***Ralstonia solanacearum* depends on catabolism of myo-inositol, sucrose, and trehalose for virulence in an infection stage-dependent manner**

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The soilborne pathogen *Ralstonia solanacearum* (*Rs*) causes lethal bacterial wilt disease of tomato and many other crops by infecting host roots and then colonizing the xylem vessels. Tomato xylem sap is nutritionally limiting but it does contain sucrose and trehalose. Transcriptomic analyses revealed that *Rs* expresses distinct sets of 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 the *in planta* fitness of bacterial mutants lacking carbon catabolic pathways expressed at either LCD or HCD. We hypothesized that the bacterium needs LCD carbon sources early in disease (root infection) while HCD carbon sources are required during late disease (stem colonization). An *Rs* *ΔiolG* mutant unable to use the LCD nutrient myo-inositol was defective in root colonization but once it reached the stem, this strain colonized and caused symptoms as well as wild type. In contrast, *Rs* mutants unable to use sucrose (*ΔscrA*), trehalose (*ΔtreA*), or both (*ΔscrA/treA*), infected roots as well as wild type but were defective in colonization and competitive fitness in tomato mid-stems and were reduced in bacterial wilt virulence. Additionally, xylem sap from tomato plants colonized by *ΔscrA*, *ΔtreA*, or *ΔscrA/treA* contained more sucrose than sap from plants colonized by wild-type *Rs*. Together, these findings suggest *Rs* metabolism is specifically

adapted for success in the different nutritional environments of plant roots and xylem sap.

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

Microbial success hinges on the ability to be niche adapted and reduce organic compounds. When facing niches with diverse and scares nutrient availabilities having a large scope of catabolism pathways is essential (Fatima & Senthil-Kumar, 2015, Siebrecht et. al., 2003). However, there is a cellular cost for utilizing large numbers of catabolic pathways (Dekel & Alon, 2005, Polz & Cordero et. al. 2018, Pfeiffer & Bonhoeffer, 2004, Gudelj et. al., 2007). Microbes use many signaling mechanisms to make informed choices about what pathways to activate and when (Wadhams & Armitage, 2004). There is a careful balance between wasting energy from improper pathway regulation and gaining energy from the consumption of carbon sources. Growth and reproduction of all living organisms relies on the optimization of these chemical interactions with their environment.

Myo-inositol, sucrose, and trehalose are all compounds that influence the plant host environment for many plant-associated

microbes. Myo-inositol is a carbon source often found in high quantities *in planta* and is the most abundant carbon source found in root nodules formed by association of *Bradyrhizobium diazoefficiens* and host pea (Sköt and Egsgaard 1984). The first of many nutrient interfaces for plant-associated microbes is root exudates where myo-Inositol is commonly found (Wang & Bergeson, 1974, Wei *et. al.*, 2015). However, sucrose is the major form of plant carbon produced through photosynthesis (Salanoubat & Belliard, 1989). It is continuously shuttled from source to sink and is essential for plant growth and development (Pollock & Farrar, 1996, Sonnewald & Willmitzer, 1992). Pathogens can modulate the host metabolism and cause plants to produce and reallocate carbon sources like sucrose (Thines *et al.*, 2000). Plants use trehalose signaling to balance nutrient levels, for example trehalose 6-phosphate influences *Arabidopsis*' sucrose and starch ratios in leaf carbon storage systems (Kolbe *et al.* 2005). Trehalose is well characterized for its roles in plant metabolic regulations and stress tolerance (Lunn *et al.*, 2014, Paul *et. al.*, 2008). Myo-inositol, sucrose, and trehalose are all compounds the many microbes can consume and could modulate host plant environment conditions to be more favorable for invading pathogens.

Ralstonia solanacearum (*Rs*) is part of a heterogeneous species complex of plant pathogenic β -proteobacteria that cause bacterial wilt disease (BWD) (Allen *et. al.*, 2005, Genin & Denny, 2012, Prior *et. al.*, 2016) This pathogen indiscriminately stifles yield for subsistence and commercial crops (Elphinstone, 2005). The pathogen's wide host range and high environmental survival contribute to the large losses seen each year. *Rs* causes disease on over 100 economically relevant crops including tomato, potato, banana, tobacco, peanut, ginger, cloves, and eggplant (Allen *et. al.*, 2005). To investigate the

Rs-tomato pathosystem we used the Asian strain GMI1000, belonging to phylotype I sequevar 18 and causes major losses to tomato growers (Prior *et. al.*, 2016).

Rs spends its life cycling between two dynamic nutritional environments: rhizosphere soil and xylem sap. The rhizosphere is teeming with a wide variety of microbes and carbon sources (Yang & Crowley, 2000). *Rs* is well adapted to surviving in this environment with >50 catabolic pathways to scavenge the many available resources, although specific pathway profiles vary within the species complex (Álvarez, B. *et al.*, 2010, Jacobs *et. al.*, 2012, Zuluaga *et. al.* 2013). *Rs* remains a relatively low titers in the bulk soil of infected fields around $<10^4$ CFU/gram (Allen *et. al.*, 2005, Hayward, 1991). *Rs* senses and chemotaxes to locate and enter plant roots via wounds or natural openings.

We know that *Rs* metabolic pathways are differentially regulated during infection with *Rs* $\Delta phcA$, a quorum sensing mutant that is a proxy for the low cell density (LCD) early infection regulatory mode. PhcA is a global regulator that mediates a trade-off from maximizing growth to producing costly virulence factors (Khokhani *et. al.*, 2017, Polz & Cordero *et. al.* 2018) Interestingly, these results suggest that *Rs* uses a set of carbon sources available in early infection (e.g. myo-inositol) but shifts preferences during late infection or high cell density (HCD) (e.g. sucrose)(Jacobs *et. al.* 2012, Khokhani *et. al.*, 2017, Lowe-Power *et. al.*, 2018). Looking at these dynamic shifts in *Rs* behavior in plant xylem sap narrowed the search for carbon sources contributing to *Rs* virulence and colonization in specific infection stages.

Within 24 hours *Rs* will travel through the developing vascular bundles to reach the xylem vessels of susceptible tomato roots (McGarvey *et. al.*, 1999, Caldwell *et. al.* 2017). Once inside

a host, *Rs* occupies the xylem sap, historically described as a nutrient-poor solution of water and minerals. The vascular vessels themselves are dead, lacking the potential nutrient sources found in living cells (Evert & Eichhorn, 2006). The xylem vascular system acts as a highway for *Rs* to spread systemically through its host. *Rs* promptly multiplies to $>10^9$ CFU/gram stem and eventually grows into aggregates in a biofilm matrix that can fill entire vessels and obstruct water flow (Gnanamanickam, 2006). The completion of the *Rs* life cycle results in the common symptoms of BWD, including wilting of the leaves, petiole epinasty, vascular discoloring, rot, and death.

Little is understood about how *Rs* grows to such high cell densities in a relatively harsh, nutrient-poor environment like xylem sap. We now know that tomato xylem sap contains enough sugars, amino acids, and organic acids to support limited bacterial growth *in vitro* (Coplin *et. al.*, 1974, Dixon & Pegg *et. al.*, 1972, Evert & Eichhorn, 2006, Fatima & Senthil-Kumar, 2015, Wang & Bergeson, 1974, White *et. al.*, 1981). These nutrients vary by plant species, season, time of day, and growing conditions (Siebrecht *et. al.*, 2003, Zuluaga *et. al.*, 2013). Infected xylem sap is enriched in metabolites, including many carbon sources that support *Rs* growth (Lowe-Power *et. al.*, 2018). This metabolomics finding suggests that the presence of *Rs* increases accessible xylem carbon. A transcriptomic analysis shows that several metabolic pathways in *Rs* are differentially regulated during infection (Jacobs *et. al.*, 2012). This work advocates that sucrose, trehalose could fuel *Rs* growth *in planta*.

Our objective was to elucidate the links between *Rs* catabolism, infection stage, and ability to colonize and cause disease on tomato crops. We hypothesize that *Rs* is adapted to two dynamic nutritional environments: rhizosphere soil and xylem sap by using specific carbon sources at different stages in infection cycle.

We analysis bacterial fitness compare to wildtype using bacteria catabolic mutants knocking out *Rs* ability to consume myo-inositol, sucrose, trehalose, and sucrose & trehalose ($\Delta iolG$, $\Delta scrA$, $\Delta treA$, and $\Delta scrA/treA$). We focused on identifying if specific carbon sources that *Rs* consumes required during BWD. We found that LCD gene (*iolG*) is required for early/root infection and HCD genes (*scrA* & *treA*) are required for late/stem infection. When determine through enzymatic assays that sucrose is require for consumption whereas trehalose is modulating plant response rather that being a primary carbon source for bacterial growth in late infection.

RESULTS

‘Omics analysis and phenotypic characterization of bacterial catabolic genes, *iolG*, *scrA*, and *treA*.

Rs (GMI1000) bacterial genes *iolG*, *scrA*, and *treA* are functional *in planta*, absolute expression being 10.15, 11.08, and 12.53 respectively (Jacobs *et. al.*, 2012). Bacterial gene *iolG* encodes an oxidoreductase 2-dehydrogenase signal peptide protein in the myo-inositol catabolism pathway. *scrA* encodes a sucrose-specific transmembrane protein or sucrose importer. *treA* encodes an alpha-trehalase signal peptide protein associated with the trehalose catabolism genes. Myo-inositol, sucrose, and trehalose can be used by GMI1000 as a sole carbon source, detected in *ex vivo* xylem sap, and found in root exudates (Lowe-power *et. al.* 2016, Wang & Bergeson, 1974, Wei *et. al.*, 2015). Tomato cultivar Bonny Best *ex vivo* xylem sap metabolites sucrose and myo-inositol are increased less than 2-fold change whereas trehalose is increase 19-fold change between healthy and GMI1000 Infected sap (Lowe-Power *et. al.* 2016). An *in planta* transcriptomic analysis between $\Delta phcA$, a quorum sensing mutant that is a proxy for a LCD locked state. *iolG* is up regulated 2.08-fold in the *phcA* background compared to wildtype corresponding with myo-inositol

consumption being up regulated at LCD. *treA* and *scrA* in the *phcA* background are both down regulated 2.92 and .54-fold compare to wildtype corresponding with trehalose and sucrose consumption being down regulated at LCD (Khokhani *et. al.*, 2017) (Table 1). To

determine whether *ΔiolG*, *ΔscrA*, and *ΔtreA* were phenotypic knockouts of our target catabolism pathways. Each strain and complement was made through double homologous recombination to create in-frame deletions or insertions using primers listed in

Table 1: *R. solanacearum* catabolic genes of interest

Carbon Source	Gene Name	¹ In planta LogFC WT vs. <i>ΔphcA</i>	² In plant Absolute Expression	³ Metabolite Fold Change (Infected/Healthy)	^{4,5} Gene Annotation	Locus
Myo-inositol	<i>iolG</i>	2.08	10.15	1.95	Oxidoreductase 2-dehydrogenase signal peptide protein	Rsc1246
Sucrose	<i>scrA</i>	-0.54	11.08	1.29	Sucrose-specific transmembrane protein	RSc1285
Trehalose	<i>treA</i>	-2.92	12.53	19.39	Alpha-trehalase signal peptide protein	RSp 0277

¹ FC indicates gene expression fold change values in *ΔphcA* relative to wild-type as described in Khokhani *et. al.* 2017.

² Gene absolute expression values were based on four biological replicates, normalized using the Robust Multichip Algorithm as described in Jacobs *et. al.* 2012.

³ Values represent the Bonny Best *ex vivo* xylem sap metabolite fold change between Healthy and *Ralstonia* (GMI1000) Infected sap as described in Lowe-Power *et. al.* 2016.

⁴ Gene annotations based on www.ncbi.nlm.nih.gov/nuccore/NC_003295.1 and www.ncbi.nlm.nih.gov/nuccore/NC_003296.1.

⁵ Alignment of carbon sources, locus, and pathways based on KEGG.

Supplemental Table 1. To determine the metabolic profile of each mutant and complement, their growth fitness was recorded when grown on sole carbon sources of glucose, myo-inositol, sucrose, and trehalose. *ΔiolG*, *ΔscrA*, and *ΔtreA*, *ΔscrA/treA* all grow like wildtype on glucose. *ΔiolG*, *ΔscrA*, *ΔtreA* and *ΔscrA/treA* differed from wildtype growth only in their respective pathways myo-inositol, sucrose, trehalose, or both sucrose and trehalose. Additionally, all complements restored mutant growth phenotype to wildtype or better than mutant (Table 2). This confirmed that in fact the mutants used in this investigation were in fact functional and specific catabolic gene deletions.

Myo-inositol catabolism is required for colonization & competitive fitness in tomato roots

When *R. solanacearum* GMI1000 strains were separately introduced directly into tomato root infection myo-inositol consumption was required for full root infection. Tomato plants were inoculated with wild-type GMI1000, *ΔphcA*, *ΔscrA*, *ΔtreA*, or *ΔiolG* onto the roots and the population size of each strain was quantified in each seedling 24 & 72 hours post-inoculation (Fig. 1). The LCD locked mutant *ΔphcA* control grew as expected, doing well early in disease and failing at late disease. We found that genes *scrA*, *treA*, and *scrA/treA* are not required during the 1st 24 hours of root colonization. However, at 24 hours the *iolG* mutant is reduced in root colonization compared to wildtype (Mann-Whitney). Within 24 hours *Rs* will travel through the developing vascular bundles to reach the xylem vessels of susceptible tomato roots. This means 72 hours post infection represents stem or late disease

(Caldwell, D. et al. 2017, McGarvey JA. et al. 1999). As expected only genes *scrA*, *treA*, and *scrA/treA* are required for 72 hours root colonization. This suggest that bacterial myo- inositol consumption is required for early colonization.

Table 2: Growth of *R. solanacearum* catabolic mutants on relevant sole carbon sources

Strains	Glucose 10mM ¹	Myo-inositol 10mM ¹	Sucrose 10mM ¹	Trehalose 10mM ¹
Wildtype	+	+	+	+
$\Delta iolG$	+	-	+	+
$\Delta scrA$	+	+	-	+
$\Delta treA$	+	+	+	-
$\Delta scrA/\Delta treA$	+	+	-	-
$\Delta iolG$ comp	+	+	+	+
$\Delta scrA$ comp	+	+	+	+
$\Delta treA$ comp	+	+	+	-/+
$\Delta scrA/\Delta treA$ comp	+	+	+	-/+

¹Growth of *R. solanacearum* strains was evaluated qualitatively as presence (+), partial restoration to better than mutant (-/+), or absence (-) following 24h incubation at 28°C in minimal medium supplemented with 10mM of the indicated sugar as sole carbon source. This experiment was replicated 4 times, with 3 technical replicates in each experiment.

Table 3: Root Competitive Index

Time	Strains	Geometric Mean	Competitive index ^c	P value ^d
24 hr	Wildtype ^a	4.50x10 ⁵	0.2956	0.0171
	$\Delta iolG$ ^b	5.21x10 ²		
	Wildtype ^a	4.88x10 ⁵	1.91784	0.1512
	$\Delta scrA$ ^b	1.00x10 ⁶		
	Wildtype ^a	1.11x10 ⁷	1.93507	0.5252
	$\Delta treA$ ^b	1.61x10 ⁸		
72 hr	Wildtype ^a	4.34x10 ⁶	0.81981	0.5954
	$\Delta scrA/treA$ ^b	7.39x10 ⁶		
	Wildtype ^a	1.62x10 ⁶	1.482	0.2017
	$\Delta iolG$ ^b	1.27x10 ⁷		
	Wildtype ^a	2.75x10 ⁸	0.86104	0.8319
	$\Delta scrA$ ^b	3.08x10 ⁸		
	Wildtype ^a	3.30x10 ⁷	1.00095	0.6379
	$\Delta treA$ ^b	5.78x10 ⁶		
	Wildtype ^a	6.98x10 ⁸	0.79637	0.8296
	$\Delta scrA/treA$ ^b	5.72x10 ⁸		

^aThis strain is the reference strain for competitive index calculations (denominator).

^bThis strain is the competing strain for competitive index calculations (numerator).

^cCompetitive index values represent the proportional competitive advantage of the competing strain over the reference strain. A competitive index of 1 indicates no competitive advantage.

^dP values indicate the level of difference in bacterial population sizes between competing strains in each paired-strain combination in liquid broth medium as determined by Paired T-test.

To specifically measure the contribution of bacterial catabolism to early infection success, we used an *in planta* root competition assay to determine whether the GMI1000 bacterial catabolic mutants was reduced in competitive fitness. Tomato plants were inoculated with a 1:1 mixture of wild-type GMI1000 and GMI1000 $\Delta scrA$, $\Delta treA$, $\Delta iolG$ directly onto

roots and the population size of each strain was quantified in each plant 24 & 72 hours post-inoculation (tab. 3). Only the $\Delta iolG$ mutant has reduced colonization compared to wildtype

(Mann-Whitney). This indicating that only the ability to catabolize root available myo-inositol provides *Rs* with a significant fitness advantage during root infection.

Figure 1: The catabolic mutants they can't consume myo-inositol ($\Delta iolG$) is defective at 24hr while $\Delta scrA$ and $\Delta treA$ mutants are defective at only 72hr. To test the hypothesis that myo-inositol unlike sucrose and trehalose is important for early disease progress. I infected tomato plants with a GmR-marked $\Delta phcA$, KmR-marked wild-type (black), markerless $\Delta iolG$ (green), markerless $\Delta scrA$ (blue), SpecR-marked $\Delta treA$ (red), and SpecR-marked $\Delta scrA/treA$ (purple) bacteria by root attachment inoculation of 4-day old BB tomato seedlings. *Rs* population sizes were quantified by serial dilution plating of ground 2 root sections harvested 24, 48, 72 hours after inoculation. This confirms the hypothesis that the $\Delta scrA$, $\Delta treA$, & $\Delta scrA/treA$ mutants can colonize tomato seedling as well as wild-type and $\Delta iolG$ mutant final to colonize tomato seedling as well as wild-type. $\Delta phcA$ is used as a low cell density lock mutant control expected to do well at early infection only. Data shown represent 3-6 biological replications, each containing 12-20 plants per treatment, and Horizontal black line represents geometric mean of each strain. Significance by Mann-Whitney ^a $p < .001$, ^b $p < .0001$, & ^c $p < .00001$

***R. solanacearum* requires a $\Delta scrA$, $\Delta treA$, and $\Delta scrA/treA$ for full virulence.**

Rs infection modulates xylem sap conditions during infection to increase *Rs* available carbon sources like sucrose. We determined strain effects of virulence by individually inoculating each strain of bacteria on to susceptible cultivar Bonny Best tomato plant and rated disease index over 14 day (Fig 2.). We found that $\Delta iolG$ is equally as virulent as wildtype GMI1000.

However, single mutants $\Delta scrA$ & $\Delta treA$ are both defective in virulence compared to wildtype. The double mutant $\Delta scrA/treA$ virulence is reduced more than both single mutant parents. This suggest that $\Delta scrA$, $\Delta treA$, & $\Delta scrA/treA$ are required for full virulence and that there is some additive virulence defect with the double mutant.

Figure 2: *R. solanacearum* needs catabolism of sucrose and trehalose for full bacterial wilt virulence.

Unwounded 17-day-old tomato plants (wilt-susceptible cv. Bonny Best) were inoculated by pouring a dilute bacterial suspension into the pot to a final pathogen concentration of 5×10^7 CFU/gram potting mix. Plants were incubated in a 28°C growth chamber and symptoms were rated daily on a 0-4 disease index. Data shown are the mean of four (wild-type $n=8$) independent experiments, each containing 15 plants per treatment. Mutants $\Delta treA$, $\Delta scrA$, and $\Delta scrA/treA$ all have lower virulence than the wild-type ($P < 0.002$, repeated measures ANOVA). Virulence of the $\Delta iolG$ catabolic mutant is not different from wild-type ($P > 0.05$, repeated measures ANOVA).

Sucrose catabolism is required for colonization & competitive fitness in tomato stems

When *R. solanacearum* GMI1000 strains were separately introduced directly into tomato vascular systems via a soil soak. Tomato plants were inoculated with wild-type GMI1000, $\Delta scrA$, $\Delta treA$, or $\Delta iolG$ into the xylem through soil drench inoculation and the population size of each strain was quantified in each plant 5

days post-inoculation (Fig. 3). We found that genes *iolG* and *treA* are not required for stem colonization. The $\Delta scrA$ and $\Delta scrA/treA$ mutants have reduced colonization compared to wildtype (Mann-Whitney). Again, the double mutant $\Delta scrA/treA$ colonization is reduced more than both single mutant parents. This suggest that $\Delta scrA$ & $\Delta scrA/treA$ are required for stem colonization and that there is some additive virulence defect with the double mutant.

Figure 3: Sucrose catabolism helps *R. solanacearum* colonize tomato stems. Unwounded 17-day-old tomato plants wilt-susceptible cv. Bonny Best were inoculated by soil-soaking with 5×10^7 CFU/gram bacterial suspension of KmR-marked wild-type(black), markerless $\Delta iolG$ (purple), markerless $\Delta scrA$ (blue), SpecR-marked $\Delta treA$ (red), and SpecR-marked $\Delta scrA/treA$ (green) bacteria. After 5 days the bacterial population size in each plant was determined by dilution plating a ground 0.1 g stem section harvested just above the cotyledon. Data shown from 4 independent experiments, each containing 12-20 plants per treatment. Horizontal black line represents geometric mean of each strain. Asterisk indicates differences from wild-type ($P < 0.05$, one asterisk; $P < 0.00001$, 4 asterisks, Mann-Whitney)

To determine the contribution of xylem carbon sources to bacterial stem success, we used an *in planta* stem competition assay to determine whether the GMI1000 bacterial catabolic mutants were reduced in competitive fitness. Tomato plants were inoculated with a 1:1 mixture of 2000 cell of wild-type GMI1000 and GMI1000 $\Delta scrA$, $\Delta treA$, $\Delta iolG$ directly into the xylem through cut petioles, and the population size of each strain was quantified in each plant 120 hours post-inoculation (Tab. 4). The $\Delta scrA$, $\Delta treA$ and $\Delta scrA/treA$ mutants have reduced colonization compared to wildtype (Mann-Whitney). This indicating that the ability to metabolize xylem sucrose provides *R. solanacearum* with a significant fitness advantage during stem infection.

Bacterial growth based on elevated sucrose concentration in *ex vivo* xylem sap.

Excess carbon sources are associated with increased *Rs* growth (Wang & Bergeson, 1974, Fatima & Senthil-Kumar, 2015, Siebrecht et. al., 2003). GMI1000 infected *ex vivo* xylem sap has increased metabolites compared to healthy *ex vivo* xylem sap (Lowe-Power et. al., 2016). To determine if catabolic mutants leave more metabolites for *Rs* consumption compared to wildtype, we use previously wildtype or mutant infected plant xylem sap as growth media for wildtype, $\Delta iolG$, $\Delta scrA$, $\Delta treA$, & $\Delta scrA/treA$ (Fig. 4). All strains grow the same on sap previously infected with $\Delta iolG$ and $\Delta treA$ compared to sap previously infected with wildtype. Most strains grow better on previously infected with $\Delta scrA$ and $\Delta scrA/treA$ sap except the $\Delta scrA$ & $\Delta scrA/treA$ mutant. This suggests that at least sucrose is modulating bacterial growth in *ex vivo* xylem sap. Interesting $\Delta scrA/treA$ sap seem to support more growth than $\Delta scrA$ or $\Delta treA$ alone.

Table 4: Competitive fitness of *R. solanacearum* catabolic mutants in tomato midstems, relative to wild type

Time (hr)	Strains	CFU/g stem (Geometric Mean)	Competitive index ^c	P value ^d
120	Wildtype ^{ae}	3.28E+07	0.9483	0.3274
	Δ iolG ^{be}	4.79E+07		
	Wildtype ^a	4.10E+09	0.5676	0.0002
	Δ scrA ^b	7.65E+09		
	Wildtype ^a	1.09E+09	0.4231	0.0009
	Δ treA ^b	4.50E+08		
	Wildtype ^a	3.47E+05	0.5000	0.0123
	Δ scrA/treA ^b	2.78E+05		

^aThis strain is the reference strain for competitive index calculations (denominator).

^bThis strain is the competing strain for competitive index calculations (numerator).

^cCompetitive index values represent the proportional competitive advantage of the competing strain over the reference strain. A competitive index of 1 indicates no competitive advantage.

^dP values indicate the level of difference in bacterial population sizes between competing strains in each paired-strain combination in liquid broth medium as determined by Paired T-test.

^eData shown is representative of 3 rather than 4 biological replications

Figure 4: *Ex vivo* xylem sap from plants previously infected with ScrA, ScrA/TreA mutants increase *ex vivo* xylem sap growth of *R. solanacearum* strains that can consume sucrose. Growth of *R. solanacearum* strains was evaluated quantitatively by calculating Area Under the curve as following 36h incubation at 28°C in *ex vivo* xylem sap. Rs strains were grown on sap pre-infected by wildtype (black), Δ iolG (green), Δ scrA (blue), Δ treA (red), & Δ scrA/treA (purple). This experiment was replicated 3-6 times, with 3 technical replicates in each experiment. (P< 0.06, one asterisk; P<0.01, 2 asterisks, Mann-Whitney)

To investigate the concentration of sucrose left in *ex vivo* xylem sap we use an enzymatic assay of previously infected xylem sap (Fig. 5). Wildtype and $\Delta iolG$ both have the same level of sucrose while $\Delta scrA$ & $\Delta scrA/treA$ have increased levels of sucrose compared to previously infected with wildtype sap. $\Delta treA$ unexpectedly also increase sucrose levels

equal to $\Delta scrA/treA$. Although previously infect $\Delta scrA/treA$ sap induces the highest wildtype growth, $\Delta scrA$ sap contains the highest sucrose concentration. Suggesting that an increase in sucrose is responsible for some increase growth and that the additive effect of *treA* and *scrA* deletions is not fully explained by increase in sucrose concentration.

Figure 5: ScrA, TreA, and ScrA/treA mutants modulate sucrose levels in xylem. Sucrose quantification by the GOD/invertase method. Standard sucrose solutions were also added to each plate in separate wells at concentrations 10-1000 umol sucrose. This allowed a calibration curve to be constructed which was used to determine the sucrose concentration in each sample. Each point represents the means of triplicate technical replication and analysis was done in triplicate biological replications. Significance by Student-Paired T-Test, * $p < .01$, ** $p < .001$.

Discussion

Nutrient varied environments during pathogenesis

To understand how *Rs* can survive and infect host in two distinct environments, we needed to understand what carbon sources are required for the pathogen to be successful in each environment. The soil or root environment has been characterized as an environment teeming with small quantities of a diverse set of nutrient sources and high levels of competition (Fatima & Senthil-Kumar, 2015, Siebrecht *et al.*, 2003, Bais *et al.*, 2006). Recently we've learned about the nutrient availability in the highly selective xylem environment. Xylem sap has *Rs*

available carbon sources and that *Rs* infection increases the concentration of several xylem metabolites (Lowe-Power *et al.* 2016, Jacobs *et al.*, 2012, Dalsing *et al.*, 2015, Coplin & Sequeira *et al.*, 1974, Chellemi *et al.*, 1998, Zuluaga *et al.*, 2013.)

We extend our understanding of the role of pathogen utilization of carbon sources in virulence by creating a series of deletion mutants in catabolic pathways, including *iolG*, *scrA*, and *treA*. These mutants were specifically targeted because they represent LCD or HCD bacterial upregulated pathways, a sole carbon source for *Rs*, and is found in plant root exudates or xylem sap. *Rs* uses a set of carbon sources available in early infection including

myo-inositol. Then *Rs* shifts preferences during late infection or high cell density (HCD) to sucrose consumption.

***Rs* myo-inositol consumption is required for root colonization**

We found that catabolism of myo-inositol is required for *Rs* infection and colonization of tomato roots by utilizing information gained from comparing the transcriptome of *ΔphcA* and wildtype GMI1000 during infection. PhcA regulator mediates a tradeoff from a broad metabolic capacity at LCD to a much narrower capacity at HCD (Khokhani *et. al.*, 2017 & Bais *et. al.*, 2006). This shift mediated by PhcA parallels the shift between early and late-stage plant infections.

Unsurprisingly *ΔphcA*, a global regulator mutant, is avirulent (Khokhani *et. al.*, 2017, Lowe-Power *et. al.*, 2018b). However, we found high populations of this mutant in root colonization assays. A *ΔphcB* mutant, which is unable to synthesize the 3-OH-MAME quorum sensing signal and thus quorum insensitive, grew like *ΔphcA* on various sole carbon sources *in vitro* (supplemental fig. 1). This confirm that *ΔphcA* is a good proxy for early and not late infection and that LCD catabolism profiles are conserved across several types of LCD lock mutations.

The myo-inositol pathway upregulated at LCD meaning our bacterial catabolic mutant unable to consume myo-inositol (*ΔiolG*) is important for LCD conditions. We reported that *ΔiolG* is reduced in root colonization but recovers and is not reduced in stem virulence. In tomato root exudate myo-inositol is induced in response to other soilborne pathogens, this could contribute to the plant pathogen defense (Kravchenko *et. al.*, 2003, Cepulyte *et. al.*, 2018). Even though myo-inositol is available in plant xylem sap, bacterial expression increases during early disease progress. This suggest that bacterial myo-inositol consumption is only required for root infection, even though myo-inositol is abundant in xylem sap.

***Rs* sucrose consumption is required for full virulence**

Sucrose catabolism is not required for *Rs* infection and colonization of tomato roots. We confirmed that LCD gene *iolG* is required for early/root infection and by knocking out bacterial sucrose consumption we determined if HCD genes are required for late/stem infection. We compared ability of *Rs* catabolic mutants to colonize the interior of tomato seedling roots 24 & 72 hpi. Only at the 72-hour time point did lacking sucrose catabolism result in reduced root colonization. By 72 hpi, wilting symptoms were already visible in the tomato seedlings, corresponding to late disease (supplemental fig. 2). This result suggests that sucrose as a food source isn't necessary for colonization during early stages of disease but plays a role in later disease. Similarly, root competition assays confirmed *ΔscrA* is just as competitive as wildtype during early infection. Overall, our data suggest that during early infection at the root, bacterial fitness is not enhanced by catabolism of sucrose.

We identified that *Rs* strain GMI1000 requires sucrose catabolism to fully wilt and kill tomato plants. The single mutants *ΔscrA* had a virulence defect compared to the wildtype strain. Quantification of *ΔscrA* colonization in mid-stem is reduced compared to wildtype. When mutant strains were co-inoculated with their wildtype marked parent directly into tomato plant mid-stem, wildtype GMI1000 outcompeted *ΔscrA*. This provides evidence that sucrose pathway is essential for virulence. To confirm that sucrose catabolism is important our enzymatic assay showed that sucrose is increase in xylem sap when mutants can't consume the metabolite. This further supports that sucrose is induced during infection and catabolized by GMI1000 to reach full pathogenesis during mid-stem colonization and infection.

Trehalose catabolism is modulating plant available carbon

Trehalose catabolism is required for *Rs* infection and colonization of tomato stems but not roots. Root competition assays confirmed *ΔtreA* is just as competitive as wildtype during early infection. Like *scrA*, *treA* only fails at colonizing seedlings equal to wildtype at 72 hours. This confirms the role of trehalose importance during late disease. The single mutants *ΔtreA* has a stem virulence defect compared to the wildtype strain. This provides evidence that trehalose pathway is essential for virulence.

Missing both trehalose and sucrose together doesn't affect root colonization or competitive fitness compared to wildtype or parent single mutants during early infection. Double mutant *scrA/treA* has reduced seedling colonization at 72 hours the beginning of late disease. The single mutants *ΔtreA* and *ΔscrA* both had slight defects but the double mutant, missing both pathways, had a significant loss in virulence compared to the wildtype strain. This is confirmation of the importance of trehalose and sucrose for full virulence at HCD.

Recent information confirms that trehalose concentration levels in tomato xylem are so low that it's unlikely that lack of trehalose consumption is affecting population size (MacIntyre *et. al.* 2019). In culture 1 mM of trehalose is required to induce measurable growth in *Rs* populations and enzymatic assays with the limit of detection of .1mM trehalose couldn't detect trehalose in wildtype, *scrA*, *treA*, or *scrA/treA* infected xylem sap (supplement fig. 4). Our result that *ΔtreA* is reduce in mid-stem competitive fitness and not stem colonization aligns with the suggestion that absence of *treA* is not contributing to virulence based on direct trehalose consumption for increased growth.

Interestingly, we support this claim by observing *ΔscrA/treA* during most assays having an additive effect compared to the single mutants *ΔscrA* or *ΔtreA*. The exception being xylem sap sugar composition modulation assays. Xylem sap previously infected with *ΔscrA* supports more wildtype growth and increases sucrose concentration however xylem sap previously infected with *ΔscrA/treA* supports more wildtype growth than *ΔscrA* parent but fails to increase sucrose concentration as high as *ΔscrA* parent. This is compounded by the finding that *ΔtreA* increase sucrose concentration compared to wildtype. We suggest that small quantities of trehalose are regulating plant carbon sources in host xylem.

Trehalose is known for its roles in plant metabolic regulations and stress tolerance (Lunn *et al.*, 2014, Paul *et. al.*, 2008). Arabidopsis plants use trehalose 6-phosphate as a balancing signal for leaf carbon storage systems, managing sucrose and starch ratios (Kolbe *et al.* 2005). Among many other metabolites, trehalose is induced 19-fold in infected compare to healthy tomato plants and bacteria *treA* is upregulated *in planta* during late disease (Lowe-Power *et. al.* 2016, Jacobs *et al.*, 2012). *TreA* may breakdown trehalose, signaling molecule, to maintain the proper balance required of enrichment of tomato xylem sap without sparking plant defense.

Link bacterial catabolism, life stage, and virulence

Ralstonia catabolic profile during given infection stages contributes to successful virulence. *Rs* faces niches with diverse and scarce nutrient availabilities and used specific carbon pathways to be success if each environment. *Rs* myo-inositol is induce in tomato root exudate and *Rs* used it to gain advantage early in infection (Kravchenko *et. al.*, 2003). During late disease *Rs* utilizes sucrose and trehalose for food and possibility to

balance plant responses in the favor of the pathogen (Kolbe et al. 2005). This economically important pathogen strategically takes advantage of myo-inositol, sucrose, and trehalose, compounds the many microbes can consume, and could modulate the environment to be more favorable for *Rs* successful virulence.

Conclusion

We take a holistic approach to understand the how the host xylem environment shapes bacterial wilt disease caused by *R. solanacearum*. We report evidence that xylem sucrose and trehalose are required for late/stem infection while myo-inositol is requiring for early/root infection. We discovery that *Rs* induces and consumes plant sucrose and that *treA* may be required for maintaining a constant sucrose concentration inside of host xylem. Together these experiments describe the primary metabolic needs of successful *Rs* cells during early and late infection. This contributes to our understanding of how this devastating plant pathogen is successful in or excluded from the nutritionally harsh xylem and root environment.

Materials and Methods

Mutant Construction

Standard protocols. Cloning, restriction digestion, sequencing, and PCR were performed using standard methods. *R. solanacearum* and *E. coli* were transformed by electroporation or natural transformation as previously described.

Services. DNA sequencing were performed at the University of Wisconsin-Madison Biotechnology Center. Oligonucleotide were synthesize by Integrated DNA technologies.

Deletions Mutants. Gibson assembly was used to create in-frame deletion using primers listed in Supplemental Table. Deletion constructs were introduced into the chromosome of wild-type *R. solanacearum* strain GMI1000 by double homologous recombination as

previously described to create GMI1000 Δ iolG, GMI1000 Δ scrA, GMI1000 Δ treA, and GMI1000 Δ scrA/*treA*, respectively.

Marking unmarked mutants. Natural transformation of plasmids containing Kanamycin or Gentamycin plasmid was used to introduced resistance marker into the chromosome of *R. solanacearum* unmarked mutants. The correct allelic replacement in each mutant and complement was confirmed by PCR and sequencing analyses.

Complementation Mutants. Splicing by overlap extension was used whole gene replacement constructs using primers listed in Supplemental Table. Complementation constructs were introduced into the chromosome of respective *R. solanacearum* mutant GMI1000 by double homologous recombination as previously described to create GMI1000 Δ iolG.COMP, GMI1000 Δ scrA.COMP, GMI1000 Δ treA.COMP, and GMI1000 Δ scrA/*treA*.COMP, respectively.

Bacterial Growth

Media. *R. solanacearum* GMI1000 (WT) was grown in Boucher-Minimal medium broth (BMM), Casamino Acids-Peptide-Glucose rich (CPG) medium liquid broth and plates.

Conditions. Plates were grown stagnate at 28°C for 48 hours prior to use. Liquid broth cultures were grown shaking at 28°C.

Antibiotics Additives. Antibiotics were added to plates and broth as needed when a marked mutant was being cultured – kanamycin at 0.5 μ L/mL of media, spectinomycin at 1 μ L/mL of media, gentamicin at 1 μ L/mL of media, tetrazolium chloride (TZC) at 2 μ L/mL of media. (Modified from Denny & Hayward et al. 2001)

Catabolic Mutant Media Growth Characterization

Growth solutions were prepared. Boucher-Minimal medium broth lacking a carbon source was use as the base solution. 10mM of carbon

source (glucose, myo-inositol, sucrose, or trehalose) was added to create sole carbon source growth media.

Strains were prepared. Each strain was grown in 3 mL of a diluted 10 mM solution of each media type. The tubes shook in a 28°C incubator overnight to test for the presence or absence of growth on each sole carbon source over 4 biological replicates.

Catabolic Mutant Xylem Sap Growth Characterization

Growth solutions were prepared. Ex vivo xylem sap was harvested as previously described⁵. However, these plants were pre-inoculated with GMI1000 Wildtype, Δ iolG, Δ scrA, Δ treA, or Δ scrA/treA which creates five types of ex vivo xylem sap that experienced different *R. solanacearum* catabolic potentials. Ex vivo sap used was normalized to bacterial colonization of 1×10^5 CFU/gram.

Strains growth. A Titertek Multiskan spectrophotometer plate reader measuring AB₆₀₀ was used to quantify bacterial growth in each media type for 24 hours. Bacterial suspensions of each strain were added to 50 μ L of each type of ex vivo xylem sap growth media in 96-well, flat-bottom, half-area plates. Bacterial suspensions were adjusted to an OD of 0.01. Absorbance curves were produced and Mann-Whitney analysis was done of area under curve values for each strain compared to wildtype.

Plant Virulence Assay.

Plants growth. Wilt-susceptible tomato plants (*Solanum lycopersicum*) cultivar Bonny Best (BB) were used for *in planta* virulence assays. BB were sown and watered every day for 14 days to promote healthy germination. The 14-day old plants were then transplanted and watered with Hoagland's Solution and water in an alternating pattern until one day prior to inoculation.

Soil soak bacterial inoculation. On the 17th day, an unwounded plant was inoculated using

a naturalistic soil soak protocol. Each plant was soaked with a 50 mL inoculum of bacterial suspension, adjusted to 10^7 CFU/mL. As a negative control, 15 BB plants of each biological replicate were inoculated with 50 mL of DI water.

Rating Disease. The disease progress of each plant was rated daily for 14 days post-inoculation (dpi) on a 0-4 disease index scale – where 0 represented no symptoms, 1 corresponded to 1-25% leaf area wilting, 2 for 26-49%, 3 for 50-74%, and 4 for 75-100% of the leaf area wilted. These virulence assays were repeated for 4 independent biological replicates and were analyzed through repeated measures of ANOVA.

In Planta Colonization & Competition

Stem Inoculations. Plants for colonization are inoculated with either the wild-type strain or the mutant using the soil soak method as described above. Plants for competition are inoculated with marked wildtype and marked mutant cell suspensions were combined in a 1:1 ratio using the petiole method as described. On the 21st day, cut petiole plants were inoculated. Each plant was inoculated with a 2000 cell Bacterial suspensions. As a negative control, 15 BB plants of each biological replicate were cut and inoculated with DI water.

Root Inoculations. Plants were inoculated with either the wild-type strain or the mutant using the root attachment method as previously described⁷.

Plants were inoculated with marked wildtype and marked mutant cell suspensions were combined in a 1:1 ratio using the root attachment method as described above.

Sterilization. Stem colonization and competition require no extra surface sterilization. Roots are sterilized by 10% bleach for 30 seconds, 70% ethanol for 30 seconds, and sterilize water for 1 min.

Quantification. Stem population sizes of each strain in tomato plants were quantified as described. Briefly, at a given time points after

inoculation, plants inoculated with each strain were segmented ~1cm, weighed, and ground in 1 ml sterile deionized water. Root population sizes of strain in tomato plants were quantified by collecting root at different time points after inoculation. Two whole seedling roots are weighed and ground in 300 ul sterile deionized water. The resulting homogenate was dilution plated on TZC plates supplemented with 100 mg l⁻¹ cycloheximide and appropriate antibiotics. *R. solanacearum* population sizes were determined as cfu g⁻¹ of plant tissue. Methods modified from Khokhani *et. al.*, 2018. All experiments were repeated at least three times. Competitive Index was calculated and analysis base on previously described methods⁸. Statistical Analysis is done using Mann–Whitney U test or Wilcoxon sign-rank test.

***Ex vivo* xylem sap collection** Xylem sap was harvested from plants 5 days post inoculation after. Plants to ensure adequate water status, plants were well watered each evening before sampling. Plants inoculated by soil soaking were detopped 2cm above the cotyledons, and plants inoculated by petiole inoculation were detopped at the site of inoculation. Root pressure allowed xylem sap to accumulate on the stump. The sap accumulated in the first 2-3 min was discarded and the stump was washed with water and gently blotted dry with a Kimwipe. Over 30 min, the accumulating sap was frequently transferred into pre-chilled 1.5 ml flip cap microcentrifuge tubes held in -20°C block or on ice. Samples were flash-frozen and stored at -80°C until analysis. Subsequently, 100 mg stem samples were harvested and stem colonization was quantified as stated above. (Modified from Lowe-Power *et. al.* 2018)

GOD/invertase enzymatic assay to determine sucrose concentration. in a 96-well plate, 85 ul distilled water, 5 uL from each

sample and 10 uL invertase were placed in each well. The invertase was prepared at a concentration of 10 mg/mL in distilled water. The plate was then sealed and placed in a water bath at 55 C for 10 min. After this period the plate was removed from the water bath and 200 IL of GOD (Bioclin kit) reagent was added, and the plate was sealed again and placed in a water bath at 37 C for 15 min. After this time, the plate was removed from the water bath and placed at room temperature for 5 min. The absorbance at 490 nm was read in a Titertek Multiskan Plus spectrophotometer, equipped for reading plates. Standard sucrose solutions were also added to each plate in separate wells at concentrations 10- 1000 umol sucrose. This allowed a calibration curve to be constructed which was used to determine the sucrose concentration in each sample. Each analysis was done in triplicate. (Modified from Teixeira *et. al.* 2012)

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