Plant pathogenic *Ralstonia* phylotypes evolved divergent respiratory strategies and behaviors to thrive in xylem

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Running title: Diversity of energy metabolism in *Ralstonia solanacearum* species complex

ABSTRACT

Bacterial pathogens in the *Ralstonia solanacearum* species complex (RSSC) infect the water-transporting xylem vessels of plants, causing bacterial wilt disease. Strains in RSSC phylotypes I and III can reduce nitrate to dinitrogen via complete denitrification. The four-step denitrification pathway enables bacteria to use inorganic nitrogen species as terminal electron acceptors, supporting their growth in oxygen-limited environments like biofilms or plant xylem. Reduction of nitrate, nitrite, and nitric oxide all contribute to virulence of a model phylotype I strain. However, little is known about the physiological role of the last denitrification step, the reduction of nitrous oxide to dinitrogen by NosZ. We found that phylotypes I and III need NosZ for full virulence. However, strains in phylotypes II and IV are highly virulent despite lacking NosZ. The ability to respire by reducing nitrate to nitrous oxide does not greatly enhance growth of phylotype II and IV strains. These partial denitrifying strains reach high cell densities during plant infection and cause typical wilt disease. However, unlike phylotype I and III strains, partial denitrifiers cannot grow well under anaerobic conditions or form thick biofilms in culture or in tomato xylem vessels. Furthermore, aerotaxis assays show that strains from different phylotypes have different oxygen and nitrate preferences. Together, these results indicate that the RSSC contains two subgroups that occupy the same habitat but have evolved divergent energy metabolism strategies to exploit distinct metabolic niches in the xylem.

IMPORTANCE

Plant pathogenic *Ralstonia spp.* are a heterogeneous globally distributed group of bacteria that colonize plant xylem vessels. *Ralstonia* cells multiply rapidly in plants and obstruct water transport, causing fatal wilting and serious economic losses of many key food security crops. Virulence of these pathogens depends on their ability to grow to high cell densities in the low-oxygen xylem environment. Plant pathogenic *Ralstonia* can use denitrifying respiration to generate ATP. The last denitrification step, nitrous oxide reduction by NosZ, contributes to energy production and virulence for only one of the three phytopathogenic *Ralstonia* species.

These complete denitrifiers form thicker biofilms in culture and in tomato xylem, suggesting they are better adapted to hypoxic niches. Strains with partial denitrification physiology form less biofilm and are more often planktonic. They are nonetheless highly virulent. Thus, these closely related bacteria have adapted their core metabolic functions to exploit distinct micro-niches in the same habitat.

INTRODUCTION

Bacteria with flexible respiratory metabolisms can continue to grow in environments with fluctuating electron acceptor availability. Oxygen is the most energetically favorable terminal electron acceptor (TEA), but it is not always available to environmental microbes in soil or to pathogenic bacteria in host tissues. Such microbes are often forced to use alternative TEAs (1). Among alternative TEAs, nitrate (NO_3^-) yields the most reductive power and is commonly available (2). Nitrate respiration can occur alone when oxygen is limiting, yielding nitrite (NO_2^-) and generating a proton motive force to make ATP. It can also be coupled with denitrification, the oxygen-sensitive three-step enzymatic reduction of nitrite to nitric oxide (NO) to nitrous oxide (N_2O) to dinitrogen gas (N_2). Separate reductases carry out each step of this pathway. Nitrate-respiring bacteria theoretically gain maximal energy by reducing NO_3^- all the way to N_2, using all inorganic nitrogen species in this pathway as TEAs.

We previously showed that a plant pathogen, *Ralstonia pseudosolanacearum* GMI1000, depends on nitrate respiration for colonization and virulence (3). GMI1000 is a member of the *Ralstonia solanacearum* species complex (RSSC), a diverse group of bacteria that colonize and block the water-transporting xylem vessels of higher plants (4). Xylem is a relatively low O_2 environment, containing about 200 μM O_2, which is hypoxic relative to the 9.4 mM O_2 in the atmosphere (3). However, xylem sap also contains 30 mM NO_3^- , which we showed is the optimal concentration for growth of strain GMI1000 (3). Respiratory reduction of NO_3^-, NO_2^-, and NO are all crucial for plant colonization and virulence of GMI1000 (3). However, it has long been known that strains in the RSSC vary in their ability to carry out the final reduction of N_2O

to inert N_2 gas (5). We wondered whether these closely related bacteria have adapted their core metabolic functions to exploit distinct micro-niches in the same habitat.

The RSSC contains four major phylogenetic lineages, phylotypes I-IV (6). Phylotypes I and III are closely related and were recently renamed *R. pseudosolanacearum* while phylotypes II and IV were named *R. solanacearum* and *R. syzygii*, respectively (7). Using a genetically diverse panel of RSSC strains, we recently showed that phylotypes I and III (hereafter phyl. I/III) are complete denitrifiers that benefit dramatically from the presence of NO_3^- during oxygen-limited growth (5). All 25 tested phyl. I/III strains are complete denitrifiers that produce N_2 gas (8, 9). In contrast, 0/35 phyl. II and 0/8 phyl. IV strains tested produced dinitrogen gas (5). Despite this substantial difference in energy metabolism, all four phylotypes in the RSSC include strains that can infect a common host, tomato.

We hypothesized that RSSC phylotypes depend on distinct respiratory mechanisms for growth *in planta*. Using bioinformatics and functional analyses, we identified intriguing metabolic and physiological differences that extend beyond the reduction of N_2O to N_2 gas. Phylotype II and IV strains (hereafter phyl. II/IV) lack the final NosZ-dependent denitrification step, and they benefit only slightly from any step in the denitrification pathway under low O_2 conditions. Intriguingly, we found that phyl. I/III strains and phyl. II/IV strains also differ with respect to biofilm formation, taxis towards low oxygen environments, and aggregation in tomato xylem. These functional differences suggest that despite causing similar wilt disease, these two subgroups have adapted to exploit different respiratory niches *in planta*.

RESULTS

Denitrification pathway gene content correlates with RSSC phylotypes. We analyzed the distribution of denitrification genes in 51 genomes reflecting the phylogenomic diversity of the RSSC (Fig. 1A-B). Except for two insect-transmitted phyl. IV strains that have reduced genomes (R24 and BDB R229) (10), all RSSC genomes contained the *nar* cluster encoding NarK1/2 nitrate transporters and the first reduction step of the pathway (NO_3^- to NO_2^-). All

strains also encoded Ani and Nor enzymes that catalyze the second and third steps of denitrification (NO_2^- to NO to N_2O), except for genome-reduced R24. No members of phyl. II/IV carry the *nos* genes that perform the terminal N_2O reduction step. In contrast, all phyl. I and 10/11 phyl. III strains have the *nosZRDFYL* cluster (Fig. 1C).

We wondered if this variation in *nos* content was associated with additional differences in proteins and regulators involved in nitrate-dependent metabolism under low O_2 conditions. We first explored this hypothesis by comparing amino acid sequences of the enzymes, transporters, regulators encoded in the three denitrification gene clusters. We selected one representative strain from each of the four phylotypes: GMI1000 for phyl. I; K60 for phyl. II; CMR15 for phyl. III, and PSI07 for phyl. IV (Fig. 1). These four strains are all soil-borne, colonize xylem vessels, and cause indistinguishable wilt symptoms on tomato plants. The catalytic subunits NarG, AniA, and NorB were relatively well conserved in all four genomes (Fig. 1C), but the NO_3^- transporters (NarK1 and NarK2), the NO_3^--responsive regulators (NarX and NarL), and accessory metabolic proteins (NarJ, NarI, MobB, and RSp1504) had lower sequence identity.

Many highly conserved regulators influence NO_3^- metabolism and denitrification in other bacteria (11). All RSSC representatives encoded the potential NO-responsive regulators NnrS (GMI1000 locus RSc3399); NsrR (RSc3397); a predicted O_2 responsive FNR (RSc1283); two FNR-like regulators (RSp0190 and RSc0966, respectively); and the sigma-54 factor RpoN1 (RSc0408) (12). In contrast, the NorAR NO binding system (RSp0958 and RSp0959) were only present in phyl. I/III (13) (Fig. S1).

The *nos* N_2O reduction genes are clustered in an apparently horizontally transferred element scattered among the Beta-proteobacteria. We investigated the presence of the *nos* gene cluster in complete genomes from diverse strains in the subclass Beta-proteobacteria. Similar to what we found in the RSSC (Fig. 1A) and prior reports in *Neisseria* spp. (14), the presence of the *nos* cluster was highly variable (Fig. S2).

In genomes of both phyl. I strain GMI1000 and phyl. III strain CMR15, the *nos* clusters are located near fragments of transposition insertion sequences (IS) (Fig. S3). These

transposition remnants are phylotype-specific and lie outside the conserved *nos* region. As is typical of horizontally acquired elements, the *nos* cluster is in different locations in the two genomes. This suggests phyl. I/III strains either acquired this locus through separate events or that genome shuffling occurred after the initial incorporation of the cluster.

While the genomic context and the flanking insertion sequences of the *nos* cluster differ between phyl. I/III strains, the cluster gene content and structure is conserved (Fig. S3). The *nos* clusters in GMI1000 and CMR15 contain all eight genes associated with NosZ function (*nosZRDFYLX* and *cco5*) (2, 15–20). Between the IS elements, there are also five conserved genes that are absent from phyl. II/IV genomes, including a SWKP family type III-secreted effector of unknown function (RSp1374).

To gain insight into the evolutionary history of the *nos* cluster, we compared 36 bacterial NosZ protein sequences (Fig. S4). Consistent with multiple horizontal gene transfer (HGT) events, NosZ protein phylogeny generally does not correspond with whole genome phylogeny. However, the RSSC NosZ sequences cluster with NosZ from close relatives, *Ralstonia pickettii* and *Cupriavidus metallidurans*. This pattern may indicate a period of vertical inheritance of the cluster followed by loss in phyl. II/IV, or that HGT is more frequent between organisms with regions of sequence homology, independent of transposition events.

RSSC strains that encode the *nos* cluster require the last step of denitrification for full virulence on tomato. Because all described RSSC strains are pathogenic to plants, but only two subgroups contain the *nos* locus, we wondered if NosZ contributes to fitness or virulence of phyl. I/III strains. We previously determined that a Δ*nosZ* mutant of phyl. I strain GMI1000 reached a lower population size than wildtype in tomato stems 3 days after petiole inoculation, but the difference was small (3). However, when we quantified population sizes earlier, at 2 days post inoculation, we found a larger growth difference (Fig. 2A). This early colonization defect suggested that N_2O reduction contributes to *in planta* success of phyl. I strain GMI1000.
To more thoroughly investigate the role of N_2O reductase in virulence for this phyl. I strain, we monitored symptom development following soil-soak inoculations of unwounded plants. This

assay revealed that NosZ, and by extension, complete denitrifying respiration, play a significant role in pathogenesis of strain GMI1000 (Fig. 2B). To determine if NosZ is also a virulence factor in the closely related phyl. III, we created a Δ*nosZ* mutant of strain CMR15 and repeated the assay. CMR15 Δ*nosZ* was similarly reduced in virulence (Fig. 2C). These results show that while strains from all four RSSC phylotypes wilt and kill tomato plants, only phyl. I/III strains require *nosZ* for full virulence.

NO_3^- enhances anaerobic growth of complete denitrifiers in the RSSC. To test whether RSSC phylotypes differ in their denitrification physiology, we selected three representative strains from each phylotype. Growth assays revealed that the *nos* cluster was required for complete denitrification to N_2 under anaerobic conditions (Fig. 3A) (5). All tested phyl. I/III strains grew to a 3 to 4-fold higher cell density when provided with NO_3^- than when this TEA was absent. In contrast, anaerobic growth of phyl. II/IV strains was unaffected by the presence of NO_3^-. Notably, Δ*nosZ* mutants of both GMI1000 and CMR15 still grew better with NO_3^- than without it [data not shown; (3)]. This indicates that the lack of N_2O reductase activity alone does not fully explain the inability of phyl. II/IV strains to use NO_3^- as a TEA under anaerobic conditions.

To determine if NO_3^- enhances growth of phyl. II/IV strains under any O_2 level, we measured growth of each representative strain in 0.1, 1.0, 10.0, and 21.0% O_2 with or without 30 mM NO_3^- (Fig. 4). At the ambient 21.0% O_2, all four RSSC strains were inhibited by NO_3^-, possibly because of NO_2^- or NO-induced oxidase inhibition (3). Growth of phyl. II K60 was also inhibited by NO_3^- in 10% O_2. Both phyl. II K60 and phyl. IV PSI07 benefited slightly from the presence of NO_3^- under hypoxic conditions (1.0% or 0.1% O_2) while phyl. I GMI1000 and phyl. III CMR15 grew much better in this condition. Overall, the phyl. II/IV strains did not grow as well on NO_3^- as phyl. I/III strains at any O_2 level.

All phyl. II/IV strains tested encode the NO_3^-, NO_2^-, and NO reductases that catalyze the first three steps in denitrifying respiration, but comparative transcriptomic analysis revealed that phyl. II strain UW551 did not express this pathway as highly as phyl. I strain GMI1000 during

growth in tomato stems (21). This suggested that denitrification may be less important for phyl. II than for phyl. I. To confirm that the Nar NO_3^- reductases in phyl. II/IV strain are functional, we measured NO_2^- production during anaerobic growth (Fig. 3A). Four of six tested phyl. II/IV strains produced detectable NO_2^- even if their growth was not enhanced by this metabolic conversion. After 4 h incubation at high cell densities (10^9 CFU/mL), phyl. II strain K60 accumulated ~40 µM NO_2^- while phyl. I GMI1000 accumulated ~90 µM.

To determine if the predicted NO reductase NorB is functional in phyl. II/IV, and to compare N_2O production by completely and partially denitrifying strains, we measured N_2O gas produced by denitrifying cultures of the four phylotype representatives. Consistent with the finding that all four strains reduce NO_3^- in low oxygen conditions, all strains produced detectable quantities of N_2O (Fig. 3B). Complete denitrifiers produced approximately 2-fold more N_2O per cell than partial denitrifiers (P=0.0065).

To determine if phyl. II/IV strains can grow in low O_2 conditions by using fermentation as an alternative to NO_3^- respiration, we used HPLC to look for fermentation end products in filtered spent culture of the four representative strains after 24 h growth in either aerobic or anaerobic conditions. We did not detect acetate, lactate, or other fermentation end products (data not shown). Moreover, fermentation usually acidifies culture media, and the pH of the RSSC culture supernatants was unaltered.

Gene enrichment analysis reveals additional metabolic differences between phyl. I/III and phyl. II/IV strains. To identify metabolic functions that may have co-segregated with the *nos* cluster and that could explain the observed differences among strains in inorganic nitrogen respiration, we screened genomes for KEGG categories that are enriched in phyl. I/III strains vs. phyl. II/IV strains (Fig. S5). The KEGG enrichment analysis suggested that the genomes of complete and partial denitrifying strains were enriched in distinct sets of aromatic degradation pathways. Phyl. II/IV strains were enriched in a Ben/Cat pathway for benzoate/catechol degradation (22). Phyl. I/III strains showed enrichment of a partial Dmp pathway that is missing the DmpB gene that catalyzes the ring opening of catechol (23). The

KEGG enrichment analysis was performed on the small set of genomes available in 2015, which was biased towards phyl. II genomes. We curated high-quality genomes that better represent the genomic diversity of the RSSC and performed BlastP searches for aromatic degradation enzymes (Fig. 5). This robust analysis confirms that the Ben/Cat pathway is specifically present in most phyl. II/IV genomes. The partial Dmp pathway is sporadically present in phyl. I/III genomes and absent from phyl. II/IV genomes. Overall, the protocatechuate degradation pathway (Pca) is broadly conserved across the RSSC (22). The hydroxycinnamic acid degradation pathway (Fcs) is broadly conserved except in the phyl. IIC lineage and the Banana Blood Disease lineage of genome-reduced phyl. IV strains. The salicylic acid degradation pathway (Nag) is broadly conserved except in the phyl. IIC and most IIA genomes and the Sumatra Disease of Clove lineage of genome-reduced phyl. IV strains.

Complete and partial denitrifiers in the RSSC have different oxygen preferences. To better understand oxygen preferences within the RSSC, we stab-inoculated strains into tubes of VDM soft agar with or without 30 mM NO_3^-. In these tubes, O_2 is available in a diffusion gradient near the agar surface. After one week, the growth patterns in these culture tubes indicated that phyl. I/III GMI1000 and CMR15 had a strong preference for anaerobic environments when NO_3^- was available, while phyl. IV had a subtle preference for lower-than-atmospheric O_2 levels (Fig. S6). A GMI1000 Δ*narG* mutant, which lacks the first denitrification step, had no tactic response to NO_3^- conditions (data not shown), which is consistent with dependence of these migration patterns on energy taxis (also known as aerotaxis) (24).

Complete and partial denitrifiers in the RSSC may have adapted to different ecological niches. Biofilms are typically hypoxic (25), so we hypothesized that phyl. I and III strains would form more robust biofilms. We tested this hypothesis using the PVC-crystal violet biofilm assay, which showed that phyl. I/III strains formed thicker biofilms *in vitro* than phyl. II/IV (Fig. 6B).

Previous SEM studies showed that the complete denitrifier GMI1000 forms dense biofilms in tomato xylem vessels (26, 27). To determine if all RSSC phylotypes form similarly

dense biofilm *in planta*, we used SEM to image xylem tissue at onset of wilt symptoms. The complete denitrifying strains in phyl. I/III colonized xylem vessels differently than the phyl. II/IV partial denitrifiers. Phyl. I GMI1000 and phyl. III CMR15 colonized many xylem vessels extensively and often formed thick biofilms on vessel walls and in the lumens (Fig. 6A). In contrast, cells of phyl. II K60 and phyl. IV PSI07 were visible in fewer xylem vessels and often formed single-cell layers on the vessel walls (Fig. 6A). During SEM sample preparation, stem cross-sections are washed in a fixative solution. We hypothesized that planktonic or loosely attached cells may disperse into the solution before the fixative can anchor them in place. To assess the relative numbers of planktonic and attached cells in plants infected with each strain, we quantified bacterial populations in homogenized stem samples and then quantified the unattached cells that streamed from cut stem sections incubated in water. As previously observed, all four phylotype representatives colonized plants similarly, reaching population sizes $>1x10^9$ CFU/g stem (Fig. S7A). There was no difference in the proportion of released cells of phyl I/III strains GMI1000 and CMR15 as well as phyl. II K60. More than 90% of these cells were retained in cut stem sections. However, almost twice as many phyl. IV PSI07 cells streamed from cut stems into the water (Fig. S7B). If PSI07 cells were more often planktonic or loosely attached, this could explain the relatively few bacteria visible in SEMs of PSI07-infected stems. Together, the PVC biofilms, *in planta* SEM images, and streaming assay results suggest that during plant infection, complete denitrification may help phyl. I/III strains form more robust biofilms on xylem walls.

To assess the effects of bacterial growth on O_2 levels in tomato xylem, we used a microprobe to directly measure O_2 concentrations in sap from plants colonized by each of the four representative strains. Bacterial wilt disease significantly reduced O_2 availability in xylem sap regardless of the infecting RSSC strain. This finding is consistent with our previous observation that infection by GMI1000 reduced xylem O_2 levels by half relative to healthy plants (3). The O_2 levels in stagnant water and xylem sap from healthy tomato plants are 230 μM O_2/L, and around 200 μM O_2/L, respectively (Fig. 7). Sap from early to mid-stage diseased plants

(disease index = 1-3) infected with any of the four representative RSSC strains contained 220 to 95 μM O_2/L. Oxygen levels were lower in sap from late-stage diseased plants, ranging from 175 to 0 μM O_2/L. There was little difference in sap oxygen levels between tested strains (Fig. 7). This suggests that phyl. II/IV and I/III strains all rapidly consume any available oxygen and thus experience similarly low O_2 during wilt pathogenesis. Nonetheless, our other genomic and functional analyses indicate that the two groups have adapted to this metabolic challenge in distinct ways.

DISCUSSION

We discovered that the four phylotypes in the RSSC have surprisingly diverse energy metabolism. Analyzing the genome of diverse RSSC strains revealed that the first three steps of denitrification are broadly conserved across the RSSC, but only phyl. I/III have the final Nos-dependent step. These bioinformatic results are consistent with our prior physiological studies showing that only phyl. I/III strains produce N_2 gas (5). Unfortunately, this means that the official taxonomic revision of RSSC species incorrectly describes the denitrification phenotypes of *R. pseudosolanacearum* and *R. solanacearum* (7), which warrants correction.

We previously hypothesized that RSSC strains may have an O_2 and NO_3^- -independent mechanism to obtain energy in VDM, such as amino acid fermentation or Stickland reactions (3). However, Stickland metabolism is rare outside of *Clostridia* (28), and our data are consistent with the conclusion that RSSC lack fermentative metabolism and are obligate O_2 and NO_3^- respirators. It is possible that the strains have such a high affinity for oxygen that they can scavenge the extremely low levels of oxygen remaining in the medium during growth in an anaerobic chamber.

If phyl. II/ IV strains lack the Nos enzymes, then why do phyl. I/III strains require *nosZ* for full virulence on tomato? Pathway blockage could cause the Δ*nosZ* mutants of GMI1000 and CMR15 to accumulate N_2O. Although N_2O has low toxicity, it is possible that these mutants experience inhibitory N_2O levels *in planta*. However, direct measurements found that these

mutants did not accumulate more N_2O than wildtype *in vitro*. Because all strains produce similar N_2O levels during anaerobic growth, the most likely explanation is that phyl. I/III strains have become dependent on Nos enzymes to generate a sufficient proton motive force to sustain their growth *in planta*. Recently, we showed that levels of the denitrification intermediate NO influence expression of the type 3 secretion system for phyl. I strain GMI1000 (29). The type 3 secretion system is a major virulence factor. It is possible that *nosZ* mutants have slightly reduced expression of type 3 secretion system genes.

Throughout the course of plant infection, RSSC pathogens experience variable levels of O_2 and reactive oxygen/nitrogen species. Here we found that infection by all representative strains similarly decreased the O_2 levels in tomato xylem sap, leading to anoxia in advanced wilt disease. Nevertheless, diverse RSSC strains require mechanisms to manage the reactive oxygen and nitrogen species during plant infections (13, 30, 31). Moreover our measurements show that although O_2 levels decrease during bacterial wilt, O_2 remains present when *Ralstonia* populations are carrying out explosive growth during early and mid-stage disease. Previously, we showed that a different phyl. II strain (UW551) requires a high affinity cbb_3-type cytochrome c oxidase for maximal growth *in planta* and in microaerophilic conditions (63). Genomes from representatives from all phylotypes of the RSSC each encode both major types of terminal oxidase families: proton pumping heme-copper oxidases and the non-pumping *bd*-type oxidases (data not shown; (64)). Nevertheless, it remains an open question as to whether the two groups of RSSC strains vary in their ability to use oxygen as a TEA.

Because phyl. I/III strains form abundant and thick biofilms on xylem vessel walls, it is possible that these strains experience much lower O_2 levels *in planta* than reflected in our measurements. Although O_2 levels in exuded sap reflect the O_2 available to planktonic bacteria *in planta*, such bulk analyses can mask the spatial heterogeneity that exists within plant hosts. Additionally, we recently used X-ray microtomography to show that phyl. I strain GMI1000 induces wilt symptoms when its dense biofilms clog half of the total xylem vessels (4). The SEM

images shown here suggest that phyl. II/IV strains may form biofilms with higher oxygen availability.

It is surprising that even though all RSSC strains experience low oxygen conditions *in planta*, phyl. II/IV strains hardly benefit from the presence of the alternate TEA NO_3^-. The differences in denitrification metabolism correlated with phenotypic differences in aerotaxis behavior and biofilm formation. When provided the opportunity to migrate to their preferred oxygen concentration in soft agar, phyl. I/III strains migrated to different depths in a NO_3^- dependent manner. In the absence of NO_3^-, these strains preferred the O_2-rich surface of the agar. When NO_3^- was present, they migrated to a deeper, less oxygen-rich band. In contrast, the phyl. II/IV strains were agnostic to the presence of NO_3^-. Among the RSSC, aerotaxis has only been studied in phyl. II strain K60 (24).

Using a population genetics test, several denitrification-related genes were identified as core RSSC genes that are under selection (32). The Tajima's D were above 2 for the accessory metabolic proteins NarI and NarJ and the NO_3^--responsive regulators NarL and NarX, suggesting that there could be functionally distinct alleles of these genes within the RSSC population. Except for a robust study of variation in quorum sensing synthases/regulators within the RSSC (33), there has been relatively little investigation of variation of regulators within the RSSC.

In addition to differences in denitrifying metabolism, our genomic enrichment analyses revealed that phyl. I/III and phyl. II/IV vary in their predicted capacity for degrading aromatic compounds. Aromatic compounds are a major class of plant defense chemicals as well as possible carbon sources. Several aromatics have been detected and quantified in tomato xylem sap during infection with phyl. I strain GMI1000: salicylic acid (~20-200 nM), benzoic acid (~10 nM), and coumaric acid (~100 nM) (34). Our analyses found that phyl. II/IV genomes were enriched in a benzoic acid/catechol degradation pathway (22). The ability to degrade other aromatic compounds significantly increases *in planta* fitness of phyl. I strain GMI1000 (35, 36). It remains to be determined whether benzoate/catechol degradation similarly contributes to *in planta* fitness of phyl. II/IV strains.

Across the domain Bacteria, the class of genes encoding energy metabolism is among those most commonly found in recently horizontally acquired regions (37). It is clear that the evolution of the RSSC has been shaped by HGT events, likely boosted by the group's natural competency (38, 39). Strains in all RSSC phylotypes take up and transfer DNA (39), although comparative genomics show phyl. I is the most recombinogenic of the phylotypes (39–42). The saprophytic life stages of phyl. I/III strains may have provided opportunities to horizontally acquire the *nos* cluster from other soil residents. Moreover, survival in the soil may exert selective pressure for complete denitrification. Like the RSSC, *Bradyrhizobium* isolates can vary in the presence/absence of the *nosZ* gene. An elegant microbial ecology study in Japan found a correlation where *nosZ*-minus *Bradyrhizobium* strains dominated in soil types that have high levels of volcanic ash (43). In contrast, *Bradyrhizobium* strains with the full denitrification pathway have higher tolerance to flooding (44, 45). Little is known about whether RSSC lineages vary in their ability to survive in different soil types, but it is known that some lineages persist better in soil than others (46, 47).

CONCLUSIONS

Our genomic, physiological, and virulence studies collectively suggest that RSSC strains in phyl. I/III and II/IV use different metabolic strategies to reach high cell densities *in planta*. Phyl. I/III strains benefit from denitrifying respiration during tomato infection, including N_2O reduction by NosZ. In contrast, phyl. II/IV strains are fully virulent, growing to similar densities in host stems and causing identical wilt symptoms even though they lack NosZ and cannot complete denitrifying respiration. Broader genomic and behavioral analyses *in planta* and *in vitro* are consistent with the model that the two groups respond differently to oxygen, a key environmental variable. The completely denitrifying strains in phyl. I/III grow better under hypoxic conditions *in vitro* and are more likely to aggregate in biofilms on host plant xylem vessel walls. Phyl. II/IV strains, which also colonize plant xylem vessels and cause wilt symptoms, have respiratory strategies that allow them to exploit environmental and host-

associated niches containing higher oxygen levels. Ongoing studies will test the intriguing hypothesis that these divergent energy strategies reflect inter-species niche partitioning.

MATERIALS AND METHODS

Strains, mutagenesis, and culture conditions. Origins and sequence accession numbers of strains used in this study are in Table S1. One representative strain for each RSSC phylotype was selected, based on ability to cause disease on the common host tomato, and on the extensive experimental data and closed genome sequences for these four strains: GMI1000, CMR15, K60, PSI07. The Δ*nosZ* GMI1000 mutant was generated using SOE-PCR to replace the *nosZ* ORF with a gentamicin resistance gene cassette as described (3). This mutation was moved into strain CMR15 with natural transformation (38). All strains were maintained in -80°C glycerol stocks and were cultured on solid CPG plates prior to growth in liquid media.

Bacterial growth was measured in modified VDM medium (0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.234 g $MgSO_4$, 2.5 g casamino acids, 50 mM sodium succinate, +/- 30 mM potassium nitrate) at 28°C under controlled O_2 conditions (3, 48). For routine culture bacteria were grown in rich CPG medium. Unless otherwise noted, media were inoculated to a starting $O.D._{600}$ of 0.001 (~$1x10^6$ CFU/mL) and endpoint data were collected 72 h post inoculation. All anaerobic growth was in BD GasPak systems in 1.7 mL tubes; $O.D._{600}$ was measured spectrophotometrically at the specified endpoint. Aerobic assays were incubated in a 28°C shaker at 225 rpm in 96-well plates sealed with breathable tape (Breathe-Easy membrane, Sigma-Aldrich). All other O_2 conditions were generated in a gas-controlled chamber (Ruskinn Invivo$_2$400, Sanford, ME) in 96-well plates sealed with breathable tape. For 96-well plate assays, a Synergy HT microtiter plate reader (Biotek Instruments, Winooski VT) was used to measure endpoint A_{600}.

To assess bacterial growth under a range of O_2 conditions, we stab-inoculated semi-solid VDM (0.2% noble agar) with ~$1x10^7$ CFU of each strain. Tubes were incubated at 28°C without shaking and visually assessed over the course of one week. In addition to growth measurements,

NO_2^- was quantified (reported as +/-) using Griess reactions (Molecular Probes, Inc. Eugene, OR) in lysed cell supernatant of anaerobic cultures following 3 h incubation at high cell densities ($O.D._{600}$ = 1.0; $1x10^9$ CFU/mL) as described (3). N_2 production was visually assessed as presence of gas bubbles over 96 h of anaerobic incubation in 1.7 mL tubes following inoculation at $O.D._{600}$ = 0.001. All assays were replicated 3 times per treatment per strain.

N_2O quantification. Cultures of representative strains from each phylotype were grown aerobically overnight in rich medium (CPG) in a 28°C shaker. Cultures were then diluted to an $O.D._{600}$ of 0.100 (~$1x10^8$ CFU/ml). 100 µl of culture was added to 100 ml of VDM media containing 10 mM NO_3^- in a 300 ml flask. For these assays NO_3^- concentration was reduced to 10 mM to avoid saturating the instrument. Flasks were sealed with a double-holed rubber-stopper with two glass tubes inserted for flushing the headspace and sampling gas. Flasks were flushed with >3 volumes of N_2 gas to create anaerobic conditions. Then glass tubes were stopped with small rubber septa. Media was not deoxygenated directly, because RSSC cultures rapidly deoxygenate media biologically (3). Cultures were incubated statically at 28°C for 24 h, at which point 100 µl of culture was removed and dilution plated to enumerate cell density. Nitrous oxide gas generated by denitrification was measured by using a needle and syringe to draw out a 300 mL gas sample from the top of the flask, while N_2 gas replaced withdrawn gas just above the culture level. Gas samples were placed in GC vials, injected into an Agilent 7890A GC System (Santa Clara, CA, USA) and analyzed as previously described (49).

Growth in host tomato plants and virulence assays. To measure pathogen growth *in planta*, 21 day-old tomato plants (wilt-susceptible cv. Bonny Best) were inoculated through a cut leaf petiole with 2000 CFU of RSSC as described (3). Two days post inoculation, 0.1 g stem tissue was collected from the midstem directly above the inoculated petiole, ground, and dilution plated to quantify bacterial population size. These assays contained 10 plants per strain.

To assess relative virulence, unwounded 21-day-old tomato plants were soil-soak inoculated with $1x10^8$ CFU of RSSC per g potting mix (Sunshine Redi-earth professional plant growth mix, Sun Gro Horticulture, Agawarm, MA). Symptoms were assessed daily using a

disease index based on percent of leaves wilted (0=healthy, 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100%) (50). Each assay contained 16 plants per treatment, and the assays were replicated three times.

Phylogenetic analysis. We selected 51 genomes that represent the genomic diversity of the RSSC (51). Using KBase (52), we built a phylogenetic tree by using the "Insert Set of Genomes into Species Tree" app. This KBase app creates an MSA from 49 well-conserved bacterial proteins and builds a tree with FastTree2 version 2.1.10 (53).

To expand the analysis beyond the RSSC, all complete genomes of Betaproteobacteria publicly available on NCBI at the time of this analysis (2012) were compared using MLST based on 31 loci as previously described (54). The ORFcore pipeline (55) and MUSCLE 3.7 (56) were used to extract, correct, align, and concatenate amino acid sequences. Fasttree v2.1.3 was used for tree construction (53). A gene was considered present if it shared >40% (or in the case of NosF >30%) amino acid identity over at least 70% of the length of the query sequence (*Pseudomonas stutzeri* CAA37) as determined with blastp in the BLAST+ package. Blastp was also used for amino acid comparisons across all available NosZ sequences on NCBI at the time of analysis (2015).

Protein sequence comparisons. Homologs of denitrification pathway genes were detected in genomes of strains GMI1000, CMR15, K60 and PSI07 using the MicroScope web interface, BLAST, and OMA (57). The corresponding protein sequences were then aligned using MUSCLE (56) and percentage identity in relation to strain GMI1000 was computed for each locus using the seqinr R package (58).

FNR binding site predictions. The Virtual Footprint Regulon Analysis program from PRODORIC predicted intergenic FNR binding sites across RSSC genomes using a weighted matrix generated from the *E. coli* FNR binding sequence (59). Sequence logos were generated with Weblogo (60), and the trimmed FNR binding site sequences from all four phylotypes. The PRODORIC Virtual Footprint Promoter Analysis program predicted the presence of FNR binding sites upstream of genes related to denitrification for the four representative strains with

closed genome sequences. Because its genome was then in draft form, the representative Phyl. II strain K60 was excluded from this analysis.

KEGG enrichment analysis. All RSSC genome sequences available in 2015were annotated using HMMer models for KEGG and Pfam as described (61). Amino acid identity (25% with 50% coverage) was used to cluster sequences *de novo* with proteinorthov5. Gene families that were over-represented in Phyl. I/III or Phyl. II/IV strains were identified using Fisher's exact test in the Python package scipy (62). The significance level was set at $P>0.05$.

Biofilm assays. Biofilm formation of each phylotype representative strain was assessed *in vitro* using the PVC plate-crystal violet stain assay (24). Values were reported as $O.D._{590}/O.D._{600}$ to normalize for minor differences in growth rates among strains.

Scanning electron microscopy. Three-week-old Bonny Best tomato plants were inoculated through a cut leaf petiole with $1x10^2$ CFU with RSSC GMI1000, K60, CMR15, or PSI07. At first sign of symptoms (DI=1), two thin slices were collected from each mid-stem following surface sterilization and were processed for SEM as described (26). Briefly, each section was incubated in primary fixative (2.5% glutaraldehyde, 2% formaldehyde, 0.003 M $MgCl_2$, 0.003 M $CaCl_2$ in 0.05 M PIPES buffer, pH 7) for 8 h, washed twice in 0.05 M PIPES buffer, incubated overnight in secondary fixative (1% OsO_4 in PIPES), rinsed in PIPES and transferred through a series of ethanol concentrations up to 100%. Samples were then dehydrated, sputter-coated in gold, and visualized with a Zeiss LEO 1530 high-resolution scanning electron microscope (Materials Sciences Center, University of Wisconsin-Madison). For each strain, 6-8 plants were assessed.

Bacterial streaming assay. Nineteen-day-old tomato plants were inoculated through a cut petiole with $1x10^3$ CFU of strain GMI1000, K60, CMR15, or PSI07 as described above. At the first sign of symptoms (DI=1), 1 cm of stem tissue from 0.5 cm below the inoculation site was excised, cut in half, and floated in 1 ml sterile water in a 24-well microplate. Samples were incubated for 90 min at room temperature with 85 rpm shaking before the stem section was removed and the escaped bacteria were quantified as $OD_{600\ nm}$ using a spectrophotometer.

Xylem sap oxygen measurement. Seventeen-day-old tomato plants were soil soak inoculated as described above with $1x10^8$ CFU/ml of either GMI1000, CMR15, K60, PSI07, or water. When symptoms first appeared, disease severity was rated, plants were de-topped and xylem sap rapidly pooled on the cut stem via natural root pressure. An oxygen microsensor probe (Unisense, Aarhus, Denmark) was immediately inserted into the pooled xylem sap and read until the signal was steady for 60 s. For an O_2 saturated water control, air was bubbled through distilled water for 5 min. The anoxic control solution was 0.1 M sodium ascorbate, 0.1 M sodium hydroxide in water. The O_2 content of stagnant deionized water was measured at each sampling point to ensure probe consistency.

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FIGURES

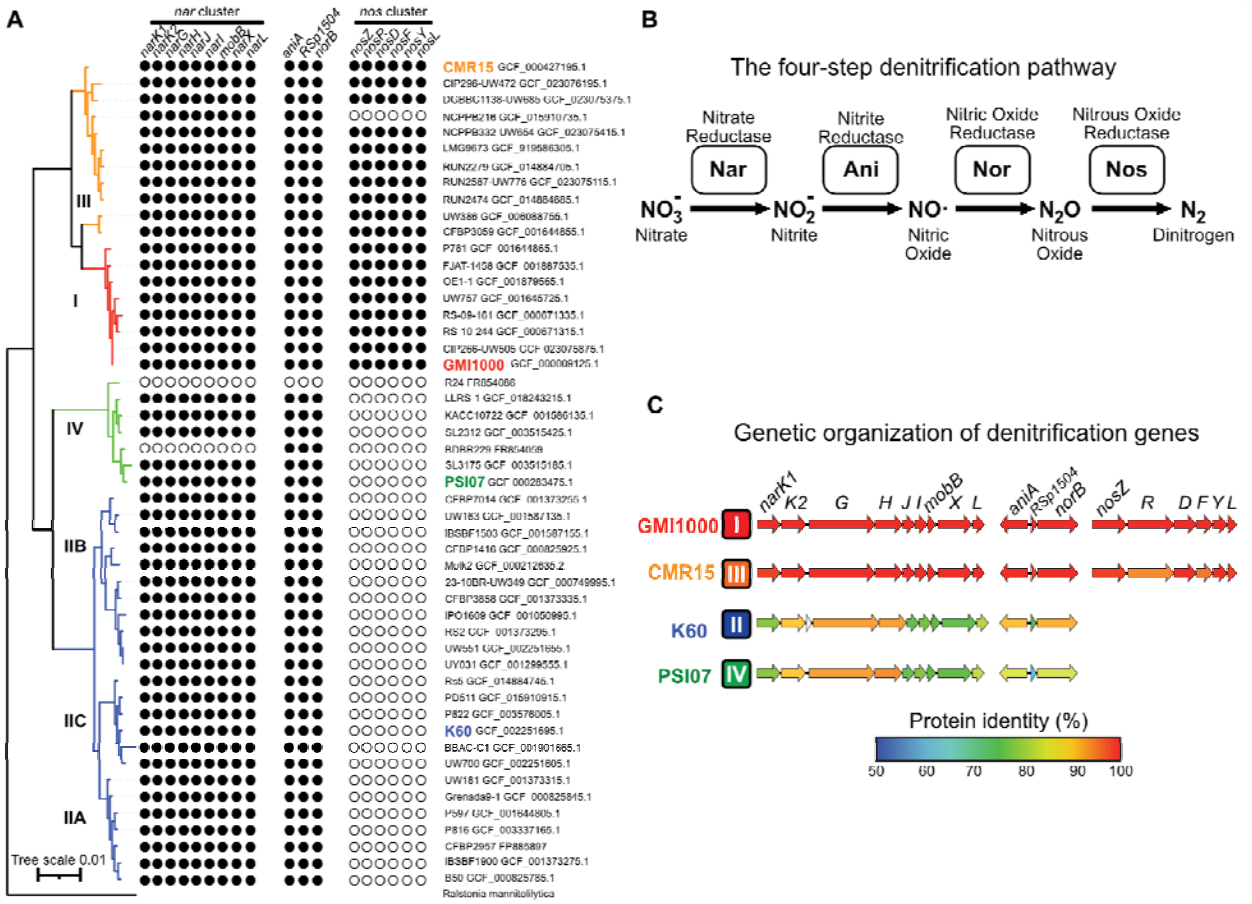


Figure 1. The first three steps of denitrification are well conserved across the RSSC, but only Phyl. I and III have the 6-gene *nos* cluster. A) A phylogenetic tree of 51 RSSC genomes that represent the described genomic diversity of the species complex. The tree was constructed in KBase using the Insert genomes into SpeciesTree app. The Newick file was modified in iToL and Affinity Designer. Denitrification genes were identified using the KBase BlastP app with a 40% identity and 70% length cut-off. **B)** The four-step denitrification pathway enzymes, substrates, and products. **C)** Using strain GMI1000 for comparison, the identity values of orthologous proteins were computed with the R package *seqinr* after aligning amino-acid sequences with MUSCLE. Protein percentage identity of each is color coded according to a 50 to 100% gradient. Phylotype is indicated to the right of each strain name. Note that the three gene clusters shown are not adjacent to each other, but they are all located on the megaplasmid.

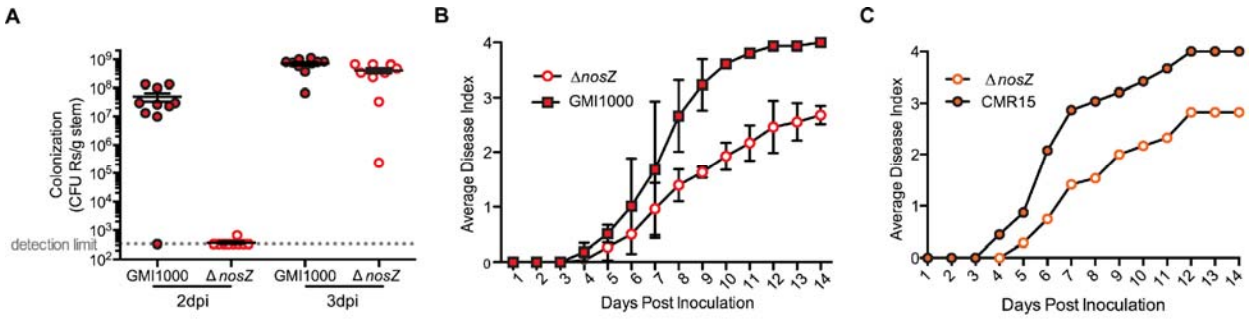


Figure 2. Strains that have *nos* genes require the complete denitrification pathway for full virulence on tomato. A) Two and three days after petiole inoculation of 21-day-old wilt-susceptible tomato plants with either wild-type GMI1000 or a Δ*nosZ* deletion mutant, stem sections were harvested and bacterial population sizes in stems were determined by serial dilution plating of ground stem sections. Each circle shows the bacterial population size in a single plant; horizontal bars represent median values; vertical bars indicate standard error of the mean. Data represent ten biological replicates per time point per strain. At both time points, population sizes of GMI1000 and Δ*nosZ* were significantly different (P=0.0066 at 2 dpi) and P=0.0150 at 3 dpi, two-tailed t-test). The 3 dpi data were published previously [3]. **B, C)** Symptom severity of RSSC-susceptible tomato plants was monitored daily following naturalistic soil-soak inoculation with $1x10^8$ CFU/g soil of either **B)** wild-type RSSC strain GMI1000 (Phyl. I) and GMI1000 Δ*nosZ* or **C)** wild-type RSSC strain CMR15 (Phyl. III) and CMR15 Δ*nosZ.* Each point indicates average symptom severity; bars in B reflect standard error of the mean of 3 assays, each with 16 plants per treatment (P<0.005, 2-way ANOVA). Representative data from one biological replicate containing 12 plants is depicted in panel C.

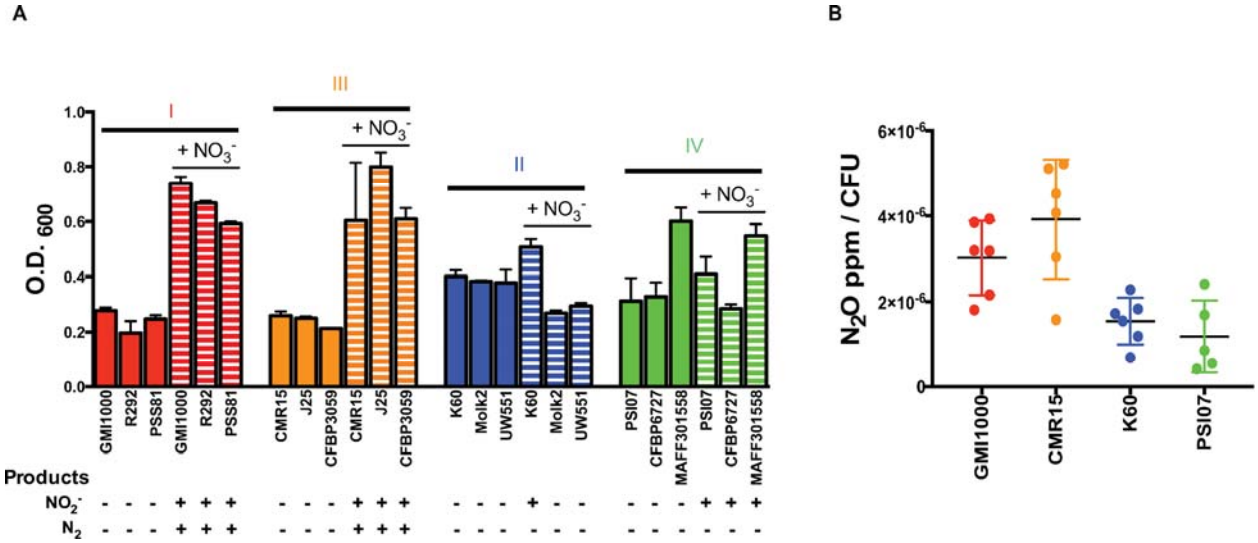


Figure 3. Presence of a functional *nos* cluster in RSSC strains correlates with ability to anaerobically grow on nitrate and produce N_2O. A) Using three representative strains per phylotype (as labeled on X-axis), cell cultures ($O.D._{600}$ 0.001) were incubated statically for 72 hours in VDM with or without 30 mM nitrate and endpoint growth was measured spectrophotometrically. Vertical bars represent standard error of the mean. Growth data were used with permission from [5]. In separate tubes, Dinitrogen (N_2) gas production was qualitatively monitored for 96 h in separate tubes. Nitrite (NO_2^-) was measured using Greiss reactions, using cultures of each strain in VDM inoculated to $O.D._{600}$ =1.0 and incubated for

3 hours anaerobically. Presence or absence of NO_2^- and N_2 are indicated with + and -. Data reflect 3 biological replicates per strain. **B)** Production of N_2O gas. Using a representative strain for each phylotype (GMI1000, K60, CMR15, PSI07), cell cultures ($O.D._{600}$ = 0.0001) were incubated anaerobically for 24 h in VDM with 10 mM NO_3^-. CFU was enumerated at time of gas collection. Data shown reflect 5 or 6 biological replicates. Asterisks indicate significantly different p-values (GMI vs K60=0.0055, GMI vs PSI07=0.0064, CMR vs K60=0.0030, CMR vs PSI07=0.0041, unpaired t-test).

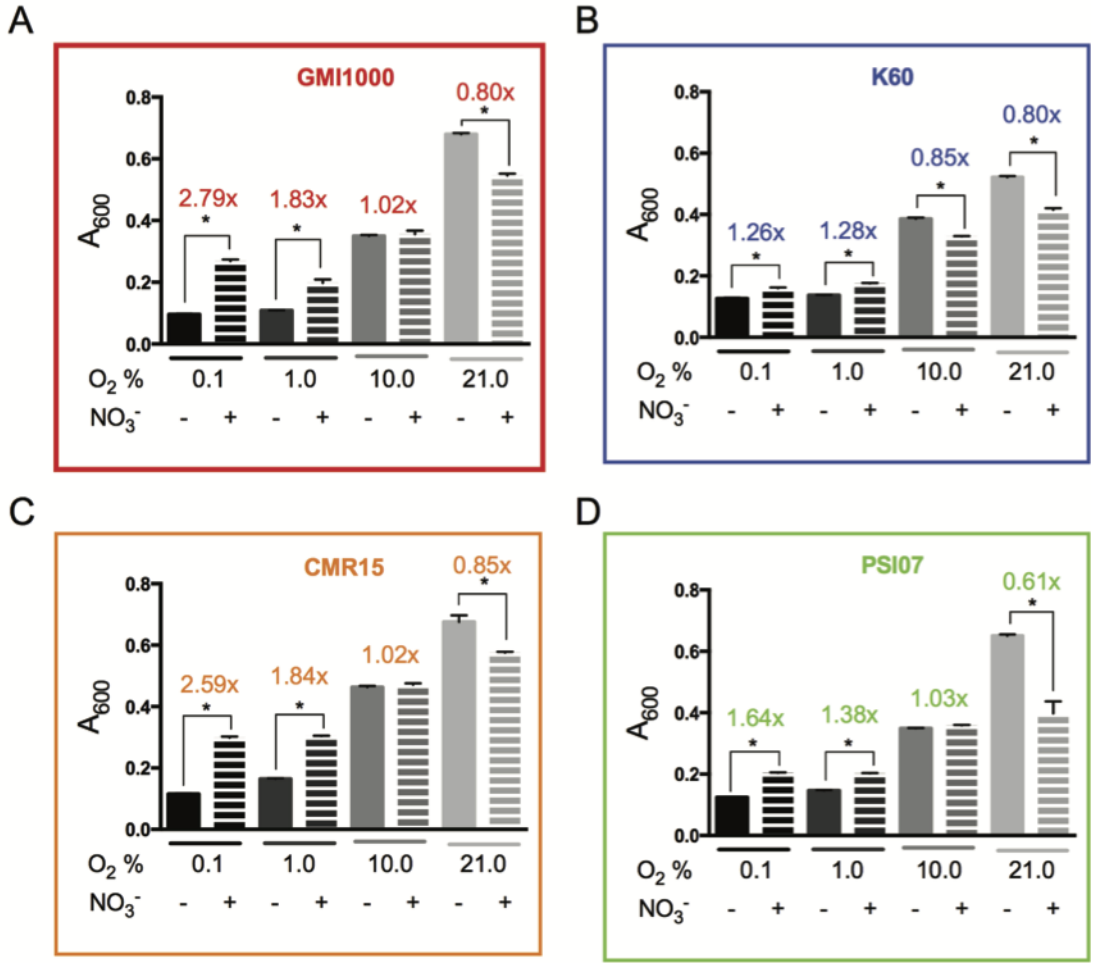


Figure 4. Nitrate enhances growth of RSSC strains under low-oxygen conditions, with the biggest impact on phylotypes I and III. Representative strains of each phylotype (A-D) were grown in denitrifying-favoring VDM broth with and without 30 mM nitrate and at various oxygen levels (0.1, 1.0, 10.0, and 21.0% oxygen). A_{600} was measured after 72 h growth at 28°C, with moderate shaking. Bars indicate standard error. Each treatment was repeated a total of 9 times. *P<0.05 by 2-tailed t-test.

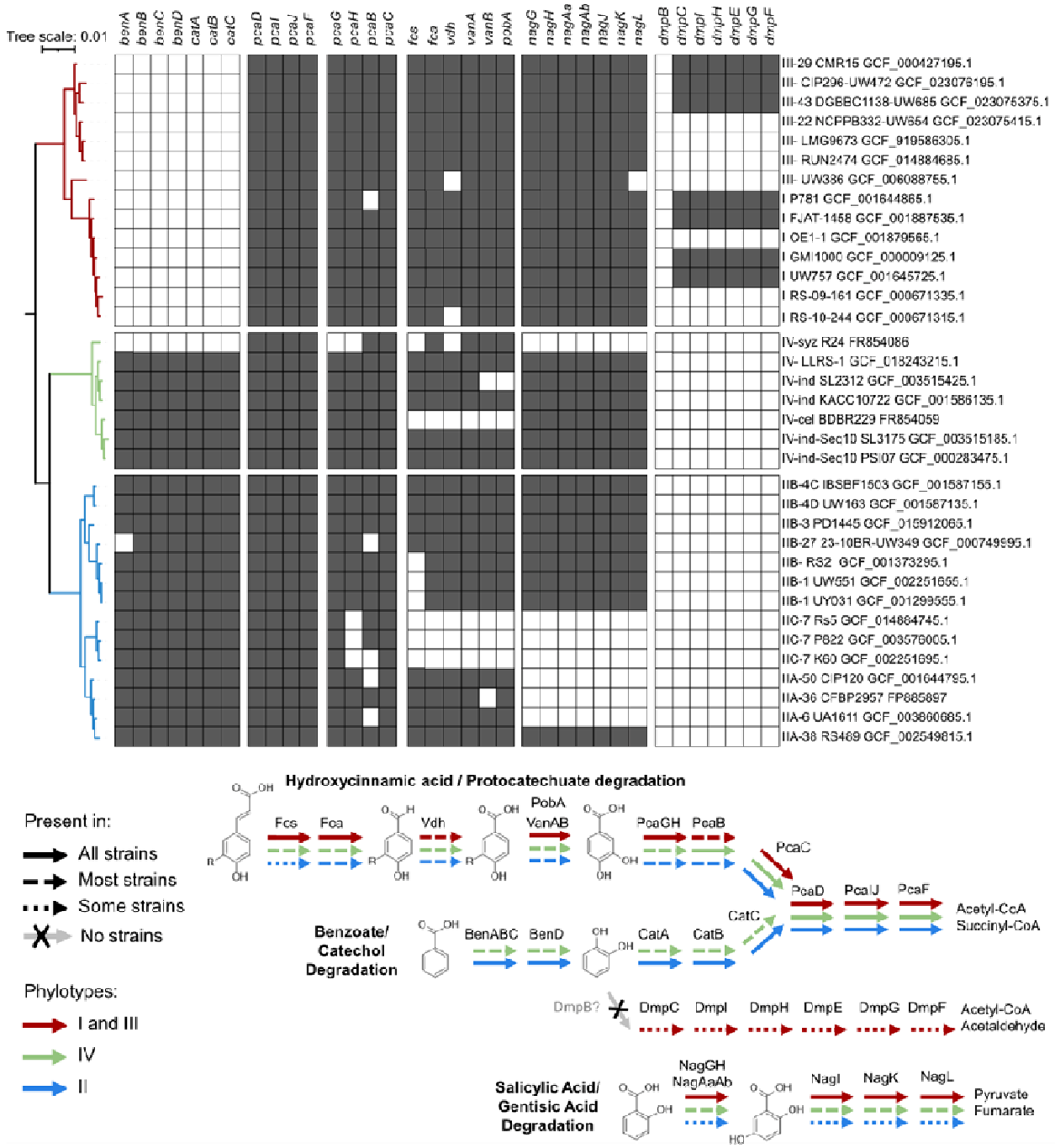


Figure 5. Variation in aromatic degradation pathways within the RSSC. Top: presence/absence of aromatic degradation genes across the species complex. Bottom: Summary of the conservation of the aromatic degradation pathways across distinct phylotypes. The benzoate/catechol degradation pathway and the hydroxycinnamic acid/protocatechuate pathways converge on the last four Pca enzymes. The phylogenetic tree was constructed based on 49 conserved genes using the KBase SpeciesTree app. The presence/absence of each gene was determined through BlastP analysis of the genomes.

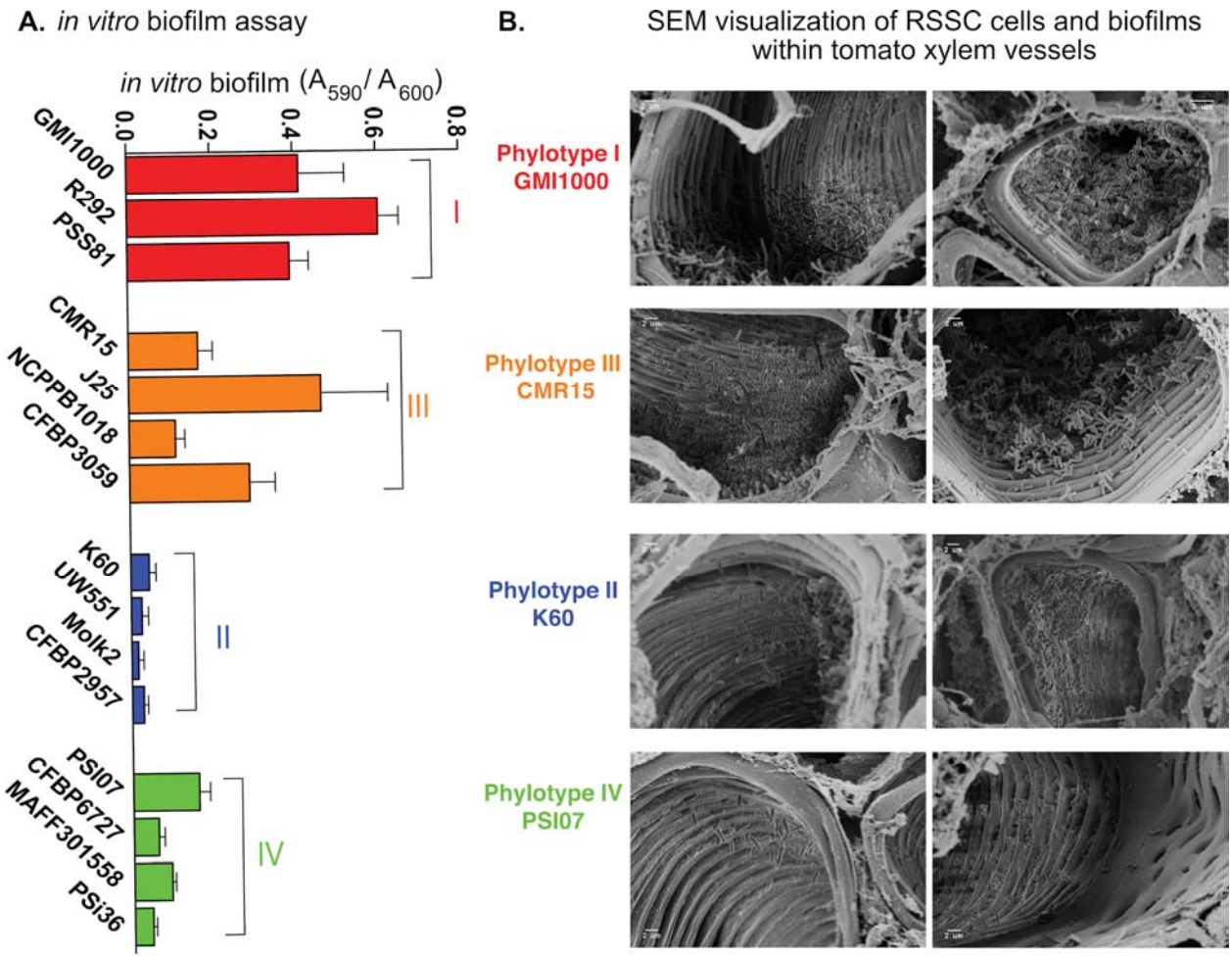


Figure 6. Complete denitrifying RSSC strains form more biofilm in culture and occupy different niches in tomato xylem during bacterial wilt disease. A) Using 3-4 representative strains per phylotype, biofilm formation was quantified using the PVC plate-crystal violet assay 96 well plates. Vertical bars represent standard error of 9 biological replicates. **B)** Representative SEM images showing stem cross-sections of tomato plants infected with a representative strain from each phylotype (GMI1000, K60, CMR15, PSI07), as indicated. Susceptible tomato plants (cv. Bonny Best) were soil-soak inoculated with ~$1x10^8$ CFU/mL bacterial suspension in 80 g soil. At the first sign of disease, stem samples were taken, sliced, fixed, plated with gold, and visualized with a Zeiss LEO 1530 high-resolution scanning electron microscope. SEM images were captured from two biological replicates of plant inoculations each, with three or four plants and two stem slices per plant.

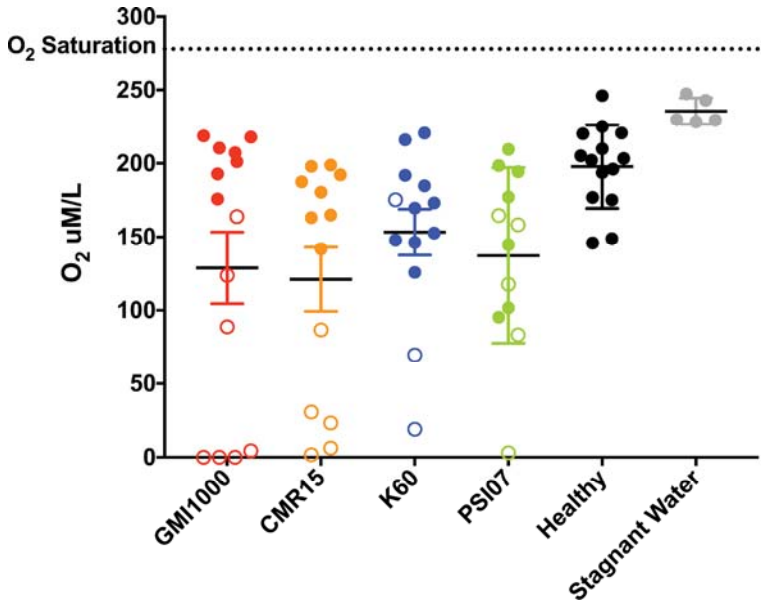


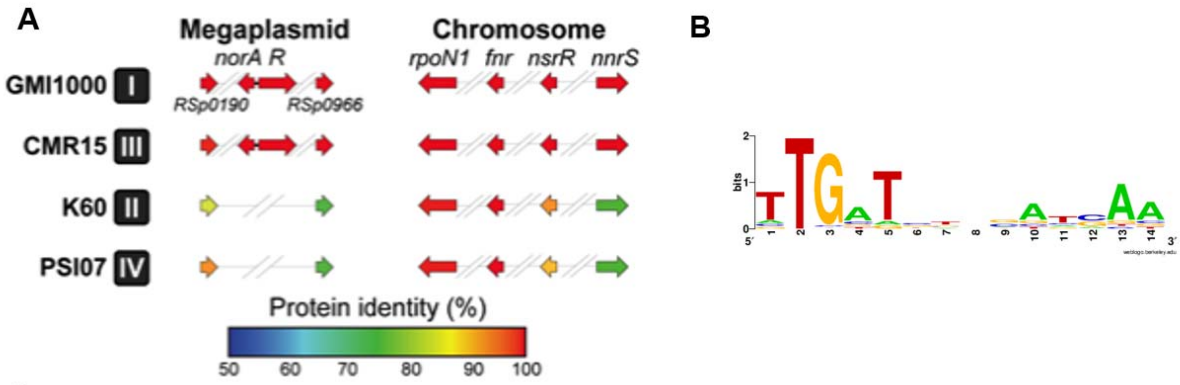
Figure 7. Xylem sap oxygen levels in healthy and RSSC-infected plants. A microsensor was used to measure oxygen levels in the xylem sap that exuded from a freshly-detopped tomato plant inoculated with water (Healthy) or with RSSC phylotype representatives, GMI1000, CMR15, K60, or PSI07. Each circle represents the O_2 level in one plant; N =12-14 plants/condition. Closed circles are levels from plants with mid-stage disease (1-74% leaf area wilted) and open circles are levels from plants with late stage disease (>75% leaf area wilted). Xylem sap from infected plants contained less O_2 than sap from healthy plants (P= 0.0443, 0.0015, 0.0125, and 0.0031 for healthy vs. GMI1000, CMR15, K60, and PSI07, respectively, Mann-Whitney test). All tested RSSC strains similarly reduced sap O_2 levels. Xylem sap from healthy plants contained less O_2 than stagnant water (P= 0.0005, Mann-Whitney test). Bars show standard error of the mean.

Supplementary Materials

Table S1. *Ralstonia* spp. strains used in this study.

Strain	Phyl	Host	Origin	Accession Number
GMI1000	**I**	**Tomato**	**Guyana**	**Genbank: NC_003295, NC_003296**
Y45	I	Tobacco	China	GenBank: AFWL00000000
F504, FQY_4	I	Tobacco	China	GenBank: NC_020799.1, 021745.1
YC45	I	Ginger	China	GenBank: CP011997.1, CP011998.1
Rs09-161	I	Eggplant	India	GenBank: CM002757.1, CM002758.1
Rs10-244	I	Pepper	India	GenBank: CM002755.1, CM002756.1
R292	I	Mulberry	China	NA
PSS81	I	Tomato	Taiwan	NA
K60	**II**	**Tomato**	**United States**	**GenBank: NCTK00000000**
B50	II	Banana	Brazil	EMBL: PRJEB7421
IBSBF1900	II	Banana	Brazil	EMBL: PRJEB8309
CFBP2957	II	Tomato	French West Indies	EMBL: FP885897, FP885907
UW181	II	Plantain	Venezuela	EMBL: PRJEB8309
Grenada9-1	II	Banana	Grenada	EMBL: PRJEB7428
Po82	II	Potato	Mexico	GenBank: CP002819, CP002820
UW179	II	Banana	Colombia	EMBL: PRJEB7426
UW163	II	Plantain	Peru	EMBL: PRJEB7430
IBSBF1503	II	Cucumber	Brazil	EMBL: PRJEB7433
CFBP6783	II	Heliconia	French West Indies	EMBL: PRJEB7432
CFBP7014	II	Anthurium	Trinidad	EMBL: PRJEB8309
CFBP1416	II	Plantain	Costa Rica	EMBL: PRJEB7434
CIP417	II	Banana	Philippines	EMBL: PRJEB7437
MolK2	II	Banana	Phillipines	GenBank: CAHW01000040
CFBP3858	II	Potato	Netherlands	EMBL: PRJEB8309
UW349	II	Potato	Brazil	GenBank: JQOI00000000.1
UW365	II	Potato	China	GenBank: JQSI00000000.1
IPO1609	II	Potato	Netherlands	GenBank: CU914168, CUP914166
UW551	II	Geranium	Kenya	GenBank: AAKL00000000
UY031	II	NA	Brazil	GenBank: CP012687.1, CP012688.1
UW491	II	Potato	Colombia	GenBank: JQSH00000000.1
RS2	II	Potato	NA	EMBL: PRJEB8309
CMR15	**III**	**Tomato**	**Cameroon**	**EMBL: FP885895, FP885896**
J25	III	Tomato	Kenya	NA
CFBP3059	III	Eggplant	Burkinia Faso	NA
PSI07	**IV**	**Tomato**	**Indonesia**	**EMBL: FP885906, FP885891**
R229	IV	Banana	Indonesia	EMBL: FP854059 to FR854085
KACC10722	IV	NA	South Korea	GenBank: CP014702.1, CP014703.1
R24	IV	Clove	Indonesia	EMBL: FR854086 to FR854092
CFBP6727	IV	Potato	Indonesia	NA
MAFF301558	IV	Potato	Japan	NA

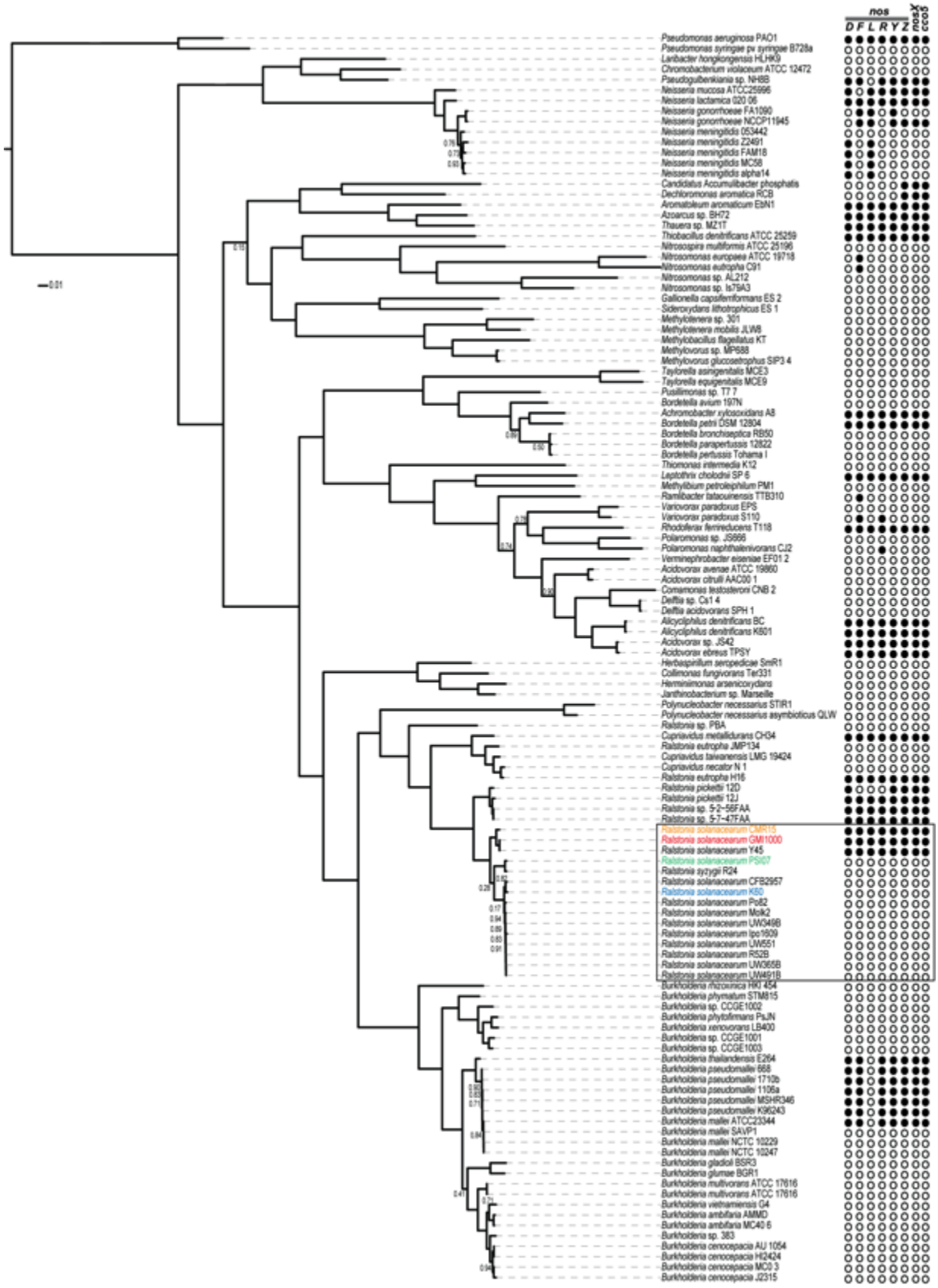
NA = not available



C

			GMI1000			CMR15			PSI07		
Gene	Locus tag in GMI1000	Gene Function	FNR Site?	Distance	Binding sequence	FNR Site?	Distance	Binding sequence	FNR Site?	Distance	Binding sequence
narK1	RSp0972	Nitrate importer	Yes	-200	TTGACGTGAATTAA	Yes	-209	TTGACATGAATTAA	Yes	-177	TTGACCGCTGTCAA
narG	RSp0974	Membrane bound nitrate reductase	Yes	-458	ATGATCCCGATCAT	Yes	-458	ATGATCCCGATCAT	Yes	-247	TTGATTCCAACCAG
nosZ	RSp1368	Nitrous oxide reductase	No			No			NA		
norA	RSp0958	Binds nitric oxide	Yes	-86	TTGATTAAAGGAAA	Yes	-86	TTGATTAAAGGAAA	NA		
norR	RSp0959	NorR family transcriptional regulator	May share with *norA*			May share with *norA*			NA		
aniA	RSp1503	Nitrite reductase	Yes	-156	TTGATGTATATCAG	Yes	-194	TTGATGTATATCAG	Yes	-223	TTGATCAACTCCAA
norB	RSp1505	Nitric oxide reductase	Yes and may share with *norB*	-96	CTGATATACATCAA	Yes and may share with *norB*	-96	CTGATATACATCAA	Yes	-201	TTGGAGTTGATCAA

Figure S1. Denitrification-related regulators and FNR predicted binding sites in genomes of phylotype representative strains in the RSSC. A) The presence or absence and % amino acid identity of all denitrification-associated regulators were determined, using the strain GMI1000 genome for comparison. The *norAR* gene pair is predicted to be nitric oxide-responsive, while *rpoN1* controls gene expression in response to nitrogen starvation and *nsrR* responds to nitrite/nitric oxide. NnrS in other systems responds to nitric oxide. The majority of the regulators, particularly FNR-like RSp0190, RSp0966 and *fnr* (RSc1283), are predicted to be oxygen-responsive. **B)** Predicted binding site consensus (logo) for the RSSC FNR-like regulon. **C)** Predicted FNR-like regulator binding sites located upstream of start site for inorganic nitrogen metabolism genes in strains GMI1000, CMR15, and PSI07.


nos
D F L R Y Z
nosX
ccoS
Pseudomonas aeruginosa PAO1
Pseudomonas syringae pv syringae B728a
Lariibacter hongkongensis HLHK9
Chromobacterium violaceum ATCC 12472
Pseudogulbenkiania sp. NH8B
Neisseria mucosa ATCC25996
Neisseria lactamica 020 06
Neisseria gonorrhoeae FA1090
Neisseria gonorrhoeae NCCP11945
Neisseria meningitidis 053442
Neisseria meningitidis Z2491
Neisseria meningitidis FAM18
Neisseria meningitidis MC58
Neisseria meningitidis alpha14
Candidatus Accumulibacter phosphatis
Dechloromonas aromatica RCB
Aromatoleum aromaticum EbN1
Azoarcus sp. BH72
Thauera sp. MZ1T
Thiobacillus denitrificans ATCC 25259
Nitrosospira multiformis ATCC 25196
Nitrosomonas europaea ATCC 19718
Nitrosomonas eutropha C91
Nitrosomonas sp. AL212
Nitrosomonas sp. Is79A3
Gallionella capsiferriformans ES 2
Sideroxydans lithotrophicus ES 1
Methylotenera sp. 301
Methylotenera mobilis JLW8
Methylobacillus flagellatus KT
Methylovorus sp. MP688
Methylovorus glucosetrophus SIP3 4
Taylorella asinigenitalis MCE3
Taylorella equigenitalis MCE9
Pusillimonas sp. T7 7
Bordetella avium 197N
Achromobacter xylosoxidans A8
Bordetella petri DSM 12804
Bordetella bronchiseptica RB50
Bordetella parapertussis 12822
Bordetella pertussis Tohama I
Thiomonas intermedia K12
Leptothrix cholodnii SP 6
Methylibium petroleiphilum PM1
Ramlibacter tataouinensis TTB310
Variovorax paradoxus EPS
Variovorax paradoxus S110
Rhodoferax ferrireducens T118
Polaromonas sp. JS666
Polaromonas naphthalenivorans CJ2
Verminephrobacter eiseniae EF01 2
Acidovorax avenae ATCC 19860
Acidovorax citrulli AAC00 1
Comamonas testosteroni CNB 2
Delftia sp. Cs1 4
Delftia acidovorans SPH 1
Alicycliphilus denitrificans BC
Alicycliphilus denitrificans K601
Acidovorax sp. JS42
Acidovorax ebreus TPSY
Herbaspirillum seropedicae SmR1
Collimonas fungivorans Ter331
Herminiimonas arsenicoxydans
Janthinobacterium sp. Marseille
Polynucleobacter necessarius STIR1
Polynucleobacter necessarius asymbioticus QLW
Ralstonia sp. PBA
Cupriavidus metallidurans CH34
Ralstonia eutropha JMP134
Cupriavidus taiwanensis LMG 19424
Cupriavidus necator N 1
Ralstonia eutropha H16
Ralstonia pickettii 12D
Ralstonia pickettii 12J
Ralstonia sp. 5-2~56FAA
Ralstonia sp. 5-7~47FAA
Ralstonia solanacearum CMR15
Ralstonia solanacearum GMI1000
Ralstonia solanacearum Y45
Ralstonia solanacearum PSI07
Ralstonia syzygii R24
Ralstonia solanacearum CFB2957
Ralstonia solanacearum K60
Ralstonia solanacearum Po82
Ralstonia solanacearum Molk2
Ralstonia solanacearum UW349B
Ralstonia solanacearum Ipo1609
Ralstonia solanacearum UW551
Ralstonia solanacearum R52B
Ralstonia solanacearum UW365B
Ralstonia solanacearum UW491B
Burkholderia rhizoxinica HKI 454
Burkholderia phymatum STM815
Burkholderia sp. CCGE1002
Burkholderia phytofirmans PsJN
Burkholderia xenovorans LB400
Burkholderia sp. CCGE1001
Burkholderia sp. CCGE1003
Burkholderia thailandensis E264
Burkholderia pseudomallei 668
Burkholderia pseudomallei 1710b
Burkholderia pseudomallei 1106a
Burkholderia pseudomallei MSHR346
Burkholderia pseudomallei K96243
Burkholderia mallei ATCC23344
Burkholderia mallei SAVP1
Burkholderia mallei NCTC 10229
Burkholderia mallei NCTC 10247
Burkholderia gladioli BSR3
Burkholderia glumae BGR1
Burkholderia multivorans ATCC 17616
Burkholderia multivorans ATCC 17616
Burkholderia vietnamiensis G4
Burkholderia ambifaria AMMD
Burkholderia ambifaria MC40 6
Burkholderia sp. 383
Burkholderia cenocepacia AU 1054
Burkholderia cenocepacia H2424
Burkholderia cenocepacia MC0 3
Burkholderia cenocepacia J2315
0.01

804

Figure S2. The *nos* cluster, containing the genes required for the last step of denitrification, has been repeatedly laterally transferred across the Betaproteobacteria. All complete Betaproteobacteria genomes from NCBI available in 2012 were compared via MLST using 31 loci as described (22). The corresponding sequences were extracted, corrected, aligned and concatenated using the ORFcor pipeline developed by Klassen and Currie (23) and MUSCLE 3.7 was used for alignment. The tree was constructed using Fasttree v2.1.3 and bootstrap values are found at the nodes. Any values above 0.95 were removed for simplicity. Nos protein sequences were identified using a strict threshold for annotation (>40% identity over 70% of the query sequence length for all but NosF, which had a cut-off of 30% because it is more diverse). *P. stutzeri* CAA37 Nos sequences were used as the query sequences using blastp in the BLAST+ package. Circles represent the 6 standard components of the nitrous oxide reductase gene cluster (listed in alphabetical order from *nosD* to *nosZ*) and 2 additional genes (*nosX* and *cco5*) that often co-segregate. White circles indicate absence of a gene and black circles indicate its presence. All strains in the RSSC are framed in the black rectangle. Strains chosen to represent each of the four phylotypes are in color: phylotype I representative, GMI1000, is in red; phylotype II K60 in blue; phylotype III CMR15 in orange; phylotype IV PSI07 in green.

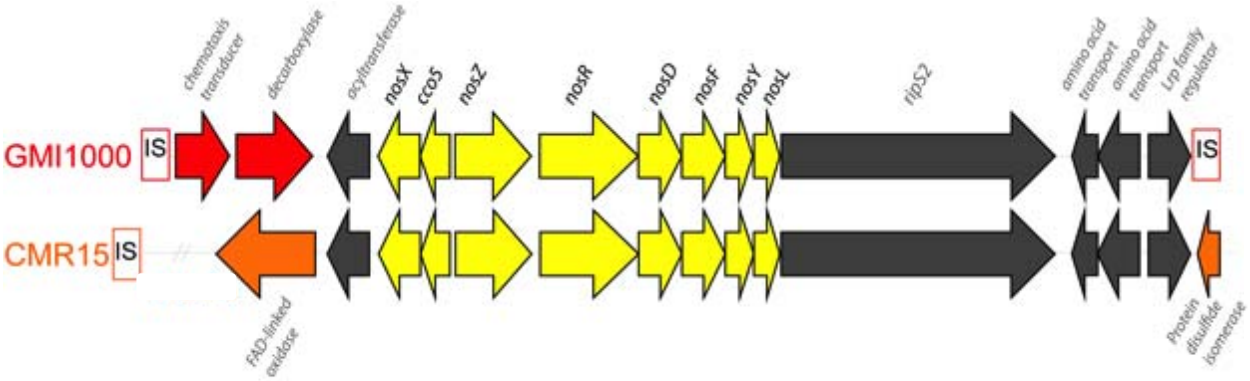


Figure S3. *R. solanacearum* strains in phylotypes I and III share a highly conserved nitrous oxide reductase gene cluster (in yellow). All genes near the *nosZ* ORF that lie between two predicted insertion sequences in phyl. I strain GMI1000 genome (locus tags RSp1362-RSp1378) are aligned to the region in phyl. III strain CMR15 (CMR15_mp30001-CMR15_mp30026). Genes found only in GMI1000 are in red while genes in orange are specific to CMR15. Predicted functions based on NCBI and Phyre2 protein structure modeling are listed in gray italics near each ORF lacking an annotation.

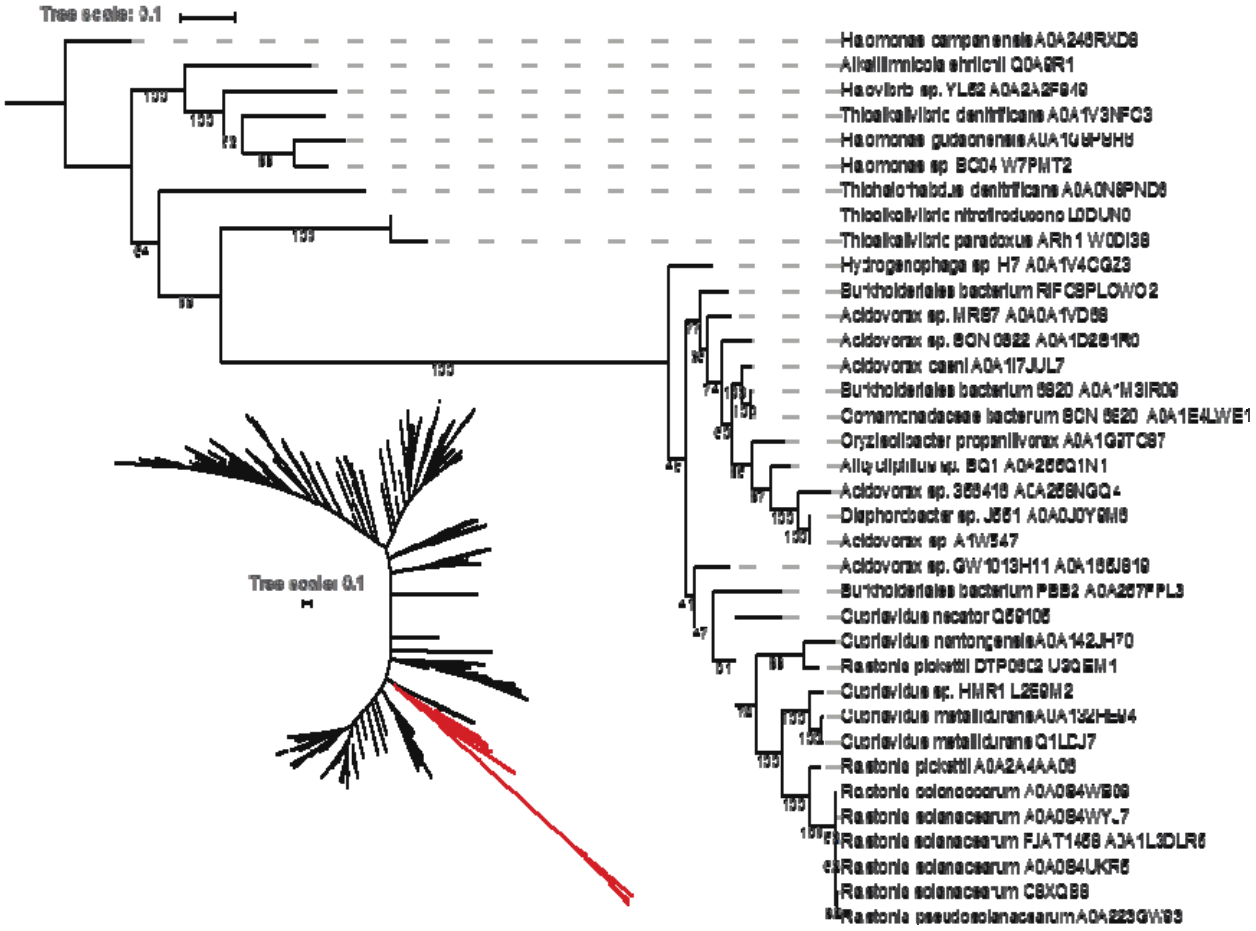


Figure S4. NosZ protein phylogeny. Inset: Full phylogeny of all NosZ protein sequences from uniprot. The clade containing *Ralstonia* is highlighted in red. The clade containing Uniprot proteins from the RSSC is shown in detail; they form a monophyletic clade of highly related sequences.

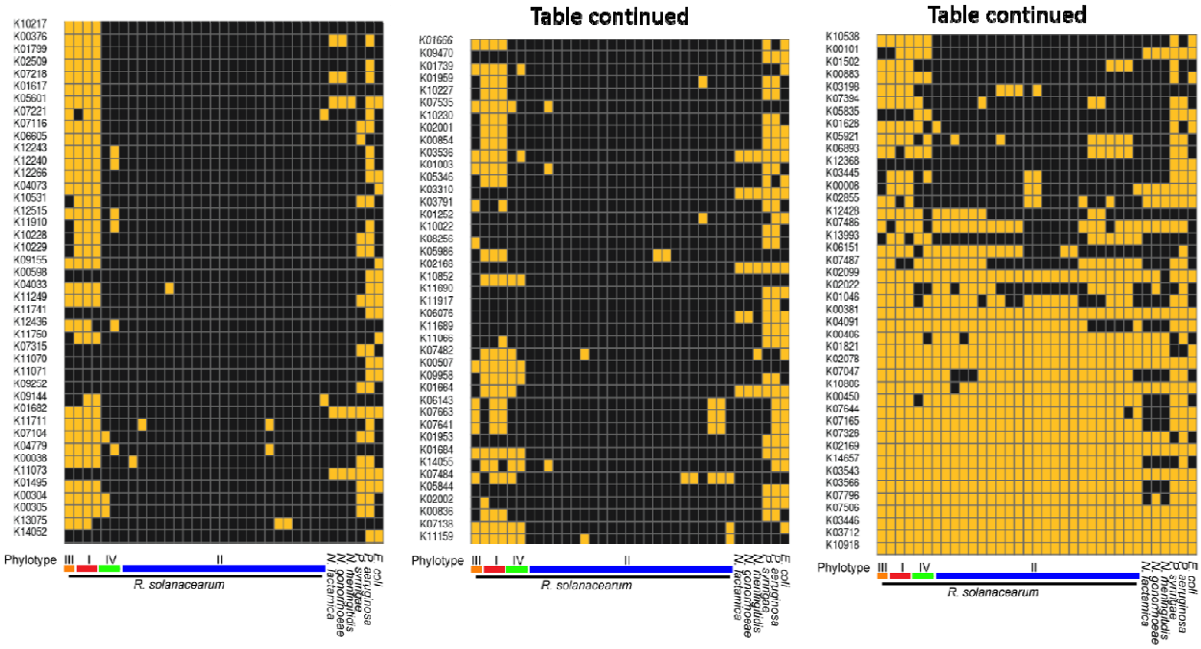


Figure S5. Over-represented KEGG groups in RSSC phylotype I and III strains. HMMer models for KEGG were used to identify the over-represented KEGG families in *R. solanacearum*. Complete denitrifiers I and III shown in red and orange respectively, while incomplete denitrifiers II and IV are shown in blue and green respectively. Presence/absence of overrepresented KEGG families depicted above as yellow or black respectively. Distantly related bacteria with and without complete denitrification were used for comparison (right side of figure).

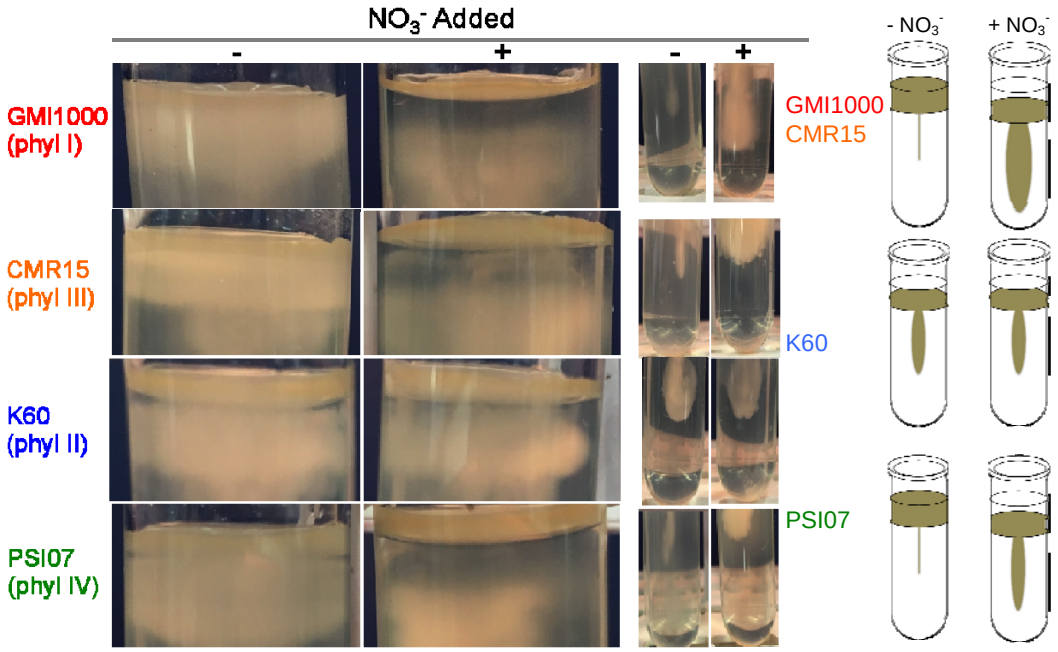


Figure S6. Oxygen preference assessments of *R. solanacearum* phylotype representatives. CPG overnight cultures of each strain were pelleted and re-suspended in water to an $O.D._{600}$ of 1.0. 10uL of these cell suspensions were stab inoculated and slowly released by a pipette into a plastic tube filled with

20 mL 0.2% semisolid VDM agar with or without nitrate added. Following one week of 28°C incubation without shaking, tubes were imaged. To the right, diagrams depict the general trends.

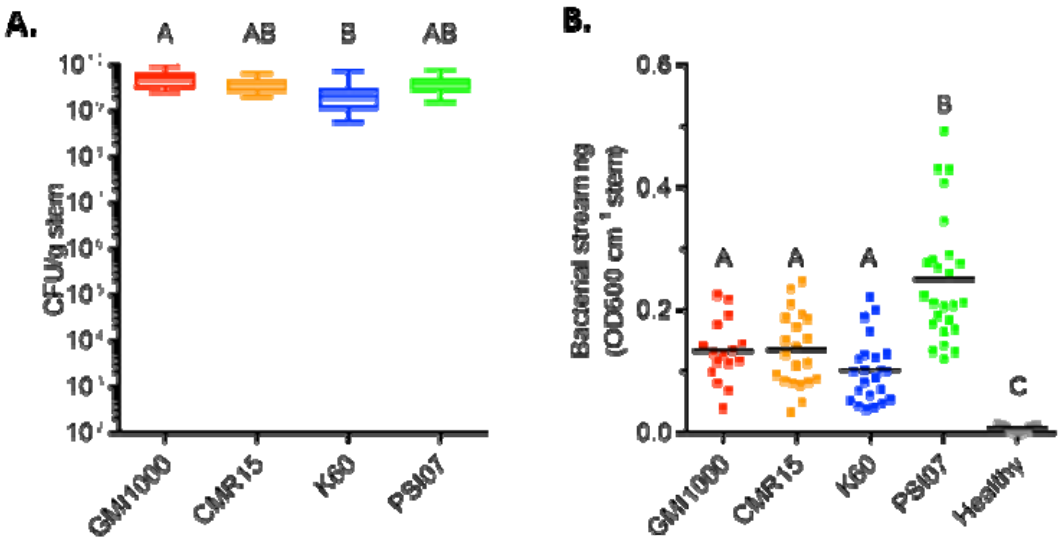


Figure S7. Complete and incomplete RSSC denitrifiers adhere to xylem differently. Representative tomato-colonizing strains (GMI1000, K60, CMR15, PSI07) were inoculated into tomato stem via cut petiole inoculation ($1x10^3$ CFU). At the first sign of wilting symptoms, **(A)** total bacterial populations in the stem were enumerated by dilution plating or **(B)** bacterial attachment to stem xylem was assessed by measuring planktonic cells that floated or swam out of cut stem tissue. One cm of stem was incubated in water with 85 rpm shaking. After 90 min, bacterial density in the water was measured by $OD_{600\ nm}$. Letters indicate $P<0.05$ by ANOVA with Tukey's multiple comparison test; (A) N=14-15 plants and (B) N=16-25 plants per condition.