**Ralstonia solanacearum** Uses Inorganic Nitrogen Metabolism for Virulence, ATP Production, and Detoxification in the Oxygen-Limited Host Xylem Environment

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**ABSTRACT** Genomic data predict that, in addition to oxygen, the bacterial plant pathogen *Ralstonia solanacearum* can use nitrate (NO$_3^-$), nitrite (NO$_2^-$), nitric oxide (NO), and nitrous oxide (N$_2$O) as terminal electron acceptors (TEAs). Genes encoding inorganic nitrogen reduction were highly expressed during tomato bacterial wilt disease, when the pathogen grows in xylem vessels. Direct measurements found that tomato xylem fluid was low in oxygen, especially in plants infected by *R. solanacearum*. Xylem fluid contained ~25 mM NO$_3^-$, corresponding to *R. solanacearum*’s optimal NO$_3^-$ concentration for anaerobic growth *in vitro*. We tested the hypothesis that *R. solanacearum* uses inorganic nitrogen species to respire and grow during pathogenesis by making deletion mutants that each lacked a step in nitrate respiration ($\Delta$narG), denitrification ($\Delta$aniA, $\Delta$norB, and $\Delta$nosZ), or NO detoxification ($\Delta$hmpX). The $\Delta$narG, $\Delta$aniA, and $\Delta$norB mutants grew poorly on NO$_3^-$ compared to the wild type, and they had reduced adenylate energy charge levels under anaerobiosis. While NarG-dependent NO$_3^-$ respiration directly enhanced growth, AniA-dependent NO$_3^-$ reduction did not. NO$_3^-$ and NO inhibited growth in culture, and their removal depended on denitrification and NO detoxification. Thus, NO$_3^-$ acts as a TEA, but the resulting NO$_2^-$ and NO likely do not. None of the mutants grew as well as the wild type *in planta*, and strains lacking AniA (NO$_3^-$ reductase) or HmpX (NO detoxification) had reduced virulence on tomato. Thus, *R. solanacearum* exploits host NO$_3^-$ to respire, grow, and cause disease. Degradation of NO$_3^-$ and NO is also important for successful infection and depends on denitrification and NO detoxification systems.

**IMPORTANCE** The plant-pathogenic bacterium *Ralstonia solanacearum* causes bacterial wilt, one of the world’s most destructive crop diseases. This pathogen’s explosive growth in plant xylem is poorly understood. We used biochemical and genetic approaches to show that *R. solanacearum* rapidly depletes oxygen in host xylem but can then respire using host nitrate as a terminal electron acceptor. The microbe uses its denitrification pathway to detoxify the reactive nitrogen species nitrite (a product of nitrate respiration) and nitric oxide (a plant defense signal). Detoxification may play synergistic roles in bacterial wilt virulence by converting the host’s chemical weapon into an energy source. Mutant bacterial strains lacking elements of the denitrification pathway could not grow as well as the wild type in tomato plants, and some mutants were also reduced in virulence. Our results show how a pathogen’s metabolic activity can alter the host environment in ways that increase pathogen success.

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Diverse bacteria are capable of respiratory nitrate reduction, the membrane-bound conversion of nitrate (NO$_3^-$) to nitrite (NO$_2^-$), and denitrification, the stepwise enzymatic reduction of NO$_3^-$ to nitrogen gas (N$_2$) via nitric oxide (NO) and nitrous oxide (N$_2$O). NO$_3^-$ reduction and denitrification enable respiration in hypoxic or anaerobic environments because nitrogen oxides can serve as terminal electron acceptors (TEAs) in place of oxygen (O$_2$) (1). Bacterial NO$_3^-$ respiration and denitrification are major drivers of the global nitrogen (N) cycle, of nutrient losses from soils, and of climate change (2-4). Though it has been extensively investigated in mutualistic N-fixing bacteria, the role of inorganic N metabolism in the virulence of plant pathogens is largely unexplored (5,6). This may be because energetic metabolism has been seen as biologically distinct from virulence. However, bacteria need energy to produce virulence factors and grow inside the host, which are both essential processes for pathogenesis (7-9). Studies of several animal-pathogenic bacteria reveal that the host environment induces microbial nitrate respiration and/or denitrification and that these bacterial inorganic N reductions affect virulence (10-12).

The soilborne plant-pathogenic bacterium *Ralstonia solanacearum* invades plants through their roots. It aggressively colonizes the xylem elements in its host’s vascular system, blocking water transport so that infected plants wilt and die (13). The bacterium
thrive in plant xylem, quickly reaching densities upwards of 10^9 CFU/g of stem tissue (14). This rapid growth is puzzling, because xylem sap is relatively nutrient poor and, presumably, low in O_2 (13, 15). R. solanacearum carries genes encoding three types of terminal oxidases (cytochrome bd oxidase, cytochrome c bb_2-type oxidase, and cytochrome c aa_3-type oxidase). We previously found that one of the bb_2-type oxidases is required for normal microaerobic growth and contributes significantly to the virulence and multiplication of R. solanacearum during plant infection (16). While these observations indicate that the pathogen uses O_2 during growth in the host, it is likely that R. solanacearum often encounters anaerobic conditions in its soil, rhizosphere, and plant habitats.

A preliminary analysis found that tomato xylem sap does contain significant levels of NO_3, which is an excellent TEA. In terms of redox potential, it is nearly as suitable as O_2 (16, 17). Additionally, in response to pathogen infection, plants, like mammals, typically produce NO, another potential electron sink for denitrifying microbes (1, 18). NO can also limit microbial growth by damaging DNA, sequestering important metal cofactors, and inhibiting terminal oxidase function to arrest aerobic respiration (19–22). High NO levels in host tissue could possibly force a plant pathogen to either use a TEA other than O_2 or halt growth.

The genome of R. solanacearum strain GMI1000 appears to encode a complete denitrification pathway, which includes a respiratory NO_3 reductase (NarG), a NO_2 reductase (AniA), a NO reductase (NorB), and a N_2O reductase (NosZ). The genome also includes a gene for a predicted flavohemoglobin (HmpX) that can detoxify NO either aerobically, by converting it to NO_3, or anaerobically, by converting it to N_2O. NorB can also convert NO anaerobically to N_2O (5). A transcriptomic analysis of R. solanacearum gene expression early in bacterial wilt disease revealed that all of these genes were highly expressed during tomato pathogenesis (17).

Together, these observations suggested that this vascular pathogen may respire and generate energy using inorganic N species as TEAs in low-O_2 and/or NO-rich microenvironments of plant xylem vessels. To test this hypothesis, we constructed a set of five single-gene deletion mutants of R. solanacearum strain GMI1000 that lacked narG, aniA, norB, nosZ, or hmpX. We analyzed the ability of these mutants to respire on NO_3, tolerate NO growth in plants, and cause bacterial wilt disease. We found that although the bacterium resired on NO_3 in the presence of O_2, NO_3 respiration contributed directly to growth only under low-oxygen or anaerobic conditions. NO_2 did not support bacterial growth; indeed, NO_2 inhibited growth in a dose-dependent fashion. Both HmpX and NorB detoxified NO under aerobic and microaerobic conditions, but only NorB could degrade NO under anaerobic conditions. During plant infection, R. solanacearum quickly consumed O_2 and actively reduced inorganic N. NO_3 respiration, denitrification, and aerobic NO detoxification all contributed to R. solanacearum’s growth in planta.

RESULTS
Ralstonia solanacearum expresses its inorganic nitrogen metabolic pathways in planta during bacterial wilt disease. A recent transcriptomic study (17) found that predicted genes for NO_3 respiration and denitrification were highly induced in R. solanacearum during tomato plant infection (Fig. 1A; see Table S1 in the supplemental material). The gene encoding the major catalytic subunit of the predicted respiratory NO_3 reductase ( NarG) was expressed at 30.6-fold-higher levels in planta than in culture in rich CPG medium. CPG is composed of peptone, casamino acids, glucose, and yeast extract (with small amounts of NO_3) (23). Similarly, genes predicted to encode the catalytic subunits of NO_2 reductase (AniA), NO reductase (NorB), and N_2O reductase (NosZ) were strongly induced in planta (86.8-fold, 51.3-fold, and 26.8-fold, respectively). In addition, the gene encoding the NO-detoxifying HmpX was also induced 43.2-fold during plant infection. The absolute expression values of these genes were among the highest in the bacterium’s in planta transcriptome (see Table S1 in the supplemental material), indicating that R. solanacearum is actively metabolizing these inorganic N species during growth in host plant xylem vessels.

The general cellular context of the R. solanacearum denitrifying pathway and associated reactions, taken from genomic predictions, is shown in Fig. 1B (1). Briefly, NO_3 can freely cross the outer cell membrane and is transported into the cytoplasm by NarK1 and/or NarK2 (24). In the cytoplasm, NarG reduces the NO_3 to NO_2. This reaction pumps protons across the membrane into the periplasm, adding to the proton motive force generated in earlier steps of the electron transport chain. This proton motive force leads to ATP production. The NO_3 importer exports NO_3 from the cytoplasm to the periplasm, where AniA reduces it to NO. This reaction, along with the rest of the denitrification process, takes place in the periplasm and does not directly add to proton motive force. Instead, periplasmic reductase enzymes aid growth in some organisms by transferring electrons to their substrates, which allows for continued metabolic flow and continued proton motive force generation via early electron trans-
port chain steps. In this way, NO is reduced by NorB. The resulting N₂O is reduced by NosZ to dinitrogen gas (N₂), which can freely dissipate into the extracellular environment.

To characterize the contributions of NO₃⁻ respiration, denitrification, and NO detoxification to *R. solanacearum*’s growth in culture, as well as to its growth and virulence in tomato plants, we generated five deletion mutant strains lacking the predicted narG, aniA, norB, nosZ, and hmpX genes, together with the corresponding complemented strains, with the exception of nosZ (see Table S2 in the supplemental material).

*R. solanacearum* uses narG-dependent nitrate respiration for growth under the low-oxygen conditions found in planta during disease. Denitrification and NO₃⁻ respiration are typically low-O₂-to-anaerobic behaviors (1). To define the O₂ concentrations that support *R. solanacearum* NO₃⁻ respiration, we measured the growth of the bacterium supplied with NO₃⁻ in a range of atmospheric O₂ levels (20.9, 15, 10, 5, 1, 0.5, 0.1, and 0% O₂). To ensure maximum gas exchange, this growth assay was carried out in 10-ml cultures growing in 125-ml flasks. We used Van den Mooter (VDM) medium with 10 mM NO₃⁻, which contains casamino acids, sodium succinate, magnesium sulfate, potassium phosphate, and KNO₃ and supports respiration of inorganic nitrogen by *R. solanacearum* (25). To determine how much NO₃⁻-supported growth was due to activity of the predicted NarG, we included the ΔnarG mutant and its complemented strain in this assay. All of the strains tested grew best when supplied with 15 or 10% O₂ (Fig. 2A). However, narG was required for full growth at O₂ levels below 1%. The growth defect of the ΔnarG strain was fully restored in the complemented ΔnarG strain.

It has been postulated that bacteria experience a low-oxygen environment in plant xylem. To the best of our knowledge, however, the O₂ levels in infected tomato plants have not been directly measured (15). To determine whether the plant xylem is a permissive environment for bacterial NO₃⁻ respiration and denitrification, we used a microprobe to measure the O₂ content of tomato plant xylem sap. The xylem sap of healthy 3-week-old tomato plants contained 146.2 μM O₂ on average (Fig. 2B). There was no difference between O₂ measurements when the probe was inserted directly into xylem vessels or inserted into xylem sap accumulated on the surface of a newly cut stem (data not shown). We therefore took measurements from xylem sap accumulated on the cut stem surface, because this reduced the likelihood of breaking the delicate microprobe. Sap from healthy plants contained O₂ concentrations similar to those in sterile, nonaerated liquid growth medium (data not shown). However, xylem sap from plants infected with wild-type *R. solanacearum* contained significantly less oxygen, with an average of 70.87 μM O₂ (P = 0.0003, t test) (Fig. 2B). These values varied widely, ranging from 2.2 to 133 μM O₂. The lower O₂ concentrations were detected in plants with severe bacterial wilt symptoms, which harbored *R. solanacearum* populations of >10⁹ CFU/g stem. The in planta data demonstrate that the bacterial wilt pathogen consumes O₂ in host tissue and therefore experiences a low-O₂ environment during plant infection, particularly at the later stages of disease. Although these in planta measurements are consistent with the *in vitro* findings described above, they cannot be directly compared. The probe measured molar concentrations of dissolved O₂, while the controlled-O₂ chamber used for the *in vitro* studies delivered a known percentage of atmospheric O₂.

Reduction of nitrate, nitrite, and nitric oxide is required for growth of *R. solanacearum* at wild-type rates under anaerobic conditions. We hypothesized that, in addition to NO₃⁻ respiration (Fig. 2A), the denitrification pathway of *R. solanacearum* is required for growth under anaerobic conditions in the presence of NO₃⁻. We tested this by measuring the optical densities (ODs) of wild-type, ΔnarG, ΔaniA, ΔnorB, and ΔnosZ *R. solanacearum* strains following 24 h of stagnant anaerobic incubation in VDM medium with NO₃⁻ (Fig. 3A) (25). We included the ΔhmpX strain as a control because hmpX is not predicted to be involved in respiratory N metabolism but only in cytoplasmic NO consumption (26, 27). All of the strains tested reached endpoint optical densities below that of the wild-type parent strain, although the optical densities of the wild-type, ΔnosZ, and ΔhmpX strains were not significantly different. The ΔnarG, ΔaniA, and ΔnorB mutants grew less than the wild-type strain (P < 0.05, t test). The ΔnorB strain had the most drastic growth defect. Adding a complementing wild-type gene fully or partially restored anaerobic growth on NO₃⁻ to all deletion mutants. The complemented ΔaniA strain did not reach wild-type levels, but this strain did grow significantly better than its ΔaniA parent (P < 0.05, t test). Under aerobic conditions, all strains grew equally well, and the results were statistically indistinguishable (P > 0.05, t test) (Fig. 3B).
Nitrate supports the growth of *R. solanacearum* in a dosedependent manner, with an optimal concentration near that found in planta. To understand whether NO$_3^-$ respiration and denitrification directly or indirectly contribute to bacterial growth, we tested the ability of wild-type *R. solanacearum* to grow under microaerobic (0.1% O$_2$) conditions in VDM medium with NO$_3^-$ or NO$_2^-$ concentrations ranging from 0 to 100 mM (Fig. 4A and 5A). While *R. solanacearum* grew no better on 10 µM or 100 µM NO$_3^-$ than in the absence of NO$_3^-$ (data not shown), the pathogen did grow better on NO$_3^-$ at 1 mM (P = 0.047, repeated measures analysis of variance [ANOVA]), 10 mM (P = 0.0098, repeated measures ANOVA), 25 and 50 mM (P = 0.0021, repeated measures ANOVA), and 100 mM (P = 0.0015, repeated measures ANOVA). The bacterium grew optimally in culture at 25 to 50 mM NO$_3^-$; however, in 100 mM NO$_3^-$, the cultures had a much longer lag time, indicating that *R. solanacearum* is inhibited by high concentrations of either NO$_3^-$ or its metabolic product, NO$_2^-$.

To connect these results to a relevant biological context, we measured NO$_3^-$ in xylem sap from uninfected and *R. solanacearum*-infected tomato plants. Sap from both groups of plants contained 25 to 28 mM NO$_3^-$ (Fig. 4B). This is similar to the NO$_3^-$ concentration that best supported NO$_3^-$-respiratory-dependent growth of *R. solanacearum* in culture.

In contrast, adding NO$_2^-$ did not enhance low-O$_2$ growth of *R. solanacearum* at any level (Fig. 5A). In fact, cell growth was inhibited by 10 mM and 100 mM NO$_2^-$, suggesting that while NO$_3^-$ respiration contributes directly to growth under oxygen limitation, denitrification only facilitates growth indirectly, by eliminating toxic levels of NO$_2^-$ or by allowing the cell to release excess reducing power.

Nitrate is directly used as a terminal electron acceptor. Adenylate energy charge measures bacterial metabolic activity as indicated by the available energy momentarily stored in the adenylate system (28). To determine the contribution of NO$_3^-$ respiration to *R. solanacearum*’s energy pool, we measured the levels of ATP, ADP, and AMP in cells of ΔnarG, ΔaniA, ΔnorB, ΔnosZ, ΔhmphX, and wild-type *R. solanacearum* growing under denitrification-conducive conditions in VDM medium plus NO$_3^-$, the cultures had a much longer lag time, indicating that *R. solanacearum* is inhibited by high concentrations of either NO$_3^-$ or its metabolic product, NO$_2^-$.

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and 0.0007, respectively, *t* test). These data suggest that these two strains are metabolically blocked and may be accumulating toxic levels of NO$_3^-$ and NO. In *E. coli*, a reading below 0.5 is incompatible with metabolic flow and eventually results in cell death (28). The energy charges of the ΔnosZ and ΔhmpX mutants (both ~0.75) were not different from that of the wild-type strain (*P* = 0.0759 and 0.1015, respectively, *t* test). These results, along with the finding that narG is essential for microaerobic or anaerobic growth and NO$_3^-$ production, indicate that *R. solanacearum* uses NO$_3^-$ as a TEA.

In culture, *R. solanacearum* produces and consumes nitrite under both aerobic and anaerobic conditions. To determine whether *R. solanacearum* performs NO$_3^-$ respiration and denitrification under aerobic conditions, we quantified the NO$_2^-$ produced by aerobic cultures in VDM medium supplied with NO$_3^-$ (Table 1). Wild-type cells produced NO$_2^-$ but ΔnarG cells did not, indicating that NO$_3^-$-reductase-mediated respiration is the source of the NO$_2^-$ and that this reaction occurs when the bacterium grows aerobically. Cultures of the ΔaniA mutant accumulated more than twice as much NO$_3^-$ as the wild type, consistent with the predicted NO$_3^-$ reductase function of AniA. Interestingly, this result shows that NO$_3^-$ respiration also occurs under aerobic conditions. However, inorganic N reduction did not contribute significantly to growth under aerobic conditions. We observed a similar pattern under anaerobic conditions (Table 1), although as expected, the cultures produced much more NO$_2^-$ in the absence of O$_2$ and, as previously described (Fig. 3), this did contribute to growth. N$_2$ gas bubbles, which indicate complete denitrification, were present only when wild-type *R. solanacearum* grew anaerobically in liquid or semisolid medium containing NO$_3^-$(-). The ΔnarG, ΔaniA, ΔnorB, and ΔnosZ denitrification mutants did not produce gas bubbles under these conditions, but complementation restored gas production (data not shown).

Wild-type *R. solanacearum* cultures produced visible gas bubbles within 2 h after inoculation at high cell densities (2.5 × 10$^6$ CFU/ml) into VDM medium with 30 mM NO$_3^-$(-). This medium was used because it supports *R. solanacearum* NO$_3^-$ respiration and complete denitrification, ending in the production of N$_2$ gas (25). Interestingly, NO$_2^-$ production had not peaked at 2 HPI (hours postinoculation) but reached a maximum at 8 HPI (see Fig. S1 in the supplemental material).

### TABLE 1 Nitrite and dinitrogen gas production under aerobic and anaerobic conditions

| Genotype | NO$_2^-$ production [µM (±SEM)] | N$_2$ production under anaerobic conditions$^a$ |
|----------|---------------------------------|-----------------------------------------------|
|          | under indicated conditions$^a$  |                                               |
|          | Aerobic                         | Anaerobic                                     |
| WT       | 43 (±8)                         | 113 (±29)                                    | +                              |
| ΔnarG    | 0                               | 0                                             | −                              |
| ΔaniA    | 113 (±14)                       | 9,066 (±976)                                 | −                              |

$^a$ NO$_2^-$ concentrations were measured by Griess reaction after 20 h of growth under static anaerobic conditions or with shaking (225 rpm) under aerobic conditions in VDM medium with 10 mM NO$_3^-$ at 28°C; the NO$_3^-$ concentrations measured include any NO present. The starting OD$_{600}$ for all strains was 0.08. Values in parentheses are standard errors of the means; the experiments included 3 biological replicates, each containing 3 technical replicates.

$^b$ N$_2$ was detected visually following static 28°C incubation of tubes containing liquid VDM medium with 10 mM NO$_3^-$ inoculated with 10 µl of *R. solanacearum* strains at a range of cell densities. The same results were obtained when the assay was repeated in semisolid VDM medium with 10 mM NO$_3^-$ and aerobic incubation at 28°C.
host plant, we measured NO$_2^-$ in xylem sap from tomato plants inoculated with wild-type *R. solanacearum*, the ΔnarG mutant, the ΔaniA mutant, or water (Fig. 5B). Xylem sap from water control plants contained no detectable NO$_2^-$.

Plants infected with wild-type *R. solanacearum* contained, on average, 466.6 μM NO$_2^-$. Xylem NO$_2^-$ concentrations increased as pathogen populations in the stem increased and as disease severity increased. Sap from ΔaniA mutant-infected plants contained, on average, 1,327 μM NO$_2^-$, 2.8-fold more than plants infected by the wild type (P = 0.0337, t test). Sap from plants infected with the ΔnarG mutant contained no detectable NO$_2^-$.

These results demonstrate that *R. solanacearum* produces NO$_2^-$ via NarG-driven NO$_3^-$ respiration during infection and that plants are not a source of NO$_2^-$ in their xylem fluid.

*R. solanacearum* uses a denitrification enzyme, NorB, and the flavohemoglobin HmpX to detoxify nitric oxide. Our results suggested that some steps in denitrification could detoxify potentially harmful inorganic N species, so we characterized the responses of wild-type, ΔaniA, ΔnorB, and ΔhmpX *R. solanacearum* strains to NO challenge. We challenged these NO metabolism-related *R. solanacearum* strains with NO and monitored recovery following NO exposure in a 96-well plate format with an automated plate reader in order to determine lag time and growth rate, measured as doubling time during log phase of each strain following exposure to NO. Strains lacking a gene involved in NO exposure recovery or detoxification would be expected to have a significantly prolonged lag time and, perhaps, a lowered growth rate or doubling time. Figure 7 shows the average growth doubling times (Fig. 7B) and lag times (Fig. 7A) of cultures treated with NO relative to those of untreated control cultures. Interestingly, NO had less effect on the culture lag times of the ΔaniA and ΔnorB denitrification mutants than on the lag time of the wild-type strain. It is likely that endogenous accumulation of NO$_2^-$ and NO in these mutants primed their remaining detoxification systems and, thus, decreased their response time to exogenous NO. However, exogenous NO had a greater effect on culture doubling time in the ΔnorB and ΔhmpX strains than in the wild-type strain. This suggests that both denitrifying (NorB) and flavohemoglobin (HmpX) enzymes degrade exogenous NO.

NO can inhibit bacterial terminal oxidases (19). To determine whether the slowed growth of the ΔnorB and ΔhmpX strains following NO exposure was due to oxidase inhibition, we directly measured O$_2$ consumption in cultures challenged with 1 mM NO (Fig. 7C) (19). Both mutant strains consumed O$_2$ significantly more slowly than wild-type *R. solanacearum*. Interestingly, the ΔnorB mutant was unable to consume the last ~3 μM O$_2$, and although the ΔhmpX mutant consumed all of the O$_2$, it did so at a much lower rate. In contrast, the ΔaniA mutant consumed O$_2$ faster than the wild type. Together, these data suggest that NorB can detoxify NO under all O$_2$ conditions, while HmpX only degrades NO aerobically (down to ~3 μM O$_2$) but does so at a higher rate than NorB. This is in contrast to what was found in *Vibrio fischeri*, where Hmp functions under both aerobic and anaerobic conditions, while Nor functions only under anaerobic conditions (29). It seems likely that accumulating NO$_2^-$ in cultures of the ΔaniA mutant activates NarB and/or HmpX. We speculate that NO$_2^-$ binds NsrR, a predicted NO$_2^-$-responsive transcriptional regulator with a putative binding site upstream from narB, thus activating the expression of narB (30). HmpX may also be under the transcriptional control of NsrR. The *R. solanacearum* genome encodes a putative NsrR, but its function has never been investigated in this species. Alternatively, NsrR may not influence the expression of norB and/or hmpX and these genes may instead be controlled by alternative NO$_2^-$-responsive regulators. Posttranslational modifications, such as tyrosine nitration, may also play a role in the activation of NarB and/or HmpX function (31).

Nitrate respiration, denitrification, and nitric oxide detoxification all contribute to *R. solanacearum’s growth in planta*. The results described above suggest that *R. solanacearum* can use its NarG-AniA-NorB-NosZ-HmpX enzymes to respire on NO$_3^-$, denitrify NO$_2^-$, and detoxify NO. In addition, we demonstrated that in tomato xylem vessels, the bacterium encounters levels of O$_2$ and NO$_3^-$ conductive to these processes. To determine whether metabolism of inorganic N species helps this pathogen colonize its host plants, we measured bacterial population sizes in tomato stems two and three days after inoculation through a cut leaf petiole. In addition to the growth of the wild-type strain, we also measured the growth of the ΔnarG, ΔaniA, ΔnorB, ΔnosZ, and ΔhmpX mutant strains and their corresponding complemented strains.
All five mutant strains reached lower population sizes in planta than wild-type R. solanacearum at both 2 DPI (days postinoculation) (data not shown) and 3 DPI (Fig. 8A). At 3 DPI, the wild-type population sizes in each plant was determined by grinding and dilution plating a 0.1-g stem section centered at the site of inoculation. Ten plants were sampled per strain; each symbol represents the bacterial population size in a single plant, and the horizontal bars indicate the median population size for each strain. The gray dotted line indicates the limit of detection; samples containing no detectable bacteria were given a value of 1. The wild-type strain grew significantly better in planta than all mutants tested; complementation restored growth of mutants, although not always to wild-type levels. Compared to the WT result, the P values for each strain’s growth were as follows: ΔnarG, P < 0.0001; ΔaniA + comp (complemented ΔnarG mutant), P = 0.6371; ΔaniA, P < 0.0001; ΔaniA + comp, P < 0.0001; ΔnorB, P < 0.0001; ΔnorB + comp, P < 0.0001; ΔnosZ, P = 0.0150; ΔhmpX, P = 0.0602; and ΔhmpX + comp, P = 0.411. (B) Virulence assay. Plants were rated daily for 14 days using a disease index of 0 to 4. Data presented are mean results from 3 to 4 independent assays, each containing 10 plants per strain. Error bars indicate standard errors of the means. Disease progress curves of the ΔaniA and ΔhmpX mutants were significantly different from those of the WT strain (P < 0.001, repeated measures ANOVA).

**FIG 8** Respiratory nitrogen metabolism and nitric oxide degradation contribute to bacterial wilt virulence and support pathogen growth in planta. Twenty-one-day-old tomato plants were inoculated with 500 CFU of the specified R. solanacearum strains through a cut leaf petiole. (A) Bacterial growth in planta. At 3 days postinoculation (DPI), the pathogen population size in each plant was determined by grinding and dilution plating a 0.1-g stem section centered at the site of inoculation. Ten plants were sampled per strain; each symbol represents the bacterial population size in a single plant, and the horizontal bars indicate the median population size for each strain. The gray dotted line indicates the limit of detection; samples containing no detectable bacteria were given a value of 1. The wild-type strain grew significantly better in planta than all mutants tested; complementation restored growth of mutants, although not always to wild-type levels. Compared to the WT result, the P values for each strain’s growth were as follows: ΔnarG, P < 0.0001; ΔnarG + comp (complemented ΔnarG mutant), P = 0.6371; ΔaniA, P < 0.0001; ΔaniA + comp, P < 0.0001; ΔnorB, P < 0.0001; ΔnorB + comp, P < 0.0001; ΔnosZ, P = 0.0150; ΔhmpX, P = 0.0602; and ΔhmpX + comp, P = 0.411. (B) Virulence assay. Plants were rated daily for 14 days using a disease index of 0 to 4. Data presented are mean results from 3 to 4 independent assays, each containing 10 plants per strain. Error bars indicate standard errors of the means. Disease progress curves of the ΔaniA and ΔhmpX mutants were significantly different from those of the WT strain (P < 0.001, repeated measures ANOVA).

**R. solanacearum needs nitrite reduction and nitric oxide detoxification for full virulence on tomato.** To assess the effects of inorganic N metabolism on bacterial wilt disease development, we measured the virulence of each mutant strain on tomato plants using the cut-petiole-inoculation assay described above. The ΔaniA and ΔhmpX mutants were significantly less virulent than the wild-type strain (P < 0.0001 and 0.0002, respectively, repeated measures ANOVA) (Fig. 8B). Although the ΔnarG, ΔnorB, and ΔnosZ mutants did not reach wild-type population sizes in tomato stems by 3 DPI, none of these mutants were reduced in virulence in this assay. Together, these data suggest that NO\textsubscript{3}\textsuperscript{–} respiration, denitrification, and NO detoxification aid R. solanacearum in initial growth in the xylem but only NO\textsubscript{2}\textsuperscript{–} reduction and NO detoxification are required for full virulence, at least when the bacteria are introduced directly into host xylem through a cut leaf petiole.
DISCUSSION

The goal of this study was to define the roles of inorganic N respiratory and detoxifying metabolism in R. solanacearum, especially during its pathogenic life inside plant host xylem. We recently showed that assimilation of NO$_3^-$ contributes to R. solanacearum’s virulence and affects the production of extracellular polysaccharide, a virulence factor (32). We previously observed that a Tat secretion-defective mutant of K60, which cannot secrete its NO$_3^-$ transporter or respire on NO$_3^-$, had reduced virulence on tomato, but this suggestive result did not prove that NO$_3^-$ respiration itself contributes to bacterial wilt pathogenesis, as the Tat mutant was also defective in the secretion of other proteins unrelated to N metabolism (33). Little was known about NO$_3^-$ reduction in this bacterium, possibly because Bergey’s Manual describes the species R. solanacearum as incapable of denitrification (34). However, comparative genomic analysis revealed that R. solanacearum strains in phylotypes I (Asia) and III (Africa) have complete denitrification pathways. Most strains in phylotypes II (Americas) and IV (Indonesia) have the three enzymes that allow them to reduce NO$_3^-$ to N$_2$O, but they lack the NosZ nitrous oxide reductase and, thus, cannot reduce N$_2$O to dinitrogen gas (N$_2$), the final step in a complete denitrification pathway (35, 36). These genomic data are supported by phenotypic analyses showing that most R. solanacearum strains can denitrify (25). Bergey’s mischaracterization apparently arose because they defined denitrification as the production of N$_2$ gas bubbles during anaerobic growth on NO$_3^-$; and they studied the type strain, K60, which belongs to phylotype II. N$_2$ bubbles are only produced by the nosZ-carrying phylotype I and III strains.

Genes encoding NO$_3^-$ respiration, NO detoxification, and denitrification were highly differentially expressed in planta, suggesting that these processes specifically adapt R. solanacearum to conditions in plant xylem tissue. We tested this hypothesis using a set of five defined deletion mutants that each lacked one of these strongly upregulated genes. Each mutant was characterized with respect to NO$_3^-$ respiration, denitrification, and NO detoxification in culture; their phenotypes functionally confirmed the sequence-based gene annotations.

The ΔnarG mutant could not respire on NO$_3^-$ and had significantly lower adenylyl energy charge levels than its wild-type parent, indicating that R. solanacearum reduces NO$_3^-$ to generate proton motive force and produce ATP. Additionally, wild-type R. solanacearum grew on NO$_3^-$ in a dose-dependent fashion. The bacterium did not grow on NO$_2^-$, suggesting that it cannot use this molecule as an energy-generating TEA. In contrast, another betaproteobacterium, Neisseria meningitidis, can grow directly on NO$_2^-$ (37). We observed low levels of background growth in VDM medium independent of NO$_3^-$ and NarG function, suggesting that R. solanacearum has NO$_3^-$-independent ways to obtain energy under anaerobic conditions, possibly via amino acid fermentation or Stickland reactions (38). Various modifications in the composition of VDM medium did not eliminate this low-level growth. However, the background growth did not obscure the significant differences between mutant and wild-type strains under any of the conditions studied.

Because O$_2$ is typically the preferred TEA, microbes tend to resort to NO$_3^-$ respiration and denitrification only under anaerobic conditions (1). N. meningitidis, however, can denitrify aerobically when reactive nitrogen species (RNS) intermediates accumulate (39). Likewise, R. solanacearum reduced NO$_3^-$, NO$_2^-$, and NO in culture under both aerobic and anaerobic conditions. However, the reduction of these inorganic N species only supported bacterial growth under low O$_2$ concentrations (<1%).

Though NO$_3^-$, NO$_2^-$, and NO reductases all functioned aerobically in R. solanacearum, the final step of denitrification, the reduction of N$_2$O to N$_2$ by NosZ, did not. This suggests that R. solanacearum NosZ only functions under complete anaerobiosis, as is true of Paracoccus denitrificans and Pseudomonas stutzeri (40, 41). Under anaerobic conditions, the growth of the ΔnosZ R. solanacearum strain was statistically indistinguishable from that of the wild type. Nonetheless, the ΔnosZ strain consistently reached lower optical densities than its wild-type parent. Though the ability to reduce N$_2$O did not contribute greatly to R. solanacearum’s anaerobic growth, this consistent trend suggests that it may make a slight contribution. This could be biologically important for infection prior to xylem colonization. We are investigating this possibility further.

R. solanacearum reached different maximum readings for OD at 600 nm (OD$_{600}$) in culture depending on the experimental conditions of the growth assays. To determine the optimal O$_2$ concentration for growth, 10-ml cultures were shaken in 125-ml flasks to ensure maximum gas exchange. To determine the relative effects of anaerobiosis on the growth of the denitrifying mutants, the culture OD$_{600}$ was measured after 24 h in 15-ml conical tubes under stagnant conditions. Finally, to determine the effects of differing nitrate concentrations on R. solanacearum, bacteria were cultured in 200-μl volumes in 96-well plates at 0.1% O$_2$. Because of these differences in culture method, we compared growth trends and differences among strains, rather than the final OD$_{600}$ endpoints.

Why do R. solanacearum NO$_3^-$ respiration and denitrification pathways operate even in the presence of O$_2$? First, the pathogen must rapidly adapt to major changes in O$_2$ tension as it moves from soil to plant and between microhabitats within the plant. Before it colonizes xylem vessels, R. solanacearum forms microcolonies on root surfaces; such microcolonies formed by Pseudomonas aeruginosa are oxygen limited (42). In planta measurements showed that over the course of infection, R. solanacearum itself lowers the O$_2$ content in xylem fluid. Active inorganic N metabolism may allow uninterrupted bacterial growth during transitions from an aerobic to an anaerobic habitat. Such transitions can be deleterious; for example, the metabolism of Dinorosobacter shibae comes to a halt while denitrification is activated during the switch from aerobic to anaerobic respiration (43). Second, R. solanacearum may use denitrification to dissipate excess reducing power, as multiple Paracoccus species do with periplasmic nitrate reductases (44).

Third, R. solanacearum could use the denitrification pathway to protect itself from the damaging RNS NO$_3^-$ and NO. These can be produced by the bacterium itself and, possibly, additionally by plant defense systems. They threaten the bacterium whether O$_2$ is present or not. Although both NorB and HmpX could degrade NO in the presence of O$_2$, HmpX was more efficient than NorB. However, HmpX was ineffective at less than 3 μM O$_3$, like Pseudomonas aeruginosa’s flavohemoglobin, which functions only under aerobic conditions (45). In Salmonella enterica and Vibrio Fischerii, Hmp is the main aerobic NO detoxification system (26, 29). Moraxella catarrhalis, which has no HmpX equivalent, relies on its NorB homolog for NO detoxification (46). R. solanacearum may
have other minor NO detoxification systems, which would be evident in a ΔnorB ΔhmpX double mutant. For example, R. solanacearum GM11000 expresses two potential cytochrome bd oxidases during disease (see Table S1 in the supplemental material); these can also act as NO detoxification systems and have been shown to contribute to virulence in Shigella flexneri (47, 48).

These explanations are not mutually exclusive, although our data directly support the detoxification hypothesis. In aerobic culture, exogenously supplied NO inhibited terminal oxidase function in the ΔnorB and ΔhmpX mutants, but strains that had intact norB and hmpX were unaffected (data not shown). Furthermore, in anaerobic culture, the ΔnorB strain had a low adenylate energy charge and grew very poorly on NO3−. This was presumably the result of toxic accumulation of endogenous NO, which could not be removed by HmpX under anaerobic conditions. When challenged with exogenous NO under low-O2 conditions, the ΔnorB strain had a longer doubling time than the wild type, confirming that this mutant is impaired in NO detoxification. Additionally, in both aerobic and anaerobic culture, the ΔaniA strain produced and accumulated NO2−, which inhibits R. solanacearum at concentrations above 1 mM. This led to an early decline in cell density, an overall lower growth endpoint, and a low adenylate energy charge. Taken together, the results of our experiments in culture suggest that inorganic N reduction and detoxification are needed to protect R. solanacearum from RNS toxicity under both anaerobic and aerobic conditions.

Genomic analyses suggest that R. solanacearum has a complex cascade of regulators that could modulate inorganic N metabolism, and several of these putative regulators are strongly expressed during wilt disease (17, 35). It appears that NO2− induces the expression of either norB or hmpX, because the ΔaniA strain, which accumulates NO2−, could detoxify NO and resist oxidase inhibition better than its wild-type parent. Our data support in silico predictions of transcriptional regulator binding sites that suggest norB expression could be controlled by NsrR, a NO2−-responsive regulator (30). Additionally, we could not fully restore the ΔaniA mutant to wild-type growth with complementation constructs that included either the aniA gene plus its upstream regulatory region or the aniA gene plus the upstream regulatory region and two downstream putative signal peptide-encoding genes, RSp1501 and RSp1502. The upstream regulatory region may also modify norB gene expression, as in silico predictions suggest (30). The detrimental effects of a second copy of this regulatory region could explain this partial complementation. In a possibly analogous situation, complementation of the aniA homolog in Pseudomonas aeruginosa was reported to be “slightly toxic” (49). We also observed that NO3− respiration and denitrification occurred mainly during late log or early stationary phase, hinting that this function could be modulated by one of R. solanacearum’s two quorum-sensing systems, as is the case in P. aeruginosa (50, 51).

The concentrations of O2, NO, and NO2− are key regulatory signals for other denitrifying bacteria (1, 30, 52). Following detailed gene expression analyses in culture, biosensing fluorescent reporter constructs could be developed to nondestructively measure the levels of these compounds experienced by R. solanacearum over the course of plant infection. We speculate that bacteria aggregated on xylem vessel walls experience higher NO and NO2− levels than planktonic bacteria in flowing xylem fluid. It would also be interesting to determine how inorganic N respiration and detoxification affect biofilm formation, maintenance, and dispersal, which are dependent on inorganic N metabolism in P. aeruginosa (53, 54).

After exploring the roles of NO3− respiration, denitrification, and NO detoxification in R. solanacearum cells growing in culture, we characterized these metabolic behaviors in the biologically relevant plant environment. Our experiments in culture showed that the bacterium can use these pathways to grow in high NO3− concentrations over a range of O2 levels. These were similar to the NO3− and O2 levels that we detected in xylem sap from healthy and diseased tomato plants. NO3−, the product of NarG-mediated NO3− reduction, was present in sap from infected plants but not in sap from healthy plants. Furthermore, the NO2− concentration in sap increased with pathogen cell density and was dependent on narG and aniA function. Similarly, during macrophage infection, Mycobacterium tuberculosis uses NarG to produce NO2−, which influences the expression of many genes (55). This offers genetic and biochemical evidence that R. solanacearum reduces NO3− and NO2− in planta and indirectly suggests that the pathogen produces NO during infection. Attempts to use a microprobe to directly measure NO in xylem sap were unsuccessful, likely because both plant and microbial cells rapidly degrade this molecule, which is also chemically unstable (21). Notably, both NO2− and NO can affect plant defense signaling (56, 57). We are currently investigating the effects of R. solanacearum inorganic N metabolism on plant defense responses.

Direct measurements in plants infected by wild-type and mutant strains showed that R. solanacearum modifies its habitat by lowering the average O2 concentration in the xylem. This is likely the result of bacterial oxidative respiration, because during tomato infection, R. solanacearum had high expression levels of genes for several predicted oxidases, including high-affinity oxidases likely to be effective under hypoxic conditions (see Table S1 in the supplemental material). The O2 levels that we measured in xylem sap probably overestimate the amount of O2 available to the pathogen in microhabitats such as bacterial aggregates on xylem vessel walls. It has been hypothesized that during intestinal infection, Escherichia coli’s anaerobic metabolism fluctuates to efficiently scavenge O2 (58). We propose that R. solanacearum similarly consumes any available O2 in xylem vessels, thereby creating an environment that requires the pathogen to use alternate TEAs, such as NO3−, during pathogenesis. Alternatively, the two TEAs may be used simultaneously during infection.

In response to pathogens, plants produce NO, an early component of the defense signal transduction pathway (57). The results of our in vitro experiments indicate that NO can also directly inhibit R. solanacearum by interfering with terminal oxidase function and that the pathogen protects itself from NO by converting the toxic molecule into either N2O (via NorB) or NO2− (via HmpX, if O2 is present) (5). The plant pathogen Dickeya d惠antii requires HmpX for full virulence on Saintpaulia plants (59). Because D. d惠antii cannot produce NO via denitrification or any other known enzymatic pathway, it must use HmpX to detoxify plant-produced NO (60). Our results suggest that R. solanacearum produces NO in culture (via denitrification) and encounters NO during infection. Do plants produce NO in response to R. solanacearum, or is the NO presumably encountered by the bacterium during infection the result of bacterial metabolism? Additionally, does the pathogen experience inhibitory levels of NO during infection, and if so, has the pathogen adapted to make use
of it? As shown above, R. solanacearum can use NO$_3^-$ as a TEA to generate energy, so HmpX may play two synergistic roles by converting the host’s chemical weapon into an energy source. This may be of particular importance in NO$_3^-$-limited environments, such as in N-limited plants growing in low-input agriculture or natural ecosystems.

These data are consistent with the theory that plant pathogens nutritionally modify their host environment during infection (17, 61). One current paradigm is that the pathogen tricks its host into producing specific metabolites for the microbe’s benefit. For example, Salmonella enterica serotype Typhimurium can use as a TEA the compound tetrasulfonate, which is produced by the host following pathogen-triggered oxidative stress (62). Our results suggest that the host environment can also be shaped by the pathogen’s own metabolic activity in ways that increase pathogen success. We are currently pursuing broader metabolomic studies to better understand how this organism alters its host environment.

None of the denitrification or detoxification mutants grew as well in tomato stems as the wild-type parent. These growth defects may be due to the mutants’ inability to degrade toxic RNS (for $\Delta$aniA, $\Delta$norB, $\Delta$hmpX, and possibly, $\Delta$nosZ strains) and an inability to access sufficient TEAs (for the $\Delta$narG strain). The partial redundancy of NorB and HmpX, which can both detoxify NO, may explain why the $\Delta$norB mutant does not have a bigger in planta growth defect and also has no virulence defect. Only the $\Delta$aniA and $\Delta$hmpX mutants had virulence defects following petiole inoculation. Because direct inoculation of bacteria into a cut leaf petiole strongly favors the pathogen, mutants must be seriously compromised to exhibit a virulence defect in this assay (63, 64). Thus, although the virulence defects of the $\Delta$aniA and $\Delta$hmpX mutants were relatively small, they are likely to be biologically relevant. Further experiments are needed to determine whether denitrification and N detoxification contribute to other steps in R. solanacearum pathogenesis, such as chemotaxis, root attachment and entry, biofilm formation and dispersal, and exit from necrotic tissue, as well as persistence in both plants and soil environments.

Taken together, these results suggest that, in host xylem, R. solanacearum experiences a high-inorganic N environment that is at least partly of its own making. To succeed under these conditions, R. solanacearum has adapted its N metabolism to take advantage of the opportunities offered by this resource and solve the problems posed by the associated growth-inhibitory RNS.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The Ralstonia solanacearum strains and plasmids used in these experiments are listed in Table S1 in the supplemental material. E. coli was grown in Luria-Bertani (LB) medium at 37°C, and R. solanacearum was cultured in CPG medium at 28°C (23) unless otherwise noted. Antibiotics were used as needed at the following concentrations: 15 μg/ml gentamicin, 25 μg/ml kanamycin, and 10 μg/ml tetracycline. A modified Van den Mooter (VDM) medium was used for low-oxygen and anaerobic growth (25). To decrease the complexity of this undefined medium, we used 0.5% (wt/vol) Casamino acids instead of yeast extract. VDM medium without NO$_3^-$ was buffered to a pH of 6.2 with 10 mM morpholineethanesulfonic acid (MES) and supplemented with NO$_3^-$ or NO$_2^-$ as specified in the figure legends. Difco Laboratories (Detroit, MI), Sigma-Aldrich (St. Louis, MO), and Fisher Scientific (Hanover Park, IL) were our chemical suppliers.

**Strain construction.** Clean deletion mutants of R. solanacearum strain GM10000 lacking the complete open reading frame (ORF) of narG, aniA, norB, nosZ, or hmpX were generated via homologous recombination using targeted deletion constructs produced with splicing by overhang extension PCR (SOE PCR) as described previously (32). The corresponding complemented strains were constructed using the R. solanacearum genomic tools of Monteiro et al. (65). Briefly, the region of interest, including the native promoter, was amplified and inserted into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). Using Gateway technology, the region was then transferred to pRCT, which does not replicate in R. solanacearum (65). The complementing pRCT was then moved into the corresponding mutant via natural transformation (66), and antibiotic resistance was used to select strains that had incorporated a single copy of the complementing genomic region into the selectively neutral attTn7 site. PCR was used to confirm incorporation of the desired DNA fragment.

**Growth assays.** To initially identify the oxygen levels at which nitrate respiration contributed to cellular multiplication, 9-ml amounts of liquid VDM medium containing 10 mM KNO$_3$ were inoculated with 1-ml amounts of overnight cultures of R. solanacearum strains adjusted to an OD$_{600}$ of 0.1 ($\sim 1 \times 10^6$ CFU/ml). The initial OD$_{600}$ of the cultures was thus 0.01. The endpoint OD was measured following 24 h of incubation in an oxygen-controlled chamber (InVivo, 400; Ruskinn) to set a specified O$_2$ concentration with N$_2$ gas and with shaking at 225 rpm. The experiment was replicated three times.

Dose-dependent growth of R. solanacearum strains in 96-well plates on NO$_3^-$ was measured continuously by a BioTek HT plate reader for 25 h in the Ruskinn InVivo$_2$ 400 at 0.1% O$_2$. VDM broth supplemented with various concentrations of NO$_3^-$ was inoculated with wild-type R. solanacearum GM1000 to an initial OD$_{600}$ of 0.01. Dose-dependent growth on NO$_3^-$ was measured identically, with the exception that KNO$_3$ was used in place of KNO$_2$.

The endpoint growth of all deletion and complemented strains was assessed both under conditions of 0.1% O$_2$ with shaking (data not shown) and under anaerobic conditions (Fig. 3) in an anaerobic jar (GasPak anaerobic system; BD). Each strain was cultured overnight in 5 ml of VDM broth with no added NO$_3^-$ and incubated at 28°C with shaking at 225 rpm. These cultures were centrifuged at 6,000 rpm for 10 min and resuspended in sterile water to an OD$_{600}$ of 0.1 ($\sim 10^8$ CFU/ml). One-milliliter amounts of these inocula were pipetted into 9-ml amounts of VDM broth containing 10 mM NO$_3^-$, bringing the initial OD$_{600}$ to 0.01. The tubes were lightly capped, to allow gas exchange, and incubated in an anaerobic jar for 22 h at 28°C. The OD$_{600}$ was then measured to determine the ability of each strain to grow without O$_2$ under these denitrification-conducive conditions. The means and standard errors of data collected from five biological replicates (each including three technical replicates) are presented in Figure 3 for each strain.

**Inorganic nitrogen and oxygen measurements.** The Griess reaction (67) was used to quantify the amount of NO$_2^-$ (and indirectly, NO) produced by R. solanacearum cells under denitrification-supportive conditions. Overnight cultures of the indicated strains were set up as described above. Ten-milliliter amounts of VDM medium with 10 mM NO$_3^-$ in 15-ml sterile conical tubes were inoculated with a final OD$_{600}$ of 0.08 (Table 1) or 0.25 (see Fig. S1 in the supplemental material) of resuspended and washed bacterial cultures. The tubes were incubated anaerobically, in BD GasPak anaerobic systems, or aerobically, as specified. At indicated time points, cells were lysed. Briefly, cells were incubated at 95°C for 10 min, moved to $\sim 20^\circ$C for 10 min, and centrifuged for 1 min to collect cellular debris. Three-hundred-microliter amounts of supernatants were transferred to 15-ml conical tubes containing 2.6-ml deionized water. One hundred microliters of Griess reagent (Molecular Probes, Inc.) was added to each tube. The OD$_{540}$ was measured after 30 min of incubation at room temperature. NO$_2^-$ was quantified by using standard curves generated with known concentrations of NO$_2^-$.

The data presented represent the mean results and standard errors of three biological replicates.
tions, each with three technical replicates. N₂ production was qualitatively monitored. If denitrification went to completion, meaning that NO₃⁻ was reduced to N₂O, N₂ gas bubbles could be seen in VDM medium with 10 mM NO₃⁻ in an OD₆₀₀ of 1.0 (~1 × 10⁶ CFU/ml). Two-milliliter aliquots of these cultures were pipetted into sterile 15-ml conical tubes and incubated for 18 to 24 h anaerobically in jars as described above. Following incubation, 100 µl of each culture was used for population size determination via the dilution plating technique. One milliliter of the remaining culture was lysed, and ATP/ADP/AMP were extracted with hot ethanol. ADP was enzymatically converted to ATP with pyruvate kinase, and AMP was converted to ATP with pyruvate kinase and adenylate kinase as described (28). Once converted, total AMP, ADP, and ATP were quantified with luciferase using the Promega ATP Enliken kit. All enzymes and adenylate standards were purchased through Sigma-Aldrich (St. Louis, MO).

Data were collected with a Centro XS2 LB 960 microplate luminometer (Berthold Technologies, Germany). Data represent the mean results of three biological replicates, each with three technical replicates and are presented as percentages of the wild-type energy charge, determined using the following formula: [(ATP) + 0.5(ADP)]/([ATP] + (ADP) + [AMP]).

**in planta growth assays.** To determine the contribution of each deleted gene to R. solanacearum's multiplication in host plant tissue, 21-day-old tomato plants were inoculated with 500 cells of the indicated bacterial strain via cut petiole as described above. Plants were grown and cared for as described above. Three days after inoculation, 0.1 g of plant tissue that included and surrounded the site of inoculation was harvested, ground, and subjected to dilution plating to determine the pathogen population size. Ten plants were sampled for each treatment.

**Virulence assays.** To determine the contribution of each deleted gene to the virulence of R. solanacearum on wilt-susceptible tomato plants, 3-week-old Bonny Best plants were infected with 500 cells of the indicated bacterial strain via cut petiole inoculations as described previously (17). Plants were grown and cared for as described above. Plants were rated daily for symptom development over 2 weeks using a scale of 0 to 4, as follows: 0, no leaves wilted; 1, 1 to 25% of leaves wilted; 2, 26 to 50% of leaves wilted; 3, 51 to 75% of leaves wilted; and 4, 76 to 100% of leaves wilted. This experiment was repeated four times with 10 plants per treatment in each replicate.

**SUPPLEMENTAL MATERIAL**
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02471-14/-/DCSupplemental.

Figure S1, TIF file, 1.6 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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