scrA$ (blue), $\Delta treA$ (red), or $\Delta scrA/treA$ (magenta). Internal bacterial population sizes were quantified by serial dilution plating of ground surface-sterilized roots harvested 24 or 72 hpi. Population size is shown as CFU + 1 to account for zero in the $\log_{10}$ scale. Each symbol represents the population size in one two-root sample; data shown represent three to six biological replicates. The number under each data column indicates the number of samples that did not contain any detectable bacteria out of the total sample number. Horizontal black lines represent the geometric mean population size of each strain. The gray dotted line indicates the assay limit of detection of 1×10^2 CFU per gram. Asterisks indicate differences from wild-type population size at the same timepoint; one asterisk (*) indicates $P < 0.001$, two (**) $P < 0.0001$, and three (***) $P < 0.00001$, Mann-Whitney test.

success at this disease stage, we compared the population sizes in tomato midstems of wild type, $\Delta scrA$, $\Delta treA$, and $\Delta iolG$ *R. solanacearum* strains 5 days after soil soak inoculation. The population sizes of the $\Delta iolG$ and $\Delta treA$ catabolic mutants were indistinguishable from those of the wild-type strain. However, neither the $\Delta scrA$ nor $\Delta scrA/treA$ mutant colonized stems as well as the wild-type strain (Fig. 2). Interestingly, the population size of the $\Delta scrA/treA$ double

mutant was significantly lower than that of the $\Delta scrA$ single mutant ($P < 0.001$, Mann-Whitney test). Together, these results suggest that sucrose consumption contributes quantitatively to *R. solanacearum* multiplication in stems but that the pathogen does not need to consume either myo-inositol or trehalose to reach wild-type population sizes at this disease stage. To more precisely explore the contributions of these sugars to bacterial success in tomato midstems, we forced the catabolic

Table 2. Competitive fitness of *Ralstonia solanacearum* catabolic mutants during tomato root endosphere colonization

Time postinoculation	Competing strains	Strain population size ^a	Competitive index ^b	P value ^c
24 h	Wild type	4.50×10^5	0.2956	0.0171*
	$\Delta iolG$	5.21×10^2		
	Wild type	4.88×10^5	1.91784	0.1512
	$\Delta scrA$	1.00×10^6		
	Wild type	1.11×10^7	1.93507	0.5252
	$\Delta treA$	1.61×10^8		
72 h	Wild type	4.34×10^6	0.81981	0.5954
	$\Delta scrA/treA$	7.39×10^6		
	Wild type	1.62×10^6	1.482	0.2017
	$\Delta iolG$	1.27×10^7		
	Wild type	2.75×10^8	0.86104	0.8319
	$\Delta scrA$	3.08×10^8		
	Wild type	3.30×10^7	1.00095	0.6379
	$\Delta treA$	5.78×10^6		
	Wild type	6.98×10^8	0.79637	0.8296
	$\Delta scrA/treA$	5.72×10^8		

^a Bacterial population sizes determined by serial dilution plating of surface-sterilized ground seedling roots, shown as geometric mean of CFU per gram of root.
^b Competitive index values represent the proportional competitive advantage of the competing strain over the wild-type GMI1000 reference strain. A competitive index of 1 indicates no competitive advantage between strains.
^c P values were determined by paired T-test. Population sizes are the geometric means of four biological replicate experiments, each containing 30 to 60 tomato seedling roots.

Fig. 2. Sucrose catabolism helps *Ralstonia solanacearum* (*Rs*) colonize tomato stems. Unwounded 17-day-old tomato plants were inoculated by soaking the soil with a 5×10^7 CFU/ml suspension of wild-type *R. solanacearum* (black), $\Delta iolG$ (green), $\Delta scrA$ (blue), $\Delta treA$ (red), or $\Delta scrA/treA$ (magenta). After 5 days, the bacterial population size in each plant was determined by dilution plating a ground stem section harvested just above the cotyledon. Population size is displayed as CFU + 1 to account for zeroes in the $\log_{10}$ scale. Each symbol represents the population size in one plant. Data represent three to six independent experiments. The number under each data column indicates the number of samples that did not contain any detectable bacteria to the total sample number. Horizontal black lines represent the geometric mean of each strain's population size. The gray dotted line indicates the assay limit of detection of 1×10^2 CFU per gram. Asterisks indicate population sizes different from the wild type (one asterisk [*] $P < 0.05$, four [****] $P < 0.0001$, Mann-Whitney test).

mutants to directly compete with the wild-type strain by co-inoculating tomato plants through a cut leaf petiole with a 1:1 mixture of wild-type GMI1000 and either $\Delta scrA$, $\Delta treA$, or $\Delta iolG$. This method bypasses roots by introducing the bacteria directly into stem xylem vessels. At 5 days after inoculation, the $\Delta scrA$, $\Delta treA$, and $\Delta scrA/treA$ catabolic mutants were out-competed by wild-type GMI1000 (Table 3). In contrast, loss of myo-inositol catabolizing ability did not affect competitive fitness under these conditions. This indicates that the ability to catabolize xylem sucrose provides *R. solanacearum* with a significant fitness advantage during stem infection and, also, that ability to degrade trehalose increases pathogen competitive fitness at midstage disease.

Sucrose and trehalose catabolism contribute additively to bacterial wilt virulence.

To assess the contributions of myo-inositol, sucrose, and trehalose across the entire bacterial wilt disease process, we measured virulence of the four catabolic mutants following a naturalistic soil-soak inoculation of tomato plants. Consistent with the finding that, by 3 days postinoculation (dpi), the $\Delta iolG$ mutant colonized roots as well as wild type, in this holistic assay, $\Delta iolG$ was as virulent as its wild-type parent (Fig. 3). However, the $\Delta scrA$ and $\Delta treA$ single mutants were each defective in virulence compared with wild type. Further, deleting both *scrA* and *treA* reduced virulence more than mutating either gene alone. This suggests that catabolism of sucrose and of trehalose contribute additively to *R. solanacearum* virulence.

Ex vivo xylem sap from $\Delta scrA$ -infected plants supports more bacterial growth.

When catabolic *R. solanacearum* mutants colonize plants, they would be expected to leave unconsumed nutrients in xylem sap. We tested this hypothesis by culturing bacteria in 50 μ l of

Table 3. Competitive fitness of *Ralstonia solanacearum* catabolic mutants during colonization of tomato stems following cut petiole inoculation

Competing strains	Strain population size ^a	Competitive index ^b	P value ^c
Wild type	3.28×10^7	0.9483	0.3274
$\Delta iolG$	4.79×10^7		
Wild type	4.10×10^9	0.5676	0.0002
$\Delta scrA$	7.65×10^8		
Wild type	1.09×10^9	0.4231	0.0009
$\Delta treA$	4.50×10^8		
Wild type	3.47×10^5	0.5000	0.0123
$\Delta scrA/treA$	2.78×10^5		

^a Bacterial population sizes determined by serial dilution plating of ground stems, shown as geometric mean of CFU per gram of stem.

^b Competitive index values represent the proportional competitive advantage of the competing strain over the wild-type GMI1000 reference strain. A competitive index of 1 indicates no competitive advantage between strains.

^c P values were determined by paired T-test. Population sizes are the geometric means of three ($\Delta iolG$) or four ($\Delta scrA$, $\Delta treA$, and $\Delta scrA/treA$) biological replicate experiments, each containing midstem sections from 30 to 60 tomato plants.

Fig. 3. *Ralstonia solanacearum* needs catabolism of sucrose and trehalose for full bacterial wilt virulence. Unwounded 17-day-old tomato plants were inoculated by pouring a dilute bacterial suspension into the pot to a final pathogen concentration of 5×10^7 CFU per gram of potting mix. Plants were incubated in a 28°C growth chamber and symptoms were rated daily on a 0 to 4 disease index. Data shown are the means of four independent experiments, each containing 15 plants per treatment, except the wild-type data, which reflect eight independent experiments. The $\Delta treA$, $\Delta scrA$, and $\Delta scrA/treA$ mutants were less virulent than wild-type *R. solanacearum* ($P < 0.002$, repeated measures analysis of variance [ANOVA]). Virulence of the $\Delta iolG$ catabolic mutant was not different from that of wild-type strain GMI1000 ($P = 0.306$, repeated measures ANOVA). Error bars show standard error of the mean disease index reading for each day.

filter-sterilized xylem sap harvested from plants that had been infected 5 days earlier with either wild type or mutant *R. solanacearum* strains. The ex vivo xylem sap was prepared by pooling harvested sap normalized to host plant colonization levels of 5×10^6 CFU per gram. Wild type, $\Delta iolG$, $\Delta scrA$, $\Delta treA$, and $\Delta scrA/treA$ strains all grew equally well on sap from plants previously infected with wild type, $\Delta iolG$, or $\Delta treA$ bacteria (Fig. 4). However, all *R. solanacearum* strains grew better on sap from plants infected with $\Delta scrA$ or $\Delta scrA/treA$ mutants, which cannot catabolize sucrose. This indicates that the increased bacterial growth was fueled by sucrose, which was present at around 270 μ M in sap from wild type-infected plants (Fig. 5). Interestingly, sap from plants infected with the $\Delta scrA/treA$ double mutant supported more *R. solanacearum* growth than sap from plants infected with $\Delta scrA$ or $\Delta treA$ single mutants. This increased growth might have been fueled by catabolism of trehalose. However, direct measurement of trehalose showed that xylem sap from wild type-infected plants contained less than 100 nM trehalose, which was the detection limit of the assay. *R. solanacearum* GMI1000 can grow on trehalose as a sole carbon source only at concentrations above 1 mM (Supplementary Table S2). Thus, the concentration of trehalose in tomato xylem sap was too low to support bacterial growth, so catabolism of trehalose as a carbon source cannot explain the additive growth defect in the $\Delta scrA/treA$ double mutant.

To directly test the hypothesis that sucrose-nonmetabolizing *R. solanacearum* mutants leave this nutrient unconsumed when they infect plants, we measured sucrose concentrations in ex vivo xylem sap harvested from plants infected by various

strains. Sap from plants infected with wild type or $\Delta iolG$ contained similar levels of sucrose, around 270 μ M, while sap from plants infected with $\Delta scrA$ or $\Delta scrA/treA$ contained 43% more sucrose than sap from wild type-infected plants (Fig. 5). Unexpectedly, sap from plants infected with the $\Delta treA$ single mutant also contained higher sucrose levels than sap from wild-type plants. Although sap from plants previously infected by $\Delta scrA/treA$ supported the best growth of wild-type *R. solanacearum* (Fig. 4), bacterial growth in sap from plants previously infected by $\Delta scrA$ was the most different from growth in sap from plants infected with wild-type *R. solanacearum*. This suggests that, while an increase in sucrose is responsible for some increased growth in ex vivo sap, the additive growth effect of the $\Delta treA$ and $\Delta scrA$ deletions is not fully explained by increase in sucrose concentration. Together, these two experiments suggest that sucrose is a key nutrient supporting *R. solanacearum* growth in xylem, while trehalose improves the xylem sap environment for *R. solanacearum* via a nonnutritive mechanism.

DISCUSSION

Whole-genome expression studies indicated that *R. solanacearum* uses density-dependent QS to mediate a tradeoff from a broad metabolic capacity at LCD to a much narrower catabolic menu at HCD (Khokhani et al. 2017; Peyraud et al. 2016). This shift parallels the transition between early- and late-stage plant infections. Comparing the growth of *R. solanacearum* and a QS mutant on many substrates revealed the counterintuitive fact that many nutrients are not accessible to *R. solanacearum* cells once

Fig. 4. *Ralstonia solanacearum* strains able to use sucrose grew better on ex vivo xylem sap from plants infected with sucrose nonmetabolizing mutants. Growth over 36 h of various *R. solanacearum* strains in filter-sterilized xylem sap, from plants previously infected by either wild-type *R. solanacearum* (black), $\Delta iolG$ (green), $\Delta scrA$ (blue), $\Delta treA$ (red), or $\Delta scrA/treA$ (magenta) strains, was measured using a plate reader and is shown as area under the growth curve. This experiment was replicated three to six times, with each experiment containing three technical replicates per treatment. Error bars indicate the standard error of the mean growth value for all nine to 18 independent growth curves. Asterisks indicate difference from growth of the wild-type strain on the same xylem sap source; one asterisk (*) indicates $P < 0.05$, two (**) $P < 0.01$, Mann-Whitney test. A trending P value of $P = 0.062$ is shown for wild-type cells growing in sap from $\Delta scrA$ -inoculated plants.

they reach HCD, including nutrients like myo-inositol that are known to be available in planta. If a regulatory circuit forces the bacterium to forgo available food sources, *R. solanacearum* must benefit from restricting its metabolic capacity to only a subset of nutrients, possibly by avoiding the cost of synthesizing many catabolic proteins. We experimentally tested this model and found that an $\Delta iolG$ mutant, which cannot use myo-inositol, is reduced in root endosphere colonization but recovers once bacteria reach HCD and $\Delta iolG$ ultimately can cause disease as well as wild type. This shows that myo-inositol is a key nutrient early in infection when *R. solanacearum* has access to its full metabolic capacity, but once the bacteria reach HCD, they do not need myo-inositol to succeed even though it is present in xylem sap.

Myo-inositol catabolism is not the only pathway that *R. solanacearum* represses at HCD. Transcriptome profiles of *R. solanacearum* $\Delta phcA$ mutants, which are genetically locked in LCD mode, indicated that wild-type *R. solanacearum* had lower expression of 255 genes, including many catabolic and transporter genes (Khokhani et al. 2017; Perrier et al. 2018). These genes encode uptake and breakdown of core metabolites like sugars, amino acids, and fatty acids but, also, secondary compounds like hydroxycinnamic acid and galacturonic acid. Some of these metabolites are available to *R. solanacearum* in the xylem. As we observed for myo-inositol, the ability to degrade hydroxycinnamic acid contributes to early root colonization by *R. solanacearum* but not to bacterial fitness in the stem (Lowe et al. 2015). However, this contribution may be explained by counter-defense rather than nutrition. Although some *R. solanacearum* strains can use hydroxycinnamic acids as a sole carbon source, they are also toxic to *R. solanacearum* at high

concentrations (Lowe-Power et al. 2016). Another potential nutrient source is provided by degradation of cell-wall components like pectin, a polymeric matrix of primarily galacturonic acid (Abbott and Boraston 2008). *R. solanacearum* degrades this complex and abundant polymer with an array of extracellular enzymes, including a pectin methylesterase and three polygalacturonases (Tans-Kersten et al. 1998). Deleting these enzymes either individually or together only slightly decreased virulence on tomato by either *R. solanacearum* GMI1000 or the phylotype I strain K60 (Gonzales and Allen 2003; Huang and Allen 2000; Liu et al. 2005; Tans-Kersten et al. 1998). However, these studies did not measure effects of pectinase mutations on root colonization. While galacturonic acid is present in xylem sap from *R. solanacearum*-infected tomato, expression of the secreted pectinases and the catabolic pathway that converts galacturonic acid to pyruvate is repressed at HCD in planta and in culture (Khokhani et al. 2017; Lowe-Power et al. 2018a; Mori et al. 2017; Perrier et al. 2018). Infection stage-specific experiments could determine if degrading metabolites like hydroxycinnamic acid and galacturonic acid contribute to *R. solanacearum* virulence early in disease.

R. solanacearum cells living in rhizospheres are likely relatively few and not at quorum density. When these LCD-mode bacteria encounter plant root exudates, they can catabolize many compounds in this rich cocktail. Exudates contain metabolites available to LCD-mode *R. solanacearum*, including sugars like myo-inositol, amino acids, and larger molecules and polymers like fatty acids and glycerophosphoinositide membrane lipids that require myo-inositol as a building block (Bais et al. 2006; Čepulytė et al. 2018; Korenblum et al. 2020; Vílchez et al. 2020; Zhálnina et al. 2018). Plant nucleic acids offer another

Fig. 5. Infection with *Ralstonia solanacearum* catabolic mutants $\Delta scrA$, $\Delta treA$, and $\Delta scrA/treA$ modulates sucrose levels in tomato xylem. Sucrose was quantified in ex vivo xylem sap using the GOD/invertase method (Teixeira et al. 2012). Each plate included 10 to 1,000 μM sucrose standards that were used to construct a calibration curve to determine the sucrose concentration in each sample. Each point represents the mean of triplicate technical replicates; analysis was done on at least three biological replicate experiments. One asterisk (*) indicates $P < 0.01$, two (**) $P < 0.001$, Students paired T-test. There was no significant difference between mutant strains $\Delta treA$ or $\Delta scrA/treA$ when compared with $\Delta scrA$ ($P = 0.23$ and $P = 0.18$, respectively, Students paired T-test).

potential nutrient source in the rhizosphere. DNA secreted from root border cells plays an important role in blocking entry of root pathogens, but *R. solanacearum* secretes DNases to cut through these underground nets (Driouch et al. 2013; Tran et al. 2016). These extracellular DNases facilitate *R. solanacearum* entry and spread within hosts, and they may also release a nutrient source in the rhizosphere. Consistent with this, an LCD-locked $\Delta phcA$ mutant grows better than wild type on guanine and adenine (Khokhani et al. 2017; Tran et al. 2016). We speculate that, like myo-inositol, many carbon and nitrogen sources that *R. solanacearum* cannot metabolize at HCD contribute to *R. solanacearum* success early in disease.

After *R. solanacearum* multiplies in the confined spaces inside plants and reaches quorum cell densities, the bacterium can use a much narrower set of metabolites, notably sucrose (Khokhani et al. 2017). Sucrose is the most common sugar in plant roots and shoots (Aubry et al. 2019). We found that *R. solanacearum* did not need the ability to catabolize sucrose for successful root colonization, but it was critically important later in pathogenesis, as is also true for phylotype II strain *R. solanacearum* UW551 (Jacobs et al. 2012). Relative to wild type, an $\Delta scrA$ mutant was reduced in colonization and competitive fitness in midstems and was less virulent. Even though xylem vessels of infected plants contain very large populations of bacteria avidly consuming sucrose, xylem sucrose levels remained surprisingly high during infection. Sucrose levels were even higher if the infecting strain could not use sucrose. These results suggest that *R. solanacearum* infection somehow causes plants to direct sucrose into xylem vessels, possibly by means of type 3-secreted effectors. It is not uncommon for plant pathogens to manipulate their hosts to acquire nutrients. *Xanthomonas* spp. use TAL effectors to upregulate host sucrose-transporting SWEET genes (Chen et al. 2010; Cohn et al. 2014; Cox et al. 2017). *R. solanacearum* effectors have been shown to directly influence host metabolism of nutrients like GABA and putrescine (Wu et al. 2019; Xian et al. 2019) and a *R. solanacearum* effector may direct host production of trehalose-6-phosphate (Poueymiro et al. 2014).

Alternatively, plants may direct sucrose to the xylem in an attempt to combat water stress and xylem blockage associated with bacterial wilt disease. This phloem-loading hypothesis suggests that plants can repair embolisms (breaks in the xylem vessel water column) by moving solutes like sugars from the phloem into the xylem. These solutes, combined with the action of plant aquaporins, can drive water osmotically into the xylem, refilling the vessel and also enriching it in nutrients (Knipfer et al. 2016; Nardini et al. 2011). While some xylem-dwelling bacterial pathogens like *Xylella fastidiosa* are known to induce embolism formation in their host, it is not known if *R. solanacearum* also has this ability (Lowe-Power et al. 2018b; Pérez-Donoso et al. 2007). Because of its clear importance to *R. solanacearum* success in the xylem, it will be important to determine the mechanism by which sucrose moves into xylem vessels during bacterial wilt disease.

Trehalose is emerging as an important factor in bacterial wilt disease. Its catabolism by the TreA trehalase is strongly upregulated at HCD both in culture and in planta (Khokhani et al. 2017; Mori et al. 2017; Perrier et al. 2018). Trehalose levels in tomato xylem increased 19-fold upon *R. solanacearum* infection, and we previously showed that the trehalose in xylem sap is produced by the plant, not by the bacterium (Lowe-Power et al. 2018a; MacIntyre et al. 2020). It is unlikely that trehalose contributes to *R. solanacearum* virulence by acting as a carbon source. Using enzymatic assays with limits of detection below the 1 mM threshold required to support *R. solanacearum* growth, we were unable to detect trehalose in xylem sap from plants infected with *R. solanacearum* wild type, $\Delta scrA$, $\Delta treA$, or $\Delta scrA/treA$ strains (data not shown). Nonetheless, an $\Delta scrA/treA$

treA double mutant did not colonize tomato plants as well as wild type or even as well as single mutants lacking either *scrA* or *treA*. Further, the double mutant had a greater virulence defect than either single mutant. In coinoculation experiments, *R. solanacearum* wild type and the $\Delta scrA/\Delta treA$ double mutant strain both consistently reached a lower mean population size when they were competing with each other in planta following cut-petiole inoculation (Table 3). No such population size difference was evident in the biologically different root endosphere competitive fitness experiment (Table 2). This result, which was consistent over four biological replicate experiments, may offer a clue about why $\Delta treA$ mutants are less successful in tomato stems even though trehalose is not a significant carbon source for *R. solanacearum* in planta. Finally, xylem sap collected from $\Delta scrA/treA$ -infected tomato plants supported more *R. solanacearum* growth than sap from $\Delta scrA$ -infected plants even though it contained less sucrose. This suggests that trehalose degradation increases xylem sap nutrient value independently of sucrose. We suspect that the small quantities of trehalose in tomato xylem sap affect plant responses in ways that increase *R. solanacearum* growth. Trehalose is known to modulate plant metabolic regulation and stress tolerance (Lunn et al. 2014; Paul et al. 2008). Its degradation may contribute to *R. solanacearum* virulence by combating osmotic stress or manipulating host physiology. *Arabidopsis* plants use trehalose 6-phosphate as a signal to balance sucrose to starch storage ratios in leaves (Kolbe et al. 2005). *R. solanacearum* may use TreA to degrade this plant signaling molecule and thus maintain an optimal nutrient enrichment of tomato xylem sap. Alternatively, in a separate study, we found evidence that trehalose can act as a DAMP (damage-inducible molecular pattern) that increases tomato defenses. *R. solanacearum* may thus degrade trehalose in xylem sap to avoid sparking plant defenses (A. M. MacIntyre and C. Allen unpublished).

When growing at LCDs in the uniform environment of a shaking culture flask, the $\Delta phcA$ mutant and wild-type cells are in the same LCD regulatory state and initially grow at the same rate. So why does the $\Delta phcA$ mutant multiply faster than wild-type *R. solanacearum* early in the plant infection process? This is likely explained by the heterogeneous microenvironments that *R. solanacearum* inhabits inside roots. During root colonization, *R. solanacearum* cells are not at uniform population density and thus are not in a uniform regulatory state. Surprisingly small populations of bacteria can attain quorum in confined conditions in which QS signal cannot diffuse away. An in-situ gene expression analysis showed that aggregates as small as 33 cells reach quorum when the epiphytic bacterium *Pseudomonas syringae* is enclosed in an EPS matrix (Dulla and Lindow 2008).

Only a few hours after inoculation, *R. solanacearum* cells have already filled some vessels or intercellular spaces in the tomato root cortex (Vasse 1995). As they multiply and spread in host tissues, *R. solanacearum* subpopulations reach quorum intermittently when QS signal accumulates in these small spaces. The resulting QS-mediated downregulation of catabolic pathways slows their growth while QS-mediated upregulation of virulence functions enables long-term success inside the host. In contrast, the $\Delta phcA$ mutant does not respond to QS signal and so it grows uniformly until it is limited by an inability to produce virulence factors.

Taken together, our results suggest that *R. solanacearum* central metabolism is highly dynamic. The catabolic profile of the pathogen changes over successive infection stages to optimize growth and virulence. As *R. solanacearum* cells experience habitats with diverse and scarce nutrients, they cast a wide net by expressing many different catabolic pathways. We show that *R. solanacearum* uses myo-inositol, one of many nutrients present in tomato root exudate, to multiply in the rhizosphere and early in plant infection. As wilt disease progresses, xylem-dwelling *R.*

solanacearum cells focus their metabolism to use sucrose as a key energy source while degrading trehalose to manipulate plant responses in ways that favor the pathogen. Studies are underway to better understand the specific roles of trehalose in plant drought stress responses and bacterial wilt disease resistance.

MATERIALS AND METHODS

Bacterial strains and culture.

Bacterial strains used in this research are listed in Supplementary Table S3. *R. solanacearum* strains were routinely cultured as previously described (Khokhani et al. 2018). Bacterial growth was measured spectrophotometrically as optical density at 600 nm (OD₆₀₀), using a Bio-Tek microplate reader.

Ex vivo xylem sap to be used as a bacterial growth medium or for sugar analysis was harvested from bacterial wilt-susceptible cultivar Bonny Best tomato plants 5 dpi as described, with modifications (Khokhani et al. 2018; Lowe-Power et al. 2018a). *R. solanacearum* population sizes were measured in each sampled plant. Only sap from plants containing 5×10^4 to 5×10^8 CFU per gram of stem was sterilized by passage through a 0.22-micron filter and was retained for growth experiments. Samples were further normalized by pooling ex vivo sap samples to ensure they represented plants carrying an average bacterial population size of 5×10^6 CFU per gram. To measure *R. solanacearum* growth in the ex vivo xylem sap used as a culture medium, a 1-μl bacterial suspension, adjusted to an OD₆₀₀ of 0.01, was added to 50 μl of pooled filter-sterilized sap in 96-well flat-bottom half-area plates and was cultured at 28°C in a spectrophotometric microplate reader, which is a microaerophilic environment. Bacterial growth curves over 24 h were generated using a Bio-Tek microplate reader, measuring spectrophotometrically as OD₆₀₀. Area under growth curve was analyzed, relative to growth of the wild-type strain, using the Mann-Whitney test.

R. solanacearum deletion mutants.

Cloning, restriction digestion, sequencing, and PCR were performed using standard methods (Castañeda et al. 2005; Heckman and Pease 2007; Monteiro et al. 2012). DNA sequencing was performed at the University of Wisconsin-Madison Biotechnology Center and oligonucleotide primers were synthesized by Integrated DNA Technologies. Gibson assembly was used to create in-frame deletion constructs with the primers listed in Supplementary Table S3. Deletion constructs were introduced into the chromosome of wild-type *R. solanacearum* GMI1000 by double homologous recombination, with *sacB* counterselection as described (Castañeda et al. 2005), to create mutant strains GMI1000Δ*iolG*, GMI1000Δ*scrA*, GMI1000Δ*treA*, and GMI1000Δ*scrA/treA*. Complementation constructs were introduced into the selectively neutral *att* site in the chromosome of the relevant *R. solanacearum* mutant by double homologous recombination, as previously described, to create the strains GMI1000Δ*iolG*.COMP, GMI1000Δ*scrA*.COMP, GMI1000Δ*treA*.COMP, and GMI1000Δ*scrA/treA*.COMP (Monteiro et al. 2012). Correct allelic replacement for each mutant and complemented strain was confirmed by PCR and DNA sequencing.

Plant assays.

Virulence assays. Plants were inoculated using a naturalistic soil-soak method (Khokhani et al. 2018). Briefly, a 50 ml of a diluted water suspension of bacteria was poured onto the soil of unwounded 17-day-old Bonny Best tomato plants growing in 4-inch pots at 28°C to achieve a final pathogen density of 5×10^7 CFU per gram of soil. Data were analyzed using repeated measures analysis of variance (ANOVA), which is used for

independent variables that are related because they are sampled repeatedly over time.

Plant colonization and competitive fitness assays. Bacterial colonization and quantification in plant stems was assessed as described (Khokhani et al. 2018). To measure the ability of *R. solanacearum* strains to colonize root endospheres, 4-day-old axenic tomato seedlings were inoculated by applying a 10-μl water suspension containing 2,000 cells to seedling roots as described (Khokhani et al. 2018). Plants were inoculated with antibiotic resistance-marked wild type and marked mutant cell suspensions combined in a 1:1 ratio, using the root inoculation method described above. To remove bacteria from root surfaces, before tomato seedling roots were ground and plated, they were surface-sterilized by treatment with 10% bleach for 30 s, 70% ethanol for 30 s, and were rinsed in sterile water for 1 min. All experiments were repeated at least three times. A competitive index of *R. solanacearum* strains was calculated as previously described (Macho et al. 2007). Data were analyzed using a Mann-Whitney *U* test or Wilcoxon sign-rank test.

Sucrose levels in xylem sap were measured using a modified GOD/invertase enzymatic assay (Teixeira et al. 2012). A 5-μl sap sample was added to 85 μl of distilled water plus 10 μl of invertase solution (10 mg/ml [Sigma-Aldrich] in water, 300 units/mg) and a sample was placed in each well of a 96-well plate. The plate was sealed and incubated in a 55°C water bath for 10 min. The plate was then removed from the water bath, 200 μl of GOD reagent (Pointer Scientific Inc.) was added, the plate was sealed again, was incubated in a 37°C water bath for 15 min, and was then placed at room temperature for 5 min. The absorbance at 490 nm was read in a spectrophotometric plate reader. Each plate included 10 to 1,000 μmol sucrose solution standards to make a calibration curve that was used to determine sucrose concentration in each sample. Each analysis was done in triplicate.

AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI reference sequences:

www.ncbi.nlm.nih.gov/nuccore/NC_003295.1 and
www.ncbi.nlm.nih.gov/nuccore/NC_003296.1

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