Expression of Nitrite and Nitric Oxide Reductases in Free-Living and Plant-Associated *Agrobacterium tumefaciens* C58 Cells†

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A number of the bacteria that form associations with plants are denitrifiers. To learn more about how the association with plants affects expression of denitrification genes, the regulation of nitrite and nitric oxide reductases was investigated in *Agrobacterium tumefaciens*. Analysis of free-living cells revealed that expression of the genes encoding nitrite and nitric oxide reductases, *nirK* and *nor*, respectively, requires low-oxygen conditions, nitric oxide, and the transcriptional regulator NnrR. Expression of *nor* was monitored in plant-associated bacteria using *nor-gfp* fusion expression. In root association experiments, only a small percentage of the attached cells were fluorescent, even when they were incubated under a nitrogen atmosphere. Inactivation of *nirK* had no significant effect on the ability of *A. tumefaciens* to bind to plant roots regardless of the oxygen tension, but it did decrease the occurrence of root-associated fluorescent cells. When wild-type cells containing the *gfp* fusion were infiltrated into leaves, most cells eventually became fluorescent. The same result was obtained when a *nirK* mutant was used, suggesting that nitric oxide activated *nor* expression in the endophytic bacteria. Addition of a nitric oxide synthase inhibitor to block nitric oxide generation by the plant prevented *gfp* expression in infiltrated nitrite reductase mutants, demonstrating that plant-derived nitric oxide can activate *nor* expression in infiltrated cells.

Denitrification is the dissimilatory reduction of nitrate to gaseous end products and is utilized as an alternative form of respiration under oxygen-limited conditions (56). Each step of this pathway is catalyzed by a separate terminal reductase, so four terminal reductases, nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos), are required for the complete denitrification of nitrate to nitrogen gas (21). In most denitrifiers two essential requirements must be met before the genes encoding these terminal reductases are expressed. First, oxygen levels must be low so that oxygen reduction is no longer an efficient means of respiration. Second, a nitrogen oxide must be present (25). Thus, when oxygen levels are low and the appropriate nitrogen oxide is present, the genes encoding the terminal reductases are expressed (2, 5, 46, 49).

The capacity for denitrification is found in a diverse array of both eubacteria and archaea (41). The ability to denitrify is especially common among the α -proteobacteria (56). A number of α -proteobacteria also have the ability to form intimate associations with eukaryotes. It is not surprising then that there are a number of α -proteobacterial denitrifiers that are also pathogens, including several *Brucella* species and *Agrobacterium tumefaciens* (3, 10, 12, 19, 39, 50). *A. tumefaciens* is a plant pathogen that can transform plants by transfer of T-DNA into the plant cell, which results in uncontrolled cell proliferation and the synthesis of compounds that can be utilized as nutrients by the infecting bacteria (8, 11).

The ability to respire nitrogen oxides could be significant for the survival and growth of free-living and plant-associated *A.*

tumefaciens cells. The ability to respire nitrate has been shown to be advantageous to bacteria in the rhizosphere since nitrate provides an alternative oxidant when oxygen concentrations are low (7). In addition, the ability to reduce nitrogen oxides may be useful for countering some of the plant defenses against plant pathogens (9, 13). Therefore, this study was undertaken to study in detail the expression of genes that encode Nir and Nor. These two sets of genes were chosen since Nir is the first step in denitrification that produces a gaseous nitrogen oxide, making it the defining reaction of this process (56), and Nor is critical in metabolism of the denitrification intermediate (26) and plant product nitric oxide (NO) (27). In addition to studies examining regulation of these genes in pure culture, the expression of the *nor* operon, which encodes Nor, was examined in cells that were either attached to plant roots or infiltrated into plant leaves. This was done using a *nor-gfp* transcriptional fusion, which was shown to be useful for detecting either exogenous or endogenously generated NO.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5 α (51) and S17-1 (43) were grown in Luria-Bertani medium. *A. tumefaciens* C58 (ATCC 33970) was grown in Sistrom's medium at 30°C (31) (Table 1). When required, Sistrom's medium was modified by using 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) as a buffer and reducing the K₂HPO₄ concentration to 200 μ M. Aerobic cultures were grown in 250-ml Erlenmeyer flasks containing either 50 or 100 ml of medium and shaken at 250 rpm. To induce denitrification in *A. tumefaciens*, flasks were sealed with rubber stoppers to limit oxygen exchange, as described previously (46, 47). When necessary, 12 mM potassium nitrate was added to the medium. Antibiotics were added to *E. coli* cultures at the following concentrations: tetracycline, 10 μ g ml⁻¹; kanamycin, 25 μ g ml⁻¹; ampicillin, 100 μ g ml⁻¹; and streptomycin and spectinomycin, 25 μ g ml⁻¹. Antibiotics were added to *A. tumefaciens* cultures at the following concentrations: tetracycline, 3 μ g ml⁻¹; kanamycin, 100 μ g ml⁻¹; and streptomycin and spectinomycin 200 μ g ml⁻¹.

Construction of strains and plasmids. Campbell insertion of a suicide vector containing an internal portion of *nirK* was used to inactivate *nirK* in *A. tumefaciens*. A 424-bp *nirK* fragment extending from residue 172 of the *nirK* open

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
DH5 α F'	Host for <i>E. coli</i> cloning; F' ϕ 80 <i>lacZ</i> Δ M15 <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> ($r_K^- m_V^-$) <i>supE44 relA1 deoR</i> (Δ <i>lacZYA-argF</i>)U169	46
S17-1	Conjugational transfer of plasmids; <i>recA thi pro hasDRM⁺ RP4:2-Tc:Mu:Km:TnZ</i>	39
S17-1 (λ pir)	S17-1 derivative with the <i>pir</i> gene product; <i>uidA::pir⁺</i> ; Km ^r	32
C58	Wild-type pathogenic strain of <i>A. tumefaciens</i>	ATCC 33970
A002	<i>nir</i> strain of <i>A. tumefaciens</i> with Campbell insertion of pSUP202 into <i>nirK</i> open reading frame; Tc ^r	This study
A006	Wild-type <i>A. tumefaciens</i> containing pSBgfp; Tc ^r	This study
A009	<i>nir</i> strain of <i>A. tumefaciens</i> containing pSBgfp; Tc ^r Km ^r	This study
A010	Wild-type <i>A. tumefaciens</i> containing pJZ383; Sp ^r	This study
A011	<i>nnrR</i> strain of <i>A. tumefaciens</i> with <i>nnrR</i> replaced by Ω Sp ^r Sm ^r cassette; Sm ^r Sp ^r	This study
A029	<i>nir</i> strain of <i>A. tumefaciens</i> containing pJZ402; Tc ^r Sp ^r	This study
Plasmids		
pUC19	Used for cloning in <i>E. coli</i> (Ap ^r)	47
pBluescript II SK (+/-)	Used for cloning in <i>E. coli</i> (Ap ^r)	1
pCR2.1	Used for TA cloning in <i>E. coli</i> (Ap ^r Km ^r)	Invitrogen
pRK415	Broad-host-range plasmid (Tc ^r)	20
pSUP202	Mobilizable suicide vector (Tc ^r)	39
pKOK6	Source of <i>lacZ</i> -Km cassette (Tc ^r Km ^r Ap ^r)	21
pHP45 Ω	Source of Ω Sp ^r Sm ^r cassette (Sp ^r Sm ^r Ap ^r)	15
pWM91	<i>sacB</i> -containing conjugative suicide vector (Ap ^r)	31
pSB7	Derivative of pWM91 with 407 bp upstream to 11 bp downstream of <i>nnrR</i> translational start and 488 bp downstream to 55 bp upstream of <i>nnrR</i> translational stop (Ap ^r)	This study
pSB8	Derivative of pSB7 with Ω Sp ^r Sm ^r cassette digested with <i>Bam</i> HI from pHP45 Ω (Sp ^r Sm ^r Ap ^r)	This study
pAnirK	Derivative of pRK415 with 426 bp upstream of <i>nirK</i> and its open reading frame (Tc)	This study
pAnnrR	Derivative of pRK415 with 205 bp upstream of <i>nnrR</i> and its open reading frame (Tc)	This study
pAnorCZ	Derivative of pRK415 with <i>norB-lacZ</i> transcriptional fusion (Tc ^r Km ^r)	This study
pAnirKZ	Derivative of pRK415 with <i>nirK-lacZ</i> transcriptional fusion containing 429 bp upstream of <i>nirK</i> (Tc ^r Km ^r)	This study
pAnirK1Z	Derivative of pRK415 with <i>nirK-lacZ</i> transcriptional fusion containing 208 bp upstream of <i>nirK</i> (Tc ^r Km ^r)	This study
pAnirK2Z	Derivative of pRK415 with <i>nirK-lacZ</i> transcriptional fusion containing 149 bp upstream of <i>nirK</i> (Tc ^r Km ^r)	This study
pAnirK3Z	Derivative of pRK415 with <i>nirK-lacZ</i> transcriptional fusion containing 78 bp upstream of <i>nirK</i> (Tc ^r Km ^r)	This study
pSBgfp	Derivative of pRK415 with <i>nor-gfp</i> transcriptional fusion	This study
pJZ383	<i>Agrobacterium</i> binary vector with constitutive expression of GFP (Sp ^r)	S. C. Winans
pJZ402	<i>Agrobacterium</i> binary vector with constitutive expression of RFP (Sp ^r)	S. C. Winans

reading frame to residue 596 was amplified from *A. tumefaciens* and cloned into pCR2.1 (Invitrogen Corp.) by TA cloning to form pSB1. pSB1 was digested with EcoRI to remove the *nirK* fragment and ligated into the suicide vector pSUP202 (43) that had been digested with the same enzyme to create pSB2. pSB2 was transformed into *E. coli* S17-1 and then conjugated into *A. tumefaciens*. Exconjugants were selected by tetracycline resistance, and inactivation of *nirK* was confirmed by testing for increased nitrite accumulation in cells grown under oxygen-limited conditions in nitrate-containing medium.

The vector used to inactivate *nnrR* was constructed by amplifying two DNA fragments, one spanning a region from 407 bp upstream to 11 bp downstream of the putative *nnrR* translational start and the other spanning a region from 488 bp downstream to 55 bp upstream of the *nnrR* translational stop. These fragments were each digested with BamHI and XbaI and then cloned into pUC19 (52) and pBluescript II SK (+/-) (1) to form pSB3 and pSB4, respectively. pSB4 was digested with BamHI and KpnI, and the resulting *nnrR* fragment was cloned into pSB3 which had been digested with the same enzymes to create pSB5. pSB5 was digested with EcoRI and PstI, and the resultant *nnrR*-containing fragment was cloned into pBluescript II SK (+/-) to form pSB6. pSB6 was digested with NotI and XhoI, and the *nnrR*-containing fragment was moved into the suicide vector pWM91 (34) to create pSB7. A streptomycin/spectinomycin-resistant cassette was inserted into pSB7 by digesting pHP45 Ω (16) and pSB7 with BamHI to

create pSB8. pSB8 was mobilized into *A. tumefaciens* using S17-1 (λ pir) (35) as a donor. The *nnrR*-deficient strain was selected by streptomycin-spectinomycin resistance and sucrose sensitivity.

For complementation experiments, the entire *nirK* coding region and its regulatory region were amplified with the upstream primer 5'-GCCGGATCCCGC CAATATCGATGAAGC-3' and the downstream primer 5'-CAGGGTACCTTTCAGGCCGAGAGA-3' (the underlined sequences in primers are restriction sites). The 1,621-bp fragment extended 426 bp upstream of the putative *nirK* translation start. The amplicon was digested with BamHI and KpnI and ligated into pRK415 (23) digested with the same enzymes to form pAnirK. A DNA fragment containing the *nnrR* coding region and its regulatory region was amplified with the upstream primer 5'-GACGAATTCAGGATACGCGGTGGTATG-3' and the downstream primer 5'-CGCGGATCCCGGCCAAGCAAATGGTCAG-3'. The resulting 941-bp amplicon included 205 bp upstream of the putative *nnrR* translation start. The amplicon was digested with EcoRI and BamHI and ligated with pRK415 cut with the same enzymes to form pAnnrR (Table 1).

The *norC-lacZ* fusion was generated by amplifying a DNA fragment with the upstream primer 5'-GCCGAATTCCTCGCCATTCTCGTTCTC-3' and the downstream primer 5'-GCCGGATCCAGCAGCGTGTGACAGTTG-3'. The 620-bp fragment contained 412 bp upstream of the putative *norC* translation

FIG. 1. Schematic genetic maps of the *nirK* (A) and *norC* (B) regions, including the various *lacZ* and *gfp* fusions used in this study. The large arrows indicate the orientations of the *nirK* and *norC* open reading frames. The bent arrows indicate the starts of translation of *nirK* and *norC*. The boxes upstream of the open reading frames indicate putative NnrR binding sites.

start. The amplicon was restricted with EcoRI and BamHI and ligated along with the *lacZ* cassette from pKOK6 (24), digested with BamHI, into the broad-host-range vector pRK415. The resulting construct was designated pAnorCZ (Table 1 and Fig. 1). The *nor-gfp* fusion also was generated using the 620-bp fragment generated during construction of the *norC-lacZ* fusion (Fig. 1). The fragment was cloned into pMF3 by digesting both the plasmid and the fragment with BamHI and EcoRI. Construction of pMF3 and the source of the *gfp* have been described previously (53).

The fragment used for construction of the *nirK-lacZ* fusion was generated using the upstream primer 5'-GCCGGTACCCGCCAATATCGATGAAG-3' and the downstream primer 5'-CGGGATCCCGGCGAGCACCATGATCGC-3'. The 1,020-bp fragment contained 429 bp upstream of the putative *nirK* translation start. The amplicon was restricted with KpnI and BamHI and ligated with the *lacZ* cassette from pKOK6 digested with BamHI into pRK415 to create pAnirKZ (Table 1 and Fig. 1). A similar approach was used to generate the three truncated *nirK-lacZ* fusions designated pAnirK1Z, pAnirK2Z, and pAnirK3Z (Fig. 1). These fusions contained 208, 149, and 78 bp upstream of the putative *nirK* translation start, respectively, and were generated using the following oligonucleotides: 5'-GCCGGTACCATGTCTAGACGCAAGCCC-3' and 5'-CGGATCCCGGCGAGCACCATGATCGC-3'; and for pAnirK2Z and pAnirK3Z 5'-GCCGGTACCTTCTCCCGCCTTCA-3' and 5'-GGGGTACCTTGTATTTCGCTCCTCC-3', respectively, and the same downstream oligonucleotide that was used for amplification of pAnirK1Z.

Plasmids pJZ383 and pJZ402 (from S. C. Winans, Cornell University, Ithaca, NY) were used in experiments requiring constitutive expression of the green fluorescent protein (GFP) and the red fluorescent protein (RFP). pJZ383 was constructed by inserting *mut3-gfp* into pPZP201 (20), and pJZ402 was constructed by inserting DsRed (BD Biosciences Clontech) into pPZP201 (Table 1).

Enzyme assays. β -Galactosidase assays were performed as described previously (47). β -Galactosidase activities were determined in duplicate with at least three independently grown cultures. In experiments in which sodium nitroprusside (SNP) was used, cultures were grown under oxygen-limited conditions in unsupplemented Sistrom's medium to an optical density at 600 nm (OD_{600}) of 0.4, and then 10-ml aliquots were removed from the flasks using a syringe and added to 12-ml serum vials. The vials were crimp sealed and incubated at 30°C for 60 min to allow the cells to deplete any oxygen present in the cultures. After this preincubation, SNP was added to a final concentration of 2 mM from a 200 mM SNP stock solution, and the vials were returned to the incubator. All β -galactosidase assays for samples to which SNP had been added were allowed to stand for at least 20 min before the absorbance was determined in order to permit fading of the purple color generated as a result of SNP. To measure Nir activity, 500 μ l of cells was removed from cultures grown under denitrifying conditions. The cells were washed two times in an equal volume of phosphate buffer (pH 7.4) and resuspended in 500 μ l of phosphate buffer (pH 7.4). Then 36 nmol of sodium nitrite was added to each tube, and the cells were incubated at

30°C. In a modification of a previously described protocol for quantifying nitrite accumulation, a colorimetric assay was used to measure the decrease in nitrite concentration over time, which correlates to Nir activity (45).

Plant growth and inoculation. The *Arabidopsis thaliana* plants used in this study were the Columbia ecotype (Col-0). *Arabidopsis* seeds were sterilized by soaking them in a solution of 50% bleach containing 0.2% Tween 20 for 8 min (32). The seeds were washed with sterile water and dispensed into sterile petri plates containing MS medium (37). The plates were covered and sealed with Parafilm, and then they were incubated at 4°C for 3 days under artificial light. To assess the interaction of *A. tumefaciens* with *Arabidopsis* seedlings, bacterial cells were grown to an OD_{600} of 0.7 to 0.9 in 50 ml of Sistrom's medium under aerobic conditions. One milliliter of cells was washed twice with sterile, deionized water (DW). Plant seedlings were also washed with DW to remove residual nitrate. After washing, the seedlings were transferred to a petri plate containing 10 ml of DW, and then cells were added to the plate to a final concentration of 8×10^8 CFU ml⁻¹. The plate was then shaken at 20 rpm at room temperature. After 30 min the seedlings were transferred to a new plate containing DW and shaken for 3 min at 60 rpm to detach loosely bound cells. Next, the seedlings were transferred to a new plate containing DW and incubated under aerobic conditions or in a sealed jar flushed with nitrogen gas to lower the oxygen concentration. Varying the duration of incubation established that clusters of fluorescent cells could be readily visualized by 10 h so all incubations described below were performed for this length of time. For binding experiments using constitutively expressed GFP and RFP, the two strains were grown separately to an OD_{600} of 0.8 in 50 ml of Sistrom's medium under aerobic conditions. After the desired cell density was reached, 1 ml of cells was withdrawn from each of the two cultures, and the samples were mixed. After washing, the mixed cells were incubated with seedlings for 1 h under aerobic conditions or under a nitrogen atmosphere.

For experiments in which *Arabidopsis* leaves were used, plants were grown for 4 weeks in plastic pots in Pro-Mix soil (Premier Horticulture Inc., Red Hill, PA). Infiltration was carried out with 200 μ l of a suspension of aerobically grown cells at a concentration of 8×10^9 cells ml⁻¹ using a syringe without a needle. The syringe barrel was placed against the surface of a leaf, and the plunger was slowly pressed to force cells into the leaf. When required, 1 mM L-N^G-monomethyl arginine (L-NMMA) (Cayman Chemical Co.) was added to the cell suspension used for infiltration. For microscopic examination of leaves, the leaves were removed from the plants and a piece of a leaf including the infiltrated region was excised using a razor blade. The leaf segments were placed in a drop of water on a microscope slide and covered with a coverslip.

Microscopic analysis. Cells were visualized with an Olympus BX61 microscope equipped with a $\times 100$ UplanApo objective, N.A. 1.35, and standard filter sets for visualizing 4',6'-diamidino-2-phenylindole hydrochloride (DAPI), GFP, and RFP. Images were captured with a Cooke SensiCam with a Sony Interline chip. The SlideBook software package (Intelligent Imaging Inc.) was used to acquire images. Figures were assembled with Adobe Photoshop, version 5.5. For infiltration experiments stacks of images were obtained and deconvoluted using the SlideBook software package (Intelligent Imaging Inc.).

To visualize bacteria attached to plants, DAPI was used. Seventy microliters of a DAPI stock solution (0.2 mg/ml) was added to a plate containing seedlings previously incubated with bacteria suspended in 10 ml of DW, and then the plate was gently shaken for 20 min. After staining, the seedlings were washed with DW for 3 min.

RESULTS

Organization of *nirK* and *nor*. Examination of the *A. tumefaciens* genome sequence (<http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html>) revealed a cluster of denitrification genes located on the linear chromosome. Missing from this cluster are genes for nitrous oxide reductase, which are absent from the genome. As expected, the denitrification proteins are very similar to orthologs in other α -proteobacterial denitrifiers with copper-containing Nir. The *nirK* structural gene, *nirK*, is clustered with *nirV*, as it is in most other bacteria containing *nirK* (22). It should be noted that in both published genome sequences *nirK* is annotated incorrectly. Members of the NirK family of proteins are highly conserved (41), and alignments of the predicted *A. tumefaciens* Nir with other orthologs indicate that the predicted sequences have N-terminal

TABLE 2. Expression of *nirK-lacZ* and *norC-lacZ* in *A. tumefaciens* strains

Strain	β -Galactosidase activity (Miller units) ^a					
	<i>nirK-lacZ</i>			<i>norC-lacZ</i>		
	Aerobic	O ₂ limited	O ₂ limited + NO ₃ ⁻	Aerobic	O ₂ limited	O ₂ limited + NO ₃ ⁻
C58	5 ± 2	805 ± 25	1,307 ± 51	3 ± 2	591 ± 21	902 ± 27
C58/MOPS ^b	ND ^c	ND	ND	4 ± 2	122 ± 16	692 ± 25
A002(<i>nir</i>) ^c	3 ± 1	231 ± 14	454 ± 19	2 ± 1	68 ± 8	290 ± 15
A011 (<i>nnrR</i>)	4 ± 2	137 ± 10	110 ± 9	1 ± 1	33 ± 5	28 ± 4
A002 + SNP ^d	ND	586 ± 22	ND	ND	353 ± 16	ND

^a Activities (means ± standard deviations) were determined in duplicate assays of at least three independently grown cultures.

^b Modified Sistrom's medium is described in Materials and Methods.

^c The information in parentheses is the genotype.

^d SNP (2 mM) was added to vials using a gas-tight syringe.

^e ND, not determined.

extensions not found in other denitrifiers (see Fig. S1 in the supplemental material).

The *nor* gene cluster consists of *norCBQD*, as it does in most denitrifiers (41). The annotations of the predicted NorC and NorQ proteins are different in the two published sequences. The translation start sites have been incorrectly assigned in one of the annotations, with additional residues added at the N-terminal end of the proteins (see Fig. S2 in the supplemental material). Adjacent to the *nor* operon is *norEF*, which are not always found in related denitrifiers (41).

After correction of the predicted *nirK* and *norC* translational start sites, each site is preceded by a purine-rich Shine-Dalgarno-like sequence (AGGA) nine bases upstream of the putative translation start codon. Examination of the *nirK* regulatory region revealed the presence of two putative sequences with similarity to the Fnr- and NnrR-like consensus sequence (TTGN₈CAA) (44, 47). These sites are centered 86.5 and 172.5 bases upstream of the putative translational start and have been designated N2 and N1, respectively (Fig. 1). A similar recognition motif is located 129.5 bases upstream of the putative translational start of *norC*.

Expression of *nirK* and *nor* operon. *A. tumefaciens* C58 grown in flasks sealed with rubber stoppers but not sparged before inoculation to remove oxygen grew to a higher optical density in medium supplemented with nitrate than in unsupplemented medium, suggesting that nitrate respiration is beneficial to cell growth (data not shown). However, in sealed vials sparged with nitrogen before inoculation very little growth was observed in nitrate-supplemented medium. In anaerobic, nitrate-supplemented medium the maximum OD₆₀₀ was only about 0.2. The maximum OD₆₀₀ of cells grown in medium lacking nitrate under the same conditions was only about 0.1. Cells incubated on solid medium under strictly anaerobic conditions showed very little growth after 7 days of incubation even if the medium was supplemented with nitrate (data not shown). These data suggest that while denitrification can be beneficial to *A. tumefaciens*, growth is poor if nitrate is the only terminal oxidant available.

As expected, the Nir activity was higher in cells grown in liquid medium under oxygen-limited conditions (0.87 U) than in cells grown aerobically (<0.05 U). Addition of nitrate to cells cultured under oxygen-limited conditions increased the Nir activity slightly less than twofold, to 1.53 U. To confirm that the variation in Nir activity was due to changes in tran-

scription of *nirK*, the activity of a *nirK-lacZ* transcriptional fusion was measured under various growth conditions. A *norC-lacZ* fusion was also constructed since *nirK* and *nor* show similar expression patterns in other denitrifiers (5, 46, 48). Expression of the fusions was lowest under aerobic conditions (Table 2). Limiting oxygen exchange during growth increased the expression of both fusions more than 100-fold. Adding 12 mM nitrate to the medium increased the expression of both fusions another 1.5- to 2-fold (Table 2).

Previous work indicated that the minimal medium used in these experiments contains trace levels of nitrate (5). To confirm that the relatively high levels of expression observed in unsupplemented medium were due to residual nitrate, a Nir-deficient strain (A002) was constructed. Strain A002 accumulated nitrite when it was grown in nitrate-supplemented medium under denitrifying conditions. Complementation with *nirK* restored Nir activity in this strain. Inactivation of *nirK* resulted in significant decreases in the expression of both the *nirK* and *norC-lacZ* fusions in unsupplemented medium (Table 2). Expression increased slightly when the strains were grown in nitrate-supplemented medium, probably due to the accumulation of nitrite, which can be chemically converted to NO (46). Expression of *nor* was also significantly lower in a modified version of Sistrom's medium containing a lower concentration of phosphate buffer, a possible source of nitrate (Table 2). The NO donor SNP was found to increase expression of *nirK-lacZ* in strain A002 in medium not supplemented with nitrate. This demonstrated that *A. tumefaciens* uses NO to regulate *nirK* and *nor* expression, as observed previously for related denitrifiers (26).

Downstream of *nirV* in C58 is a gene encoding a product that exhibits significant levels of identity to members of the NnrR family, which are transcriptional regulators required for expression of *nirK* and *nor* in certain denitrifiers. Insertional inactivation of this gene resulted in an inability to reduce nitrite, as shown by nitrite accumulation when cells were grown under oxygen-limited conditions in nitrate-supplemented medium (data not shown). This indicated that this gene encodes an NnrR ortholog. The presence of *nnrR* in *trans* restored Nir activity in the NnrR-deficient strain A011. Inactivation of *nnrR* prevented a nitrate-dependent increase in *nirK-lacZ* expression under low-oxygen conditions (Table 2), which is consistent with the observed nitrite accumulation. However, a ~30-fold increase in *nirK-lacZ* expression was observed as cells shifted

TABLE 3. Expression of truncated *nirK-lacZ* fusions

Fusion	Fusion truncation (bp) ^a	O ₂ limited		O ₂ limited + nitrate	
		β -Galactosidase activity (Miller units) ^b	% of nirKZ activity	β -Galactosidase activity (Miller units)	% of nirKZ activity
nirKZ	–429	805 $\pm$ 25	100	1,307 $\pm$ 51	100
nirK1Z	–208	711 $\pm$ 19	88	1,114 $\pm$ 33	85
nirK2Z	–149	778 $\pm$ 11	97	974 $\pm$ 27	75
nirK3Z	–78	18 $\pm$ 2	2	16 $\pm$ 3	1

^a Distance upstream of the the translation start site for *nirK* (Fig. 1A).

^b Activities (means $\pm$ standard deviations) were determined in duplicate assays of at least two independently grown cultures.

from aerobic conditions to oxygen-limited conditions. The *norC-lacZ* fusion showed a similar pattern of expression in strain A011; the expression under oxygen-limited conditions was much lower than the expression in the wild type but higher than the expression in cells grown aerobically (Table 2).

There are two putative NnrR binding sites upstream of *nirK*. To determine if both of these sites are required for *nirK* expression, a series of truncated *nirK-lacZ* transcriptional fusions were constructed. For the truncation experiments, a *nirK-lacZ* fusion, which began 429 bp upstream of the putative translational start, was used as a control (Fig. 1). Expression of the nirK1Z and nirK2Z fusions, both of which contained N1 (Fig. 1), was only slightly lower than the control expression (Table 3). The shortest fusion, nirK3Z, which did not include either N1 or N2, had less than 5% of the activity of the control fusion (Table 3). These results demonstrated that N2 is required for *nirK* expression, while N1 is not. It is likely that N2 is required for expression of *nnrS*, whose transcription orientation is divergent from that of *nirK*.

Expression of *nor* in cells attached to roots. While most denitrifiers are free living, *A. tumefaciens* has the ability to form intimate associations with plants. Little work has been done to determine if denitrification genes are expressed when denitrifiers interact with their eukaryotic hosts. Since nitrate is a critical source of nitrogen and can accumulate to high levels in plant cells, it is possible that this plant-derived nitrate could activate expression of denitrification genes (36). Recent work has shown that plants also produce NO as an antimicrobial compound, which could also activate *nirK* or *nor* expression (27). To test if *A. tumefaciens* activates denitrification genes when it is in association with plants, an NO-responsive *gfp* fusion was constructed using the *nor* regulatory region to control expression. The *nor* regulatory region was chosen since it exhibits lower expression in A002 than *nirK* exhibits (Table 2) and should therefore give minimal expression of GFP when a Nir-deficient strain is present in an environment lacking nitrogen oxides.

Expression of the *nor-gfp* fusion, on plasmid pSBgfp, was initially monitored in free-living cells. A pSBgfp-containing strain of *A. tumefaciens* (A006) was very weakly fluorescent under aerobic conditions (Fig. 2B). Limitation of oxygen did not increase the fluorescence significantly above the fluorescence under aerobic conditions until the cells reached the stationary phase, at which point the cells became fluorescent. Inclusion of nitrate in the medium allowed cells in the late exponential phase to become fluorescent (Fig. 2D). When pSBgfp was introduced into strain A002, only weak fluores-

cence was observed when the strain (A009) was grown under nitrate-supplemented, oxygen-limited conditions (data not shown). To test if *nor-gfp* was responsive to exogenous NO, strain A009 was mixed with SNP under oxygen-limited conditions. Under these conditions the cells were nearly as fluorescent as wild-type cells grown with a low level of oxygen in nitrate-containing medium (data not shown).

Since the *nor-gfp* fusion could be used to detect nitrate if it was used in the wild-type strain or to detect exogenous sources of NO if it was used in the Nir-deficient strain, experiments were done to assess the *gfp* expression of cells incubated with *Arabidopsis* seedlings. In the initial experiments we examined expression of bacteria that were attached to roots. Since oxygen is an important factor in *nor* expression, seedlings in the attachment studies were incubated either aerobically with gentle shaking or in a sealed jar flushed with nitrogen. Irrespective of the strain used, very few fluorescent cells were visible when incubation was carried out under aerobic conditions. Therefore, all results discussed below are results of experiments carried out under low-oxygen conditions.

When strain A006 cells were incubated with *Arabidopsis* seedlings, bacteria could be seen in close association with roots after 1 h, the earliest time at which seedlings were examined. This is consistent with previous results (40). Examination of seedlings showed that fluorescent cells were undetectable until about 6 h, and continued incubation until 10 h resulted in increased numbers of fluorescent cells (Fig. 3). Of the 10 seedlings in each assay performed under a nitrogen atmosphere, typically 1 to 4 were found to have fluorescent cells attached to their roots (data not shown). Even in the seedlings with fluorescent cells, most of the roots did not exhibit visible fluorescence. To determine if the low yields of fluorescent cells were due to low attachment efficiencies, DAPI staining was used to detect bound, nonfluorescent cells. It was found that all seedlings examined had attached bacteria, but most of the attached cells were not fluorescent (see Fig. S3 in the supplemental material). As a control, similar experiments were carried out with *Rhodobacter sphaeroides* containing an *nnrS-gfp* fusion, whose expression is also nitrogen oxide dependent (53). When this strain was used, the number of bacteria attached to the roots decreased significantly, and no fluorescent cells were observed (data not shown).

Attachment experiments were also performed with strain A009. However, to ensure that inactivation of Nir did not affect attachment, the attachment efficiencies of wild-type and Nir-deficient strains were compared. To compare the binding of C58 and the binding of A002, plasmids constitutively express-

FIG. 2. Microscopic analysis of free-living cells of A006 grown under either aerobic or nitrate-amended oxygen-limited conditions. (A) Phase-contrast micrograph of A006 cells grown under aerobic conditions. (B) Fluorescence micrograph of the field of cells shown in panel A. (C) Phase-contrast micrograph of A006 cells grown under nitrate-amended oxygen-limited conditions. (D) Fluorescence micrograph of the field of cells shown in panel C.

ing GFP and RFP were introduced into C58 and A002, yielding strains A010 and A029, respectively (Table 1). The two strains were grown separately to similar densities, mixed with each other, and incubated with seedlings for 1 h under aerobic conditions or under a nitrogen atmosphere. Microscopic examination of the roots showed that approximately equal numbers of strain A010 and strain A029 bound to the root surface and hairs under both conditions (see Fig. S4 in the supplemental material). Therefore, inactivation of *nirK* appeared to have no significant effect on the ability of *A. tumefaciens* to bind to plant roots regardless of the oxygen tension. While *nirK* inactivation did not decrease binding, it did decrease the occurrence of fluorescent cells. The total number of fluorescent clusters of bacteria visible for A009 was much lower than the numbers in experiments performed with A006 (data not shown).

These results suggest that both wild-type and Nir-deficient cells can bind to roots but only relatively few cells become fluorescent, even under low-oxygen conditions. Assuming that attached cells are viable, it seems likely that under low-oxygen conditions it is the lack of nitrogen oxides that limits expression of the fusion. To test this hypothesis, attachment was moni-

tored under low-oxygen conditions in medium containing 12 mM nitrate. Under these conditions all seedlings inoculated with A006 were found to have fluorescent cells attached to their roots (see Fig. S5 in the supplemental material). Similar experiments were performed with A009. As predicted based on experiments with free-living cells, inclusion of nitrate in the incubation medium did not increase the number of fluorescent cells above the number seen in medium lacking nitrate (see Fig. S5 in the supplemental material). Under aerobic conditions, addition of nitrate did not change the frequency of fluorescent clusters for either A006 or A009 (data not shown).

Expression of *nor* in infiltrated cells. Fusion expression was also monitored in cells infiltrated into intact plant leaves. For these experiments strains A006, A009, and C58 were separately infiltrated into leaves. After infiltration, the plants were placed under light, and sections of leaves were removed and examined microscopically at 0.5, 3, and 6 h after infiltration. When infiltrated with C58, leaves did not show increasing levels of fluorescence relative to background levels even after 6 h of incubation, although many bacteria could be observed (Fig. 4A and B). Prolonged incubation of the control strain for 24 h gave the same results. Leaves containing A006 did show

FIG. 3. Micrographs of *Arabidopsis* root tips incubated with strain A006 after 10 h of incubation under an N_2 atmosphere. (A) Bright-field image of roots incubated with A006. (B) Fluorescence image of the same location on the root shown in panel A. (C) Bright-field image of a root hair magnified ($\times 5$) from a specific site in panel A. (D) Fluorescence image of the root hair shown in panel C.

an increase in fluorescence over time. No fluorescence was visible at 0.5 h, but by 3 h some fluorescent bacteria were observed. At 6 h significant numbers of fluorescent bacteria were visible, and the majority of cells in each field were fluorescent (Fig. 4D). Since no significant fluorescence was observed in the control experiments, the fluorescence was due to *nor*-dependent *gfp* expression. Unlike the results of experiments involving cells attached to roots, the number of fluorescent cells visible in the experiments with A009 was similar to the number of fluorescent cells observed with A006 (Fig. 4F). As with A006, fluorescence was not observed after 0.5 h, and only a few fluorescent cells were observed at 3 h. However, by 6 h the majority of the cells were fluorescent.

The observation that A009 cells infiltrated into leaves became fluorescent suggests that it is plant-derived NO that activates expression of the *gfp* fusion. NO synthesis in plants has been shown to be inhibited by inhibitors of the mammalian NO synthase (30). These compounds do not inhibit NO production by denitrifiers since they are analogs of arginine, the source of nitrogen for the NO produced from NO synthase. Therefore, A009 was coinfiltrated with 1 mM L-NMMA, a known inhibitor of nitric oxide synthase of plants (55). After 6 h of incubation the levels of fluorescence in the leaves were similar to the levels observed with control strain C58 (Fig. 4H), demonstrat-

ing that *gfp* expression in strain A009 is dependent on NO synthesis by the plant.

DISCUSSION

Genome sequencing has revealed that *A. tumefaciens* strain C58 is a partial denitrifier that lacks nitrous oxide reductase and has a cluster of denitrification genes in an ~ 60 -kb region on the linear chromosome (19, 50). Denitrification by C58 increased the maximum optical densities of cultures when oxygen was initially present. However, cells grew poorly when they were grown in strictly anaerobic, nitrate-supplemented medium. A similar phenotype has been observed for *R. sphaeroides* 2.4.3 (28). It is not clear if other related denitrifiers also grow poorly under anaerobic conditions since the procedures used to make cultures anaerobic can frequently leave low levels of oxygen in the medium, which, combined with nitrate supplementation, seems to be the optimal conditions for induction of denitrification genes. As has been observed previously for other α -proteobacterial denitrifiers, the periplasmic nitrate reductase of C58 does not appear to contribute to the limited growth observed under anaerobic conditions since no growth was observed when the Nir-deficient strain was grown anaerobically (14, 15, 29, 46).

FIG. 4. Micrographs of plant leaves infiltrated with either C58, A006, or A009. Following infiltration, plants were incubated for 6 h under artificial light. (A) Bright-field image of a leaf infiltrated with C58. (B) Fluorescence image of the region of leaf shown in panel A. (C) Bright-field image of a leaf infiltrated with A006. (D) Fluorescence image of the region of leaf shown in panel C. (E) Bright-field image of a leaf infiltrated with A009. (F) Fluorescence image of the region of leaf shown in panel E. A majority of the infiltrated A006 and A009 cells were fluorescent. (G) Bright-field image of a leaf infiltrated with A009 and 1 mM L-NMMA. (H) Fluorescence image of the region of leaf shown in panel G.

The regulation of *nirK* and *nor* was dependent on changes in oxygen levels and the presence of nitrogen oxides (Table 2). The observation that insertional inactivation of *nirK* decreased expression of both the *nirK* and *norC-lacZ* fusions, even in unsupplemented medium (Table 2), demonstrated that a nitrogen oxide must be present for maximal expression of these genes. While nitrate is the nitrogen oxide in the medium that induces gene expression, it is the production of NO that is key to *nirK* and *nor* expression, as shown by the requirement for Nir and the observation that SNP increased expression of *nirK-lacZ* in A002 (Table 2).

While NnrR is critical for activating expression of *nirK* and *nor*, inactivation of *nnrR* did not eliminate the response of these genes to a decrease in oxygen. Under aerobic conditions both fusions showed essentially the same expression in wild-type and *nnrR* mutant backgrounds (Table 2). Under oxygen-limited conditions the *nirK* fusion increased expression >100-fold in a wild-type background but still increased expression ~30-fold in the *nnrR* mutant (Table 2). A similar pattern was observed with the *nor* fusion. The increase observed in the NnrR mutant is in contrast to what has been observed for *R. sphaeroides* 2.4.3, which also utilizes a copper-containing Nir. Inactivation of *nnrR* in strain 2.4.3 eliminates any oxygen- or nitrogen oxide-dependent expression of *nirK* or *nor* (47). It is not clear what causes the increase in expression in the absence of NnrR in *A. tumefaciens*. One possibility is that an FNR-like regulatory protein has some affinity for the putative NnrR binding site and allows increased expression under low-oxygen conditions.

Since experiments with free-living cells demonstrated that significant expression of *nirK* and *nor* required both low levels

of oxygen and a nitrogen oxide, it was of interest to determine if these conditions occur when *A. tumefaciens* interacts with plants. Plants have the capacity to produce nitrate, nitrite, or NO (27, 36) so any or all of these compounds could induce GFP expression (53). By comparing expression of the *nor-gfp* fusion in wild-type and Nir-deficient strains it is possible to determine whether exogenous nitrate or NO activates gene expression. Nitrate is the most likely nitrogen oxide inducing GFP during attachment to roots since cells lacking Nir were only infrequently observed to be fluorescent. It is not clear how nitrate was supplied to the cells that became fluorescent, but the source must have been the roots themselves since no nitrate was present in the medium. When nitrate was present in the medium, nearly all of the attached cells became fluorescent, which is consistent with the hypothesis that nitrate is a limiting factor in GFP expression (see Fig. S5 in the supplemental material).

The expression of *nirK* and *nor* has also been examined in plant-associated *Bradyrhizobium japonicum* cells (33). Both genes were shown to be expressed in *B. japonicum* found in root nodules, but supplementation with nitrate did not increase expression of either gene. In *A. tumefaciens* small amounts of nitrate are sufficient to induce high levels of *nirK* and *nor* expression (Table 2). This may explain why supplementation with nitrate did not increase *nirK* and *nor* expression in *B. japonicum* nodules. It is possible there is sufficient nitrate in the root nodules to render expression of these genes unresponsive to nitrate supplementation. While *nirK* and *nor* were expressed in plant-associated *B. japonicum*, recent gene array experiments showed that the levels of *nirK* and *nor* transcripts did not increase in *Sinorhizobium meliloti* in root nodules (4).

A second array study with *S. meliloti* showed that while transcription of *nirK* and the *nor* operon increased under low-oxygen conditions, the levels of only *nirV* and *norB* transcripts increased in nodules (6). The variability in expression of *nirK* and genes in the *nor* operon seen here with *A. tumefaciens* and with other root-associated denitrifiers suggests that conditions in the rhizosphere are not always favorable for denitrification.

While *nirK* and *nor* may not always be expressed in the rhizosphere, it seems likely that if nitrate is present, it could be used to support respiration. However, studies have shown that mutants of *Pseudomonas fluorescens* lacking Nir grow as well as the wild type in the rhizosphere (18). Similar experiments with a Nar-deficient *P. fluorescens* strain showed that this strain grew slower than the wild type, indicating that Nar is key for growth of this bacterium. This result is inconsistent with the observation that the Nir-deficient strain of *A. tumefaciens* does not grow under anaerobic conditions. One explanation for this is that *P. fluorescens* contains a membrane-bound Nar but *A. tumefaciens*, like many other α -proteobacteria that utilize a copper nitrite reductase, utilizes a periplasmic Nar (17). The membrane-bound Nar supports anaerobic growth by itself, but the periplasmic enzyme does not (42). This suggests that in denitrifiers with a periplasmic Nar, Nir and Nor are more critical for respiration. This conclusion is supported by the decreased ability of Nir- and Nor-deficient strains of *B. japonicum* to form nodules (33).

While expression in root-associated cells was relatively infrequent, infiltrated cells were uniformly fluorescent (Fig. 4D). In further contrast to root-associated cells, there was no significant decrease in the fluorescence of the infiltrated Nir-deficient strain, suggesting that exogenous NO was present (Fig. 4F). The source of this NO was shown to be the plant NO synthase since the NO synthase inhibitor L-NMMA inhibited GFP expression. Recent studies using NO-sensitive fluorescent dyes demonstrated that NO did accumulate in leaves after infiltration with nondenitrifying bacteria (55). However, the response was relatively slow, requiring 3 h before NO accumulated to detectable levels (55). Expression of GFP after infiltration of *A. tumefaciens* cells containing *nor-gfp* required a similar period of time. This confirms that NO production by plants is a relatively slow process and makes it unlikely that NO is an initial signal produced in response to infection. A slower response is more consistent with a role in defense against infection. Lipopolysaccharides have been shown to induce expression of the NO synthase in *A. thaliana* that is probably involved in defense against bacterial infection (54). It seems likely that infiltration of *A. tumefaciens* into *Arabidopsis* leaves induced the innate immune response through detection of bacterial products like lipopolysaccharide, leading to NO generation.

Even though *A. tumefaciens* does not typically infect leaves, it is likely that it encounters NO production when it infects other areas of the plant. The results reported here demonstrate that if this occurs, *A. tumefaciens* responds by expressing Nor. This should allow the bacteria to mitigate the impact of one part of the host defenses and at the same time permit respiration of an alternative, plant-derived terminal oxidant since oxygen is likely limiting during growth in a plant host (38). Previous work showed that cells unable to denitrify due to loss of the TAT transport system were much less virulent than

wild-type cells (12). However, due to the pleiotropic nature of the *tatC* mutation, the relative importance of the denitrification defect in the decreased virulence of this strain is not known.

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