Duplication of Plasmid-Borne Nitrite Reductase Gene *nirK* in the Wheat-Associated Plant Growth–Promoting Rhizobacterium *Azospirillum brasilense* Sp245

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In the plant growth–promoting rhizobacterium *Azospirillum brasilense* Sp245, nitric oxide produced by denitrification could be a signal involved in stimulation of root branching, and the dissimilatory nitrite reductase gene *nirK* is upregulated on wheat roots. Here, it was found that Sp245 did not contain one copy of *nirK* but two (named *nirK1* and *nirK2*), localized on two different plasmids, including one plasmid prone to rearrangements. Their deduced protein sequences displayed 99.2% identity but their promoter regions and upstream genetic environment differed. Phylogenetic studies revealed that *nirK1* and *nirK2* clustered next to most β -proteobacterial sequences rather than in the vicinity of other *Azospirillum* spp. and most α -proteobacterial sequences, regardless of whether DNA or deduced protein sequences were used. This points to past horizontal gene transfers. Analysis of the number of nonsynonymous and synonymous substitutions per site indicated that *nirK* has been subjected to neutral selection in bacteria. The use of transcriptional fusions with *egfp*, encoding an enhanced green fluorescent protein variant, revealed that both *nirK1* and *nirK2* promoter regions were upregulated in vitro under microaerobiosis or the presence of nitrite as well as on wheat roots. The analysis of *nirK1* and *nirK2* mutants revealed that the two genes were functional. Overall, results suggest that *nirK* has been acquired horizontally by *A. brasilense* Sp245 from a distant relative and underwent subsequent duplication; however, both paralogs remained functional and retained their up-regulation by the plant partner.

Additional keywords: genome plasticity, nitrite reduction, PGPR.

Many plant-associated bacteria can perform denitrification, during which there is conversion of nitrate or nitrite to gaseous nitrogen oxides and finally to dinitrogen as the end product (Zumft 1997). This pathway is utilized as an alternative form of respiration under oxygen-limited conditions and is relevant for plant root colonization (Ghiglione et al. 2000), because such conditions occur readily in the rhizosphere (Højberg et al.

1999). A key step in this pathway is the formation of nitric oxide (NO) mediated by nitrite reductase. Two main classes of dissimilatory nitrite reductase (Nir) exist among denitrifying bacteria: the haem-cytochrome *cd₁* type encoded by *nirS* genes and the copper-containing type encoded by *nirK* genes (Zumft 1997).

Dissimilatory nitrate reduction diminishes the amount of nitrate available for plant nutrition. In addition to its significance for nitrogen acquisition by the plant, denitrification by plant-associated bacteria also may have implications for plant development (Lamattina et al. 2003). Indeed, NO produced directly or indirectly by the α -proteobacterial plant growth–promoting rhizobacterium (PGPR) *Azospirillum brasilense* Sp245 when colonizing tomato roots is involved in the stimulation of lateral root formation (Creus et al. 2005). Therefore, NO production might be another plant-beneficial trait displayed by *Azospirillum* PGPR in addition to associative nitrogen fixation (Bally and Elmerich 2006), phytohormone production (Costacurta and Vanderleyden 1995; Dobbelaere et al. 2001), deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (Blaha et al. 2006), and the biological control of phytoparasitic plants (Miché et al. 2000) or bacteria (Bashan and de-Bashan 2002). In model greenhouse experiments, wheat–*Azospirillum* spp. associations were shown to perform either denitrification or N₂ fixation, depending on the availability of NO₃[−] and O₂ (Neuer et al. 1985).

A better understanding of plant-beneficial traits is important because *Azospirillum* spp. can increase growth and yield of different plant species and are used as inoculants in cereal production (Costacurta and Vanderleyden 1995; Dobbelaere et al. 2001). In the case of NO synthesis, little is known of the genetic determinants involved. Recently, the implementation of a global promoter-trapping approach based on differential fluorescence induction (DFI) in the presence of wheat seed extracts revealed a dissimilatory nitrite reductase gene, *nirK*, in the wheat isolate *A. brasilense* Sp245 (Pothier et al. 2007). This gene also was upregulated when the PGPR colonized wheat roots. NO is important for plant growth promotion and this was the first identification of *nirK* in *A. brasilense*; therefore, further work was carried out to characterize this gene.

In the course of this work, it was found that *A. brasilense* Sp245 possesses a second *nirK*-like gene, raising the question of its phylogenetic origin and whether these two genes are functional or not. Therefore, the objective of this study was to characterize the two *nirK*-like genes in *A. brasilense* Sp245. First, the two genes were mapped on the genome and their sequences analyzed. Second, their expression was monitored on

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plant roots using transcriptional fusions of their promoter region with *egfp*, encoding an enhanced green fluorescent protein variant. Third, inactivation mutants were constructed and compared based on NO₂⁻ consumption and N₂O production.

RESULTS

Evidence for two *nirK* genes in *A. brasilense* Sp245.

A *nirK* polymerase chain reaction (PCR) product first was obtained with primers F3905/F3906 (Table 1) designed from the partial *nirK* sequence already available (Pothier et al. 2007). Using this *nirK* PCR product as a probe on digested genomic DNA of *A. brasilense* Sp245, two Southern hybridization signals were obtained (Fig. 1), implying that the *nirK* gene was present in two copies. Because genes encoding denitrification enzymes may be located on plasmids (Zumft 1997), plasmids of *A. brasilense* Sp245 were separated using a modified Eckhardt gel technique (Fig. 2A) and hybridized with the *nirK* probe (Fig. 2B). Six replicons were found (Fig. 2A, lane 2), with sizes estimated at 150, 250, 750, 800, and >1,000 kb (for two replicons, the largest not always visible on Eckhardt gels is likely to be the chromosome). Two hybridization signals were observed, corresponding to the 250-kb plasmid and either the 750- or 800-kb plasmid (Fig. 2B, lane 2). Therefore, *A. brasilense* Sp245 contains two *nirK* genes localized on two different plasmids.

Determination of entire *nirK* sequences of *A. brasilense* Sp245.

To obtain the entire sequence and flanking regions of the *nirK* gene (termed *nirK1*) induced by wheat seed extracts (Pothier et al. 2007), first a genomic library of strain *A. brasilense* Sp245 was constructed and screened by PCR using primers F3787 and F3789 targeting *nirK1* and its promoter, respectively (Table 1). Amplification products of the expected size were obtained with 5 of 650 library clones. From these clones, named pR2.62, pR2.63, pR2.64, pR2.65, and pR2.66, four different restriction patterns were retrieved; however, they all shared restriction fragments of similar sizes (data not shown). Second, the entire sequence of *nirK1* of *A. brasilense* Sp245 and its surrounding environment (i.e., a region spanning over 2.4 kb) were determined by genome walking using DNA from pR2.62 (Fig. 3A).

Because only one *nirK* genomic region was obtained, the Sp245 library was screened again, using primers (F3905/F3906) amplifying an internal fragment of *nirK*. This led to the identification of another clone called pR2.67, in which the presence of *nirK* was confirmed by Southern experiments using

the *nirK* internal probe (obtained with primers F3905 and F3906) and by sequencing. However, when PCR reactions were performed on pR2.67 with primers (i.e., F3787/F3789) targeting the previously characterized promoter of *nirK1*, no amplification product was obtained, suggesting that the two *nirK* genes did not share the same promoter region. The entire sequence of this second copy of *nirK* (termed *nirK2*) and its flanking regions (a region spanning over 3.6 kb) was determined by genome walking using pR2.67 as a template (Fig. 3B).

Genomic rearrangements affecting the replicon bearing *nirK2*.

Previous work based on plasmid profiling also had revealed six plasmids in *A. brasilense* Sp245, but with a large discrepancy in size for certain plasmids (Kamneva et al. 2001; Katzy et al. 1998). Therefore, the plasmid content of the strain was compared (Fig. 2A) after having collected Sp245 from four other laboratories studying *Azospirillum* spp. (i.e. Knoxville [G. Alexandre, University of Tennessee], Paris [C. Elmerich, Institut Pasteur], Rehovot [Y. Okon, The Hebrew University], and Leuven [J. Vanderleyden, Catholic University]). Whereas all five

Fig. 1. Evidence for two *nirK* genes in *Azospirillum brasilense* Sp245. Southern hybridization of genomic DNA of *A. brasilense* Sp245. A 240-bp internal fragment of *nirK* was used as a probe. Lanes 1 and 2, DNA from Sp245 digested with *Bam*HI and *Eco*RI, respectively.

Table 1. Primers and polymerase chain reaction conditions

Primer name	Primer sequence	Nucleotide target ^a	Reference
F2603 (forward)	5' CGGTTTACAAGCATAAAGC 3'	Multiple cloning site of pOT1e	Pothier et al. 2007
F2604 (reverse)	5' CATTTCCTCCTCCACTAG 3'	Multiple cloning site of pOT1e	Pothier et al. 2007
F3787 (reverse)	5' GATCCGGGCGCGTTCGTCATGG 3'	coding region of <i>nirK</i>	This work
F3789 (forward)	5' TCTCGCGCTGGATGTCGCCAG 3'	Promoter region of <i>nirK1</i>	This work
F3905 (forward)	5' TGTACGCCGAACCGAAGGTC 3'	Coding region of <i>nirK</i>	This work
F3906 (reverse)	5' TCATGGACACGTTACCGTG 3'	Coding region of <i>nirK</i>	This work
F4437 (forward)	5' GTGTCCTCAACATCCTGTGCG 3'	Coding region of <i>carR</i>	This work
F4438 (reverse)	5' CCTTGCCGTGTGAAGCGCAT 3'	Coding region of <i>carR</i>	This work
F5302 (forward)	5' TGGGCAACATCGATTACGAG 3'	Promoter region of <i>nirK1</i>	This work
F5303 (forward)	5' CTGCGACTGCATCGATGCGA 3'	Promoter region of <i>nirK2</i>	This work
F5595 (reverse)	5' TGTCCAGATAGCCCAGTAGC 3'	Km ^r cassette of pKNOCK-Km	This work
F5597 (forward)	5' ACTTCCTCACTCTCACAGGCA 3'	Promoter region of <i>nirK1</i>	This work
F5598 (forward)	5' AACGGAAGTGATCGGAGCGT 3'	Promoter region of <i>nirK2</i>	This work
F5635 (forward)	5' GCCCCAAGACCCACAACGT 3'	Coding region of <i>nirK</i>	This work
F5636 (forward)	5' TGAACGAGAATTCCTTGGTCTG 3'	Coding region of <i>nirK</i>	This work

^a Km^r = resistant to kanamycin.

strains of Sp245 host three plasmids of 150, 750, and 800 kb and two replicons >1,000 kb (Fig. 2A, c, f, g, h, and o, respectively), several discrepancies were observed for the other plasmids, as follows. First, all strains of Sp245 except ours contain a

160-kb plasmid (i.e., Fig. 2A, d); second, a smaller plasmid of variable size could be observed in three Sp245 strains (i.e., Fig. 2A, a or b); third, only our strain carries a 250-kb plasmid (i.e., Fig. 2A, e).

Fig. 2. Analysis of genomic rearrangements affecting the replicon bearing *nirK2*. **A**, Electrophoretic plasmid profiles of *Azospirillum brasilense* Sp245 strains kept in different laboratories; for example, in Lyon (lane 2), Knoxville (lane 3), Paris (lane 4), and Rehovot (lane 5). The marker strain *Rhizobium etli* CFN42 also is displayed (lanes 1 and 6). The plasmid profile of the Sp245 strain from Leuven (*not shown*) is identical to that of the Rehovot strain (lane 5). **B**, Southern hybridization of the plasmid profiles shown in panel A, using a 240-bp internal fragment of *nirK* as a probe.

Fig. 3. Physical maps of the two *nirK* genes from *Azospirillum brasilense* Sp245 and their respective environment. **A**, Physical map of a 2.4-kb region containing *nirK1* that was obtained by genome walking using DNA from pR2.62. ORF = open reading frame and FNR = fumarate-nitrate reduction. **B**, Physical map of a 3.6-kb region containing *nirK2* that was obtained by genome walking using DNA from pR2.67. Stripped boxes correspond to putative transmembrane helices.

	10	20	30	40	50	60	70	80	90	100	
A.brasil1	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MKTKTFLATV 10
A.brasil2	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MKTKTFLATV 10
H.denitri	MIFHRFGAVVGLMAALLSVPAHADQHQMAQKAPADAPAMKDKSKSHEEEKDPTAGAHAPVVFTLRTGIAEGRMVYIGVGGDIDHKINPTLVVHE 100										
N.multifo	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	NSGVTYRDPDVTFTLRDIDIGEGKLVFVGESGTIAGKINPDLVNP 71
N.gonorrh	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MAEQQA 29
A.faecali	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MAEQQAISRR 10
R.sullae	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MTNTLQMTTR 10
A.cyclocl	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MTEQLQMTTR 10
A.xylosox	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MNALR 5
P.chloror	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MSVFR 5
R.sphaero	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	MFTRR 5
	110	120	130	140	150	160	170	180	190	200	
A.brasil1	SAAALIALS-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	KVKEPAVDIVQIFAAPEAEAGKR 58
A.brasil2	SAAALIALS-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	KVKEPAVDIVQIFAAPEAEAGKR 58
H.denitri	GETVQVNI/VNNEGQHQHVVVDQYAARSIVNGKNASSTFSVPSKVGEFNYYCSIAHRQAQMEGNIQVLPGNRAEMKSSGADITREPADPGP--IGPR 198										
N.multifo	GAIVQINLVNGDGAMHDIAIPELGAKSDNITAKGARTSMVFRVRTGTFEYLCTLPGHKAAGMFGRLIVGK-PPEEAKGYVTNVAQDFRAGSEP--VGTR 168										
N.gonorrh	ETPAAS-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	AGELPVIDAVITTHAPEVPP-AIDRD 74
A.faecali	TILAGAAAG-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	EIALPRQKVELVDFPFMAHSAQVAG 70
R.sullae	TMITGAIVAG-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	EIALPRKRLDIVKPFVIVHTQKAG 71
A.cyclocl	TMLAGAAAG-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	DISTLPRVKVDIVKPFVIVHTQKAG 72
A.xylosox	PTILAAIAAF-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	ADKLPHTKVTLVAFPCVHPHEQATKS 52
P.chloror	SVLGACVILG-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	AELGLQRVKVDIVAPPLVHPHEQVVS 52
R.sphaero	AAIVGAALALA-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	DLSNLPVKVHTLVFPFPAHAHEQVVAAS 66
	210	220	230	240	250	260	270	280	290	300	
A.brasil1	APRTHNVVITTTTEVEARIL-IGITYTYMTFNKNVPGFVNVNAGDITNVSMNAPGSIMNHSIDFHAATGGLGGGQITQAEFGTKKEFSFKAMAPGVVY 157										
A.brasil2	APRTHNVVITTTTEVEARIL-IGITYTYMTFNKNVPGFVNVNAGDITNVSMNAPGSIMNHSIDFHAATGGLGGGQITQAEFGTKKEFSFKAMAPGVVY 157										
H.denitri	QNTNRIDLETVEVKGQIL-DNITYTYMTFNGKVPGEFLRVVSGDVELLHKKHSDLMVSVDFHETGPGGAAAFITQDPGEETVVIFKALIPGIYVY 297										
N.multifo	ASRHLTINLEATDIEGQLS-DKKLYKYMTFNKNVPGFLLRKVGDITINLASASSTNHSIDFHAATGPGGGAATQAPGETKSTFTKSLHPGLFVY 267										
N.gonorrh	YEAKRVKMETVEKTHKMD-IGVEYRYMTHTGVPGRMIRVREGDITVEEFSNNPSTVFHNVDFHAATGCGGGAATFTAPGETSTTFKALIPGLIY 173										
A.faecali	GPRVFEFTMVIIEKKIVIDAGTEVHAMENGVPGLMNVHQLIYELTLINPENTNLMNIDFHAATGLGGGGLTINPGEKTLIFKATIPGFVY 170										
R.sullae	GPRVFEFTLTIIEKKLVIIDAGTEVHAMENGVPGLMNVHQLIYELTLINPENTNLMNIDFHAATGLGGGGLTINPGEKTLIFKATIPGFVY 171										
A.cyclocl	GPRVFEFTMTIIEKKLVIIDAGTEVHAMENGVPGLMNVHQLIYELTLINPENTNLMNIDFHAATGLGGGGLTINPGEKTLIFKATIPGFVY 172										
A.xylosox	GPRVFEFTMTIIEKKLVIIDAGTEVHAMENGVPGLMNVHQLIYELTLINPENTNLMNIDFHAATGLGGGGLTINPGEKTLIFKATIPGFVY 152										
P.chloror	FPKVVQFRMSIIEKKLVIIDAGTEVHAMENGVPGLMNVHQLIYELTLINPENTNLMNIDFHAATGLGGGGLTINPGEKTLIFKATIPGFVY 152										
R.sphaero	GFVINEFEMRIIEKVVQIL-EDAYLQAMTFLSIPGLMNVHQLIYELTLINPENTNLMNIDFHAATGLGGGGLTINPGEKTLIFKATIPGFVY 165										
	310	320	330	340	350	360	370	380	390	400	
A.brasil1	HCAITFM--MAHLIANGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	NLP-AAEDDYDGLVNEKFGYVFNFGVGLVKDKFL 239
A.brasil2	HCAITFM--MAHLIANGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	NLP-AAEDDYDGLVNEKFGYVFNFGVGLVKDKFL 239
H.denitri	HCAITPS--VPIHITNGMGLLVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	GTSGEGMDYKELINEKPEYFTFNGSVGSLTRSHPL 380
N.multifo	HCAITFM--MAHLIANGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	NVRGLQESLENILAENPCHVFNCTITLALVMTM 350
N.gonorrh	HCAVAP--VGMHLIANGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	GAQGLQPFDMKVAEQFEYVFNFGVGLVAGGNAL 256
A.faecali	HCAFFG--VYFAHVTSGAGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	QVRESEYVMOGEITTVKSF-----
R.sullae	HCAFFG--VYFAHVTSGAGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	QVRESEYVMOGEITTVKSF-----
A.cyclocl	HCAFFG--VYFAHVTSGAGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	QVRESEYVMOGEITTVKSF-----
A.xylosox	HCAFFG--VYFAHVTSGAGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	QVRESEYVMOGEITTVKSF-----
P.chloror	HCAFFG--VYFAHVTSGAGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	QVRESEYVMOGEITTVKSF-----
R.sphaero	HCAFFG--VYFAHVTSGAGGAGLVVVEEGGLP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	QVRESEYVMOGEITTVKSF-----
	410	420	430	440	450	460	470	480	490	500	
A.brasil1	KASVGEIVRIIFGVGGGNFTSSBHVIGEIFDRVHNLGGLDTPFLKNVQTVSVAFGGATMVEFKVDYPCRYALVGHHSR-ATHGLIGILEVCKALDSII 338										
A.brasil2	KASVGEIVRIIFGVGGGNFTSSBHVIGEIFDRVHNLGGLDTPFLKNVQTVSVAFGGATMVEFKVDYPCRYALVGHHSR-ATHGLIGILEVCKALDSII 338										
H.denitri	YASVGEIVRIIFGVGGGNFTSSBHVIGEIFDRVHNLGGLDTPFLKNVQTVSVAFGGATMVEFKVDYPCRYALVGHHSR-ATHGLIGILEVCKALDSII 479										
N.multifo	ENATQNNVRIYFGVGGNLTSSBHVIGEIVHNVYDQASLTSPPLTDVQITLVAGGATIVEFKVDYPCRYALVGHHSR-MEKGLAGYITVCKANAEIF 449										
N.gonorrh	KAKAGEIVRMVYVNGGGRNVSSBHVIGEIFDRVHNLGGLDTPFLKNVQTVSVAFGGATMVEFKVDYPCRYALVGHHSR-ATHGLIGILEVCKALDSII 354										
A.faecali	TAPVGEIVLVHVS--QNRDTRRELIGGHGQVWETGKFNPFEDVDQETWFLFGGAGAAFYTFQPCGIYAYVHNLIDBAEDGAAAHFKVTGENDNLM 367										
R.sullae	TAPVGEIVLVHVS--QNRDTRRELIGGHGQVWETGKFNPFEDVDQETWFLFGGAGAAFYTFQPCGIYAYVHNLIDBAEDGAAAHFKVTGENDNLM 368										
A.cyclocl	TAPVGEIVLVHVS--QNRDTRRELIGGHGQVWETGKFNPFEDVDQETWFLFGGAGAAFYTFQPCGIYAYVHNLIDBAEDGAAAHFKVTGENDNLM 369										
A.xylosox	TAPVGEIVLVHVS--QNRDTRRELIGGHGQVWETGKFNPFEDVDQETWFLFGGAGAAFYTFQPCGIYAYVHNLIDBAEDGAAAHFKVTGENDNLM 349										
P.chloror	TSKVGESLVFIHS--QNRDTRRELIGGHGQVWETGKFNPFEDVDQETWFLFGGAGAAFYTFQPCGIYAYVHNLIDBAEDGAAAHFKVTGENDNLM 349										
R.sphaero	KAVGGINLVFVHS--QPKRDSRELIGGHGQVWETGKFNPFEDVDQETWFLFGGAGAAFYTFQPCGIYAYVHNLIDBAEDGAAAHFKVTGENDNLM 363										
	510	520	530								
A.brasil1	LDKSGDSRKQLGEMKH-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	354
A.brasil2	LDKSGDSRKQLGEMKH-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	354
H.denitri	HEGPAKQ-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	486
N.multifo	KP-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	451
N.gonorrh	TQKLSDTAYAGSGAASAPASAPASAPASASEKSVY-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	392
A.faecali	TSVLAPSGT-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	376
R.sullae	TTVRSPSGS-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	377
A.cyclocl	TSVVKPSM-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	378
A.xylosox	KQIKAPAPIPR-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	360
P.chloror	TQVKAPGPIVEFKQ-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	363
R.sphaero	EQVWPVGLTG-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	374

When plasmid profiles were hybridized with the *nirK* probe, which targets an internal part of *nirK*, a hybridization signal corresponding to the 750- or 800-kb plasmid (i.e., plasmid f or g) was observed with all five strains of Sp245 (Fig. 2B). In addition, a second hybridization signal was detected for four Sp245 strains including ours, on plasmid a, b, or e. To ascertain which of the two replicons harbors *nirK1*, a gene found 6.7 kb downstream of *nirK1* (i.e., *carR*) in the *A. brasilense* Sp245 genome sequencing project was used as a probe. This regulatory gene is involved in global control of carbohydrate catabolism in *A. brasilense* (Chattopadhyay et al. 1994). Only one hybridization signal was observed, corresponding to one of the 750- or 800-kb plasmids (data not shown).

Comparative analysis of *nirK* flanking sequences in *A. brasilense* Sp245.

In our strain of *A. brasilense* Sp245, the region upstream of *nirK1* and *nirK2* differed. Indeed, analysis of 861 bp upstream of the start codon revealed low identity (36.6%) between the two promoter regions and their upstream genetic environments. In the case of *nirK1*, an open reading frame (ORF) transcribed in the same direction could be identified 1,093 bp upstream (Fig. 3A). The deduced protein sequence of 96 amino acids in length (10.45 kDa) presented significant homology with sequences of oxidoreductase NAD-binding domains found in globins, the highest identity (64%) being obtained with *Methylobacterium* sp. strain 4-46 (GenBank accession number ZP_01846193). The analysis of a bigger region surrounding *nirK1* in the *A. brasilense* Sp245 genome sequencing project indicated that *nirK1* is not part of an operon.

Upstream of *nirK2* and in opposite orientation, two putative ORF displaying similarities to genes involved in denitrification could be identified (Fig. 3B). The deduced protein sequence of the first ORF beginning 496 bp upstream from *nirK2* was found to be 150 amino acids long (molecular mass of 16.87 kDa). It showed significant homology with small subunit precursors NorC (cytochrome c subunit) of nitric oxide reductases, the highest identity (81%) being obtained with the α -proteobacterium *Rhizobium* sp. strain G-179 (GenBank accession number AAC79450; mistakenly identified as a pseudomonad until now). The second ORF was found 14 bp upstream of *norC* in the same orientation (Fig. 3B). The deduced protein sequence of this ORF (363 amino acids long, molecular mass of 40.35 kDa) displayed significant homology with large subunit precursors NorB (cytochrome b subunit) of nitric oxide reductase, the highest identity (69%) being obtained with the α -proteobacterium *Magnetospirillum gryphiswaldense* MSR-1 (GenBank accession number CAM75374). Eight transmembrane helices were predicted for NorB.

Comparison of *nirK* sequences of *A. brasilense* Sp245.

Despite divergent upstream regions between *nirK1* and *nirK2* in our strain of *A. brasilense* Sp245, promoter analysis revealed several common features for *nirK1* and *nirK2*, as follows. In both cases, there were two consecutive TTG and ATG initiation

codons in the same frame, upstream of the coding region. The ATG was chosen as the most probable initiation because there is a potential ribosome binding site (RBS) close by. Along with a predictive transcription start at a relevant location, further analysis revealed consensus, σ^{70} -relevant -10 and -35 promoter sequences. Finally, the sequence wyTTGAC-(N₄)-GTCAArw, which is a recognition motif for the fumarate-nitrate reduction (FNR) transcriptional activator and is present in other *nirK* promoters, also was shown upstream of the two genes.

In our strain of *A. brasilense* Sp245, the *nirK1* and *nirK2* ORF were 1,066 bp long and have a G + C content of 64 and 63%, respectively. They shared 96% identity at the DNA level, with discrepancies at 41 positions located over the entire sequence.

The deduced NirK1 and NirK2 sequences corresponded to polypeptides of 354 amino acid residues sharing 99.2% identity (99.4% similarity), because many mutations were silent (Fig. 4), with a molecular mass of 37.7 kDa (unprocessed precursor). Typical of periplasmic metalloenzymes, the NirK1 and NirK2 products displayed a signal peptide at the N-terminal end (very high probability of $P = 0.999$ in both cases) and a putative cleavage site between the 26th and 27th residues (probability of $P = 0.601$ in both cases). The deduced sequences for *nirK1* and *nirK2* presented significant homology with those of other copper-type nitrite reductases, the best 'expect' value being obtained with *Hyphomicrobium denitrificans* A3151 (GenBank accession number BAC00912), with 59% identity in both cases.

When the two deduced NirK protein sequences were aligned with those of other copper-containing dissimilatory nitrite reductase (NirK) orthologs, the high degree of amino acid identity among the NirK sequences (excluding the signal peptide sequences) was striking (Fig. 4). Generally, copper-containing nitrite reductases fold in a trimeric structure in which a monomer (approximately 37 kDa) contains two copper atoms. One of these is designated type 1 and is required for electron transfer. The other center is designated type 2 and is the site of nitrite binding and reduction. On the basis of the alignments, type 1 and type 2 copper ligands are conserved in the deduced amino acid sequence of both NirK1 and NirK2: H₁₁₈, C₁₅₉, H₁₆₇, and M₁₇₂ are ligands to the type 1 center whereas H₁₂₃, H₁₅₈, and H₃₁₄ are ligands to the type 2 center (*A. brasilense* numbering) (Zumft 1997). Similarly, the active sites D₁₂₁ and H₂₆₃ (*A. brasilense* numbering), which are required for NO₂⁻ reducing activity, are conserved in both genes (Zumft 1997). These observations further confirm that the genes identified in *A. brasilense* Sp245 encode members of the NirK family of copper-containing dissimilatory nitrite reductases.

Phylogenetic analyses of *nirK* genes in *A. brasilense* Sp245 and other bacteria.

Neighbor-joining (NJ) phylogenetic trees were generated for *nirK* (data not shown) and for NirK (Fig. 5) after alignment of 66 eubacterial and archaeal sequences. These trees revealed that NirK1 and NirK2 clustered next to most β -proteobacterial

Fig. 4. Alignment of NirK1 and NirK2 deduced protein sequences from *Azospirillum brasilense* Sp245 with those of common copper-containing dissimilatory nitrite reductase *nirK* orthologues. Identical residues are indicated by white letters in black background; similar residues conserved in 60% of the sequences are indicated by black letters in gray background. The type 1 and type 2 copper ligands are indicated by 1 and 2, respectively. The black arrows indicate amino acid residues involved in a hydrogen-bond network around the type 2 copper and playing a role in proton donation to the substrate. Putative proteolytic cleavage site for removal of the signal peptide from NirK1 and NirK2 is indicated by scissors. NirK1 and NirK2 sequences are identical, with the exception of different residues at positions 277 and 182 and similar residues at positions 115 (*A. brasilense* numbering). Sequences from top to bottom were from *A. brasilense* Sp245 NirK1 (*A.brasil1*) and NirK2 (*A.brasil2*), *Hyphomicrobium denitrificans* A3151 (*H.denitri*, accession number BAC00912), *Nitrosospira multiformis* ATCC 25196 (*N.multifo*, YP_412685), *Neisseria gonorrhoeae* FA 1090 (*N.gonorrh*, AAW89933), *Alcaligenes faecalis* S-6 (*A.faecali*, P38501), *Rhizobium sulae* HCNT1 (*R.sulae*, Q60214), *Achromobacter cycloclastes* IAM 1013 (*A.cyclocl*, AAB19386), *A. xylosoxidans* GIFU1051 (*A.xylosox*, BAA33678), *Pseudomonas chlororaphis* ATCC 13985 (*P.chloror*, CAA79939), and *Rhodobacter sphaeroides* 2.4.3 (*R.sphaero*, AAB05767).

sequences rather than in the vicinity of the two other NirK *Azospirillum* sequences available and most α -proteobacterial sequences, regardless of whether DNA (data not shown) or deduced protein sequences (Fig. 5) were used. The only other α -proteobacterial sequence close to those of *A. brasilense* Sp245 was that of *H. denitrificans* A3151, which was in a neighboring subcluster. Similar findings also were found for trees generated by maximum parsimony (MP) and maximum likelihood (ML) methods (data not shown). These results point to past horizontal gene transfers from β -proteobacteria to α -proteobacteria, such as *A. brasilense* Sp245.

When estimating the numbers of nonsynonymous substitutions per site (dN) and synonymous substitutions per site (dS) for *nirK*, using the Kumar method under MEGA, it appears that dN (0.748 ± 0.038) was equivalent to dS (0.732 ± 0.044). This finding points to neutral selection for *nirK* in bacteria.

Functionality of the two *nirK* genes in *A. brasilense* Sp245.

Because the promoter regions of *nirK1* and *nirK2* differ, they were cloned from our strain of *A. brasilense* Sp245, each as a DNA fragment approximately 800 bp in size, and fused to *egfp* in plasmid pOT1e to obtain transcriptional fusions. Under in vitro conditions, the expression levels of *PnirK1-egfp* and *PnirK2-egfp* were low under fully aerobic conditions; under microaerobic conditions (i.e., tubes entirely filled with medium), the increase in fluorescence was 8.6-fold for *PnirK1-egfp* and 7.3-fold for *PnirK2-egfp* (Fig. 6A and B). Moreover, the expression of *PnirK2-egfp* could be induced by 5 mM of nitrate or nitrite under microaerobiosis (data not shown), which are conditions known to trigger the transcription of *nirK1* (Pothier et al. 2007).

Inactivation of *nirK* coding sequence was carried out by single homologous recombination using the pKNOCK tool. In the

Sp245 strain from Knoxville, which bears only *nirK1*, the mutant obtained grew slowly under denitrifying conditions but could no longer reduce NO_2^- based on the Griess-Ilosvays reaction (data not shown). In our Sp245 strain, a *nirK1* mutant and a *nirK2* mutant were obtained, but development of a *nirK1 nirK2* double mutant failed. The two mutants still could denitrify, because i) they still were able to consume NO_2^- and ii) there was N_2O accumulation in the headspace when acetylene was used to block the N_2O reductase (data not shown). These results demonstrate that both *nirK1* and *nirK2* are functional and that the presence of a single *nirK* gene is sufficient for denitrification in *A. brasilense* Sp245.

Activity of *nirK* genes in planta.

When a rifampicin-resistant derivative of *A. brasilense* Sp245 (i.e., Sp245-Rif) harboring either the *PnirK1-egfp* or the *PnirK2-egfp* transcriptional fusion was inoculated singly onto spring wheat (cv. Fiorina) seedlings under gnotobiotic conditions (Pothier et al. 2007), microscopy observations at 7 days revealed that inoculant cells were fluorescent on roots. Fluorescence was found in both single cells and numerous sparse clumps of cells, which were located especially in the root hair zone (Fig. 7A and D). The two fusions also were expressed on roots i) when winter wheat (cv. Soissons) was used instead of spring wheat or ii) when analysis was done at 10 days instead of 7 days (not shown). Therefore, it appears that both *nirK* genes are upregulated when *A. brasilense* Sp245 colonizes wheat roots.

DISCUSSION

In this study, dissimilatory nitrite reduction in *A. brasilense* Sp245 was shown to be catalyzed by two functional NirK iso-

Fig. 5. Neighbor-joining phylogenetic tree based on partial NirK sequences (accession numbers in parenthesis). The tree was inferred using the Kimura-2 parameter; bootstrap values (1,000 replicates) are shown when above 50%, and the scale bar represents the percentage of substitutions per site. The deduced protein sequence of *nirK* from *Thermobifida fusca* YX was used as outgroup. Symbols α , β , and γ indicate the corresponding proteobacterial subdivisions, and sequences from *Azospirillum* are shown using asterisks.

Fig. 6. Flow cytometry analysis of *nirK* transcriptional fusions. Relative intensity of green fluorescence (FL1-H) during flow-cytometry analysis of **A**, the *PnirK1-egfp* transcription fusion *Azospirillum brasilense* Sp245-Rif(pR3.8) and **B**, the *PnirK2-egfp* transcription fusion *A. brasilense* Sp245-Rif(pR3.9) grown in ABmal medium under aerobic (gray area; control) or microaerobic conditions (solid line). Representative results for one of three replicates are shown.

enzymes that are plasmid encoded. For the various species studied so far, the genes implicated in the denitrification process can be found either tightly clustered on the chromosome or plasmid-borne, even in the case of *nirK* (Chan and McCormick 2004; Schwintner et al. 1998).

A. brasilense Sp245 is the third example of a bacterium possessing two isofunctional nitrite reductases. Indeed, this already was reported for *Thauera* spp., a β -proteobacterium isolated from a high-nitrate-removal-rate reactor (Etchebehere and Tiedje 2005), and in the α -proteobacterium *Rhodopseudomonas palustris* (Larimer et al. 2004). In *Thauera* spp., however, the two *nirS* paralogs clustered distantly from one another, suggesting that one had been acquired horizontally. In contrast, here, NirK1 and NirK2 clustered similarly (as in *Rhodopseudomonas* spp.) and displayed high identity (99.2%). Phylogenetic analyses showed that both differed from most other α -proteobacterial NirK sequences, including that of other *Azospirillum* strains (Kloos et al. 2001). Therefore, it appears that horizontal acquisition of *nirK* by *A. brasilense* Sp245 (probably from a β -proteobacterium) took place prior to a gene duplication event. Indeed, *nirK* seems prone to horizontal transfer, especially in comparison with the nitrite reductase-encoding gene *nirS* (Heylen et al. 2006). The G + C content of *nirK1* and *nirK2* (64 and 63%, respectively) are in the range expected for *Azospirillum* spp.

Regions flanking *nirK1* and *nirK2* paralogs are different. Genes encoding homologues of small- and large-subunit precursors of nitric oxide reductase (i.e., NorC and NorB) were identified upstream of *nirK2*, and these genes displayed typical α -proteobacterial phylogenetic features (not shown). The proximity of *nirK* with *nor* genes also is documented in denitrifying

α -proteobacteria such as *Brucella* and *Sinorhizobium* spp. (Philippot 2002), whose *nirK* sequences were found in the large α -proteobacterial cluster (Fig. 5). On this basis, it is tempting to speculate that once *nirK* was acquired horizontally by *A. brasilense* Sp245 from β -proteobacteria, it recombined with an earlier α -proteobacterial *nirK* allele adjacent to the *norB-norC* genes (i.e., the current *nirK2* organization). If so, the *nirK* duplication event would have taken place afterward.

Unlike for *nirK2*, *nor* genes are not present upstream of *nirK1*. Instead, an ORF was found of which part of the deduced protein sequence corresponded to an oxidoreductase NAD-binding domain present in globins. In the case of globins, the enzyme may have NO dioxygenase activity, which is required for maintenance of a low, nontoxic NO concentration in the cell (Poole and Hughes 2000). However, it is not possible from the Sp245 sequence alone to establish that the neighboring gene encodes a globin oxidoreductase. Therefore, it cannot be concluded that genes next to *nirK1* are involved in denitrification, such as the ones next to *nirK2*.

Transcriptional fusions indicated that both *nirK1* and *nirK2* promoter regions of *A. brasilense* Sp245 were upregulated in vitro under conditions favorable for denitrification, as well as during colonization of spring wheat roots (under experimental conditions where *nirK1* is not expressed if the plant is replaced by polyvinylidene fluoride inert material) (Pothier et al. 2007). Thus, the two genes display similarity in their expression patterns despite divergent promoter regions, a result contrasting with the different regulation of dual *nirS* genes in *Thauera* spp. (Etchebehere and Tiedje 2005). This could imply (Zhang 2003) that the duplication event in *A. brasilense* Sp245 was rather recent in evolutionary terms and that functional diver-

Fig. 7. In planta expression of *nirK* transcriptional fusions analysed by a confocal laser scanning microscope. Expression of **A**, the *PnirK1-egfp* transcription fusion *Azospirillum brasilense* Sp245-Rif(pR3.8) and **D**, the *PnirK2-egfp* transcription fusion in *A. brasilense* Sp245-Rif(pR3.9) was monitored in the root hair zone of spring wheat roots at 7 days after inoculation of seedlings (fluorescent emission analysis). **B** and **E**, Images of the root obtained in transmitted light. **C**, Overlay of **A** and **B** and **F**, overlay of **D** and **E**; *rs* = root surface and *rh* = root hair.

gence has not yet proceeded extensively. Indeed, by mutating *nirK1* or *nirK2*, it appeared that the presence of either *nirK1* or *nirK2* was sufficient for maintaining denitrification in *A. brasilense* Sp245. In the Sp245 strain from Knoxville, inactivation of the sole *nirK* gene (i.e., *nirK1*) abolished denitrification but did not completely prevent growth, showing that cells lacking NirK activity still are viable and able to grow slightly under denitrifying conditions, a result already documented in other taxa (Baek and Shapleigh 2005; Philippot et al. 1995; Rees et al. 1997).

When assessing all bacterial sequences together, it appears that *nirK* has been subjected to neutral selection, which means that there is a low level of selective constraint on *nirK* diversification. Previous clustering of *nirK* clone sequences revealed some relations with the type of environment from which the sequences were obtained (Braker et al. 2000; Heylen et al. 2006; Priemé et al. 2002; Sharma et al. 2005). Perhaps this reflects the importance of physical proximity for horizontal gene transfer (Heylen et al. 2006). This also might be significant in terms of the particularity of denitrification processes in different environments. In the case of *A. brasilense* Sp245, the presence of two active nitrite reductases might be advantageous for root colonization, because environmental conditions in the rhizosphere are propitious for denitrification (Binnerup and Sørensen 1992; Højberg et al. 1999). Indeed, denitrification enables the bacterium to maintain energy metabolism when confronted with oxygen-deprived rhizosphere conditions (Philippot et al. 1995). In addition, NO produced by denitrifiers also may affect competing bacteria such as *Bacillus subtilis*, by causing stress and growth inhibition (Choi et al. 2006), and perhaps also dispersal of biofilm formed by neighboring bacteria such as pseudomonads (Barraud et al. 2006). Such effects could not be assessed in the current gnotobiotic conditions; however, whether or not they could favor Sp245 in its competition for root colonization sites deserves further attention.

Implication of NO in the plant hormone signaling network, especially in the indole-3-acetic acid biosynthesis pathway, is now widely documented (Lamattina et al. 2003). The promotion of lateral root formation of tomato inoculated by *A. brasilense* Sp245 previously was shown to require NO; however, whether the plant benefited directly from the NO produced by

bacteria or whether the association could modify NO production by root cells remains to be established (Creus et al. 2005). Nitric oxide also was produced during the *Medicago* spp.–*Sinorhizobium meliloti* interaction, raising the hypothesis of a role for NO in symbiosis signaling (Baudouin et al. 2006; Pii et al. 2007).

By analyzing the plasmid profile of Sp245 strains obtained from different laboratories, genomic rearrangements likely to have affected the smallest replicon (previously called the 75-MDa plasmid by Vieille and associates [1989]) (i.e., plasmid b on Fig. 2A) were revealed. Based on the principle of parsimony and the comparison of plasmid sizes, the most likely scenario of plasmid rearrangements is that replicon b (present in the Sp245 strains from Rehovot and Leuven; Fig. 2A, lane 5) could i) undergo a deletion event but without losing *nirK* (resulting in plasmid a in the Sp245 strain from Paris; Fig. 2A, lane 4), ii) form a cointegrate with the 160-kb replicon d (resulting in the *nirK* plasmid e in our Sp245 strain; Fig. 2A, lanes 2), or iii) be lost (in the Sp245 strain from Knoxville) (i.e., the one used in the current genome sequencing project; Fig. 2A, lane 3). Indeed, the presence of a single *nirK* copy in the latter, corresponding to *nirK1* according to its promoter region sequence, was confirmed by Southern hybridization (data not shown) and by searching *nirK* in the draft genome sequence of Sp245 (not available at the onset of this study). In summary, one of the plasmids bearing *nirK* can experience major genomic rearrangements. These rearrangements might be accompanied by the disappearance of genetic material, as can be suspected when assessing Eckhardt gels, which provides a likely explanation for the lack of a *nirK2* sequence in the *A. brasilense* Sp245 genome sequencing project. However, because the history of strain exchange patterns of Sp245 between these different laboratories has not been kept, the aforementioned scenario of rearrangements is only putative. Plasmid rearrangements have been described previously for Sp245, such as the loss of plasmid b (Vieille et al. 1989) or modification of plasmid profile after introduction of a RP4 replicon and long-term storage in rich medium (Katzy et al. 1998). Genomic rearrangements, such as plasmid loss or formation of cointegrate, also have been described in several *Azospirillum* strains undergoing phenotypic switch (Vial et al. 2006). In *A. lipoferum* 4B,

Table 2. Bacterial strains and plasmids used in this study

Strains and plasmids	Description ^a	Reference or source
Strains		
<i>Azospirillum brasilense</i> Sp245	Strain isolated from roots of wheat (Brazil) and kept in Lyon	Baldani et al. 1983
<i>A. brasilense</i> Sp245-Rif	Root- proficient, spontaneous rifampicin-resistant mutant of Sp245	Pothier et al. 2007
Sp245 <i>nirK1</i>	<i>nirK1</i> mutant of Sp245; Km ^r	This study
Sp245 <i>nirK2</i>	<i>nirK2</i> mutant of Sp245; Km ^r	This study
<i>Rhizobium etli</i> CFN42	Strain used as marker for plasmid profiles	Romero et al. 1991
Plasmids		
pGEM-T easy	Cloning vector for polymerase chain reaction products	Promega
pKNOCK-Km	Mobilizable plasmid, suicide vector for <i>A. brasilense</i> ; Km ^r	Alexeyev 1999
pOT1e	Promoter probe vector based on pBBR1-MCS-5 replicon; contains promoterless <i>egfp</i> and MCS between two transcriptional terminators; Gm ^r	Allaway et al. 2001
pR2.54	<i>PnirK1-egfp</i> transcription fusion in pOT1e; Gm ^r	Pothier et al. 2007
pR2.62 to pR2.66	Cosmid pWEB carrying <i>nirK1</i> from <i>A. brasilense</i> Sp245	This study
pR2.67	Cosmid pWEB carrying <i>nirK2</i> from <i>A. brasilense</i> Sp245	This study
pR3.6	1,183-bp amplicon (F5302/F3906) with 858 bp upstream of the <i>nirK1</i> ATG start codon cloned in pGEM-T easy; Ap ^r	This study
pR3.7	1,139-bp amplicon (F5303/F3906) with 814 bp upstream of the <i>nirK2</i> ATG start codon cloned in pGEM-T easy; Ap ^r	This study
pR3.8	<i>PnirK1-egfp</i> transcription fusion in pOT1e, 966-bp <i>Clal/Sall</i> fragment from pR3.6 with 848 bp upstream of the <i>nirK1</i> ATG start codon cloned in <i>Clal/Sall</i> -digested pOT1e; Gm ^r	This study
pR3.9	<i>PnirK2-egfp</i> transcription fusion in pOT1e, 920-bp <i>Clal/Sall</i> fragment from pR3.7 with 802 bp upstream of the <i>nirK2</i> ATG start codon cloned in <i>Clal/Sall</i> -digested pOT1e; Gm ^r	This study
pR3.10	268-bp internal amplicon of <i>nirK</i> (F5635/F5636) cloned in pGEM-T easy; Ap ^r	This study
pR3.12	290-bp <i>NotI</i> fragment from pR3.10 cloned in <i>NotI</i> -digested pKNOCK-Km	This study

^a Km^r, Gm^r, and Ap^r = resistant to kanamycin, gentamycin, and ampicillin, respectively.

the appearance of nonswimming variants was correlated with the loss of a 750-kb plasmid, that plasmid bearing the *acdS* gene whose product allows deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (Prigent-Combaret et al. in press). Thus, genome plasticity of *Azospirillum* strains may lead to the loss of genetic determinants such as *acdS* or *nirK*, which encode products that can be involved in the plant growth-promoting effect of *Azospirillum* spp.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains and plasmids used in this study are listed in Table 2. *A. brasilense* was grown at 28°C in Luria-Bertani (LB) medium (Sambrook et al. 1989) with NaCl at only 5 g liter⁻¹ (i.e., LBm), or in AB medium after replacing glucose with malic acid (5 g liter⁻¹; Sigma-Aldrich, Saint Quentin Fallavier, France) as sole carbon source (i.e., ABmal) (Chilton et al. 1974). *Escherichia coli* was grown in LB medium at 37°C. *Rhizobium etli* CFN42 was grown in tryptone-yeast extract medium supplemented with 6 mM CaCl₂. Liquid cultures were incubated on a rotary shaker at 180 rpm (50 rpm for microtiter plates). One-third of the volume of the tubes was filled to ensure fully aerobic conditions. To implement oxygen-limited conditions (microaerobiosis), the tubes were filled entirely with medium. Anaerobic growth was performed in 150-ml plasma flasks containing 38 ml of LBm medium supplemented with NaNO₂ to a final concentration of 2 mM. Anaerobiosis was obtained by using a vacuum pump and flushing with helium (three times). Plasma flasks then were inoculated with 2 ml of an aerobic overnight culture. Antibiotics (Euromedex, Souffelweyersheim, France) were added at the following final concentrations, unless otherwise specified: ampicillin (Ap) at 100 µg ml⁻¹, gentamycin (Gm) at 25 µg ml⁻¹, kanamycin (Km) at 40 µg ml⁻¹, and rifampicin (Rif) at 50 µg ml⁻¹.

Plasmid transfer and PCR conditions.

Bacterial conjugations were performed by biparental mating. To this end, 500-µl cell suspensions of *A. brasilense* Sp245-Rif recipient cells grown overnight in LBm containing Ap and 500 µl of *E. coli* S17.1 donor cells grown overnight in LB containing Gm or Km were mixed and collected by centrifugation. Cell pellets then were resuspended in 100 µl of LBm, placed onto LBm agar, and incubated for 20 h at 28°C. Cells were resuspended in 1 ml of 10 mM MgSO₄ and transconjugants were selected on LBm or ABmal containing Ap, Rif, and Gm (for pR3.8 or pR3.9) or Km (for pR3.12).

PCR primers (synthesized by Invitrogen, Cergy-Pontoise, France) are listed in Table 1. PCR amplifications were performed according to the *Taq* polymerase manufacturer (Invitrogen) in 25 µl using 50 ng of template DNA or using 1 µl of *E. coli* cells for library screening. The amplification cycle consisted of an initial 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at annealing temperature, and 30 s (or 90 s for large amplicons) at 72°C; followed by a final 7-min extension at 72°C. When used as probes and for sequencing, PCR products were purified using QIAquick PCR purification (Qiagen, Courtaboeuf, France).

Plasmid profiles and Southern hybridizations.

Plasmid profiles were determined by a modified Eckhardt agarose gel electrophoresis technique, as described (Vial et al. 2006), using *Azospirillum* cells grown in ABmal medium and electrophoresis at 50 V for 24 h. *R. etli* CFN42 was used as marker for estimation of plasmid size. For Southern hybridization experiments, genomic and cosmid DNA digested with either *Bam*HI, *Eco*RI, *Pvu*II, or *Sal*I, or plasmids, were blotted

to GeneScreen Plus nylon membrane (Perkin-Elmer, Zaventem, Belgium) with a vacuum blotter (Bio-Rad Laboratories, Marnes-la-Coquette, France). DNA was fixed by exposing to a 312-nm transilluminator for 4 min. Prehybridization, labeling, and purification of probes as well as hybridization were performed as previously described (Vial et al. 2006).

Sequencing and sequence analysis.

Sequencing was performed by Genome Express (Meylan, France) and sequences were analyzed with BLASTN and BLASTX algorithms on the National Center for Biotechnology Information website (Altschul et al. 1997). Identification of ORF and conversion to protein sequences were done with CUPLOT v1.0 using the *Azospirillum* codon usage database. Prediction of promoters was done with BPROM (SoftBerry, Mount Kisco, NY, U.S.A.) and manual refining then was implemented. Inverted repeat sequences near or within the predicted promoter regions were identified and examined for similarity to known binding motifs for transcriptional regulators of denitrification genes using the program FUZZNUC. Transcription starts were predicted using prokaryote neural network promoter prediction v2.2. Signal peptide and cleavage sites were predicted using SignalP v3.0 whereas transmembrane helices were detected with TMHMM v2.0.

DNA sequences and deduced protein sequences were aligned using the multiple alignment software CLUSTAL X v1.83. Between 141 and 913 amino acids were used for phylogenetic analyses using the software package MEGA v4 (Molecular Evolutionary Genetics Analysis). Phylogenetic distances were calculated by the NJ method using the Jukes-Cantor parameter model for DNA and Poisson correction for protein in MEGA. Consensus NJ trees were evaluated by bootstrap analyses based on 1,000 resamplings of each data set.

Accession numbers for sequence data obtained in the current work are EU221420 and EU221421. Preliminary data of the *A. brasilense* Sp245 genome sequencing project were used to confirm sequencing or ORF prediction. Additional *NirK* sequences from archaea and bacteria were obtained from the National Center for Biotechnology Information databases.

Determination of *nirK1* and *nirK2* sequences.

Total genomic DNA of *A. brasilense* Sp245 was obtained as described (Sambrook et al. 1989). A genomic library of *A. brasilense* Sp245 was constructed in *E. coli* Epi-100 T1^R using a pWEB cosmid-cloning kit (Epicentre, Madison, WI, U.S.A.) according to the manufacturer's instructions, except that sonication was performed for 1 min using an S-line apparatus (Bioblock, Illkirch, France) to shear genomic DNA. The 650 colonies obtained were transferred individually to microtiter wells and screened by PCR using primers F3787/F3789 or F3905/F3906 (Table 1). Sequencing of DNA of interest was performed by genome walking.

Construction of *egfp* transcriptional fusions with *nirK1* and *nirK2* promoters.

All standard cloning steps were carried out as described previously (Sambrook et al. 1989). Restriction enzymes were supplied by Fermentas (Burlington, Canada). The *PnirK1-egfp* and *PnirK2-egfp* transcription fusions were constructed by PCR amplification of *PnirK1* (1,183-bp amplicon, of which 858 bp were upstream of the *nirK1* start codon) and *PnirK2* (1,139-bp amplicon, of which 814 bp were upstream of the *nirK2* start codon), and subcloning into pGEM-T easy (Promega, Charbonnières, France). Each of the PCR primers targeting the promoter regions of *nirK1* and *nirK2* (F5302 and F5303 respectively) (Table 1) used to this end introduced a *Clal* restriction site and were used in combination with F3906

targeting *nirK*. White colonies were screened by PCR with primers F3906 (Table 1) and M13rev for correct orientation in pGEM-T easy (sense strand of *lacZ*). These constructs resulting in pR3.6 and pR3.7 (for *PnirK1* and *PnirK2*, respectively) were digested with *Clal* and *Sall*, cloned into the *Clal/Sall*-digested plasmid pOT1e and transferred into chemically competent cells of *E. coli* S17.1 λ pir (Sambrook et al. 1989). The resulting constructs, pR3.8 for *PnirK1* and pR3.9 for *PnirK2*, carry 848 and 802 bp, respectively, upstream of the start codon and both contain 118 bp of the coding region, which was confirmed by sequencing with primers F2603 and F2604 (Table 1). These constructs were introduced into *A. brasilense* Sp245-Rif by biparental mating (as described above).

Construction of *nirK1* and *nirK2* mutants.

To construct *nirK1* and *nirK2* mutants, a 268-bp *nirK* internal fragment was amplified by PCR with primers F5635/F5636 (Table 1) and subcloned into pGEM-T easy (Promega). White colonies were screened by PCR with primers F5635/F5636 for correct insertion in pGEM-T easy, resulting in pR3.10. The insert of plasmid pR3.10 was digested with *NotI* and cloned into the *NotI* site of pKNOCK-Km (Alexeyev 1999), resulting in pR3.12 after transfer into chemically-competent cells of *E. coli* S17.1 λ pir (Sambrook et al. 1989). For validation of the correct insertion of *nirK* upstream of the Km-resistance cassette in pR3.12, sequencing using primer F5595 was performed before introduction into *A. brasilense* Sp245-Rif and the Sp245 strain from Knoxville by biparental mating (as described above). Transconjugants resulting from a single recombination event of pR3.12 were selected on ABmal containing Rif and Km (for Sp245-Rif) or Amp and Km (for the Sp245 strain from Knoxville). These transconjugants were screened by PCR with primers F5595/F5597 for *nirK1* inactivation and F5595/F5598 for *nirK2* inactivation (Table 1). PCR amplicons were sequenced in order to check the recombination events.

Analysis of promoter activity by flow cytometry and epifluorescence microscopy.

Flow cytometry was used to measure fluorescence levels of *A. brasilense* Sp245 cells carrying either pR3.8 (*PnirK1-egfp*) or pR3.9 (*PnirK2-egfp*). Flow cytometry was performed with a FACSCalibur apparatus (Becton Dickinson Biosciences, Pont de Claix, France) fitted with a 15-mW argon laser emitting at 488 nm. FACSFlow solution (Becton Dickinson Biosciences) was used as sheath fluid, as previously described (Pothier et al. 2007).

Microscopy observations of cultures were made using an Axioskop 40 epifluorescence microscope (Carl Zeiss S.A.S., Le Pecq, France) with standard phase-contrast lenses. Fluorescent cells were observed with separate illuminations at 450 to 490 nm and detection with long-pass emission filter LP-520 nm.

Analysis of denitrification ability of *nirK* mutants.

Denitrification ability of *nirK* mutants was tested based on dissimilatory nitrite and nitric oxide reductase activities and compared with those of the wild type. To this end, the two *nirK* mutants and the wild-type strain were studied with nitrite as sole electron acceptor under anaerobic conditions, as described above. To monitor NO reduction to N₂O, which accumulated in the headspace of the plasma flasks in the presence of 10% acetylene, N₂O was determined by gas chromatography (Agilent Technologies, Massy, France). N₂O accumulation indicated a NO reductase activity and, hence, a NirK⁺ phenotype of the mutant. NO₂⁻ consumption was monitored colorimetrically by the Griess-Ilosvays reaction (Merck, Darmstadt, Germany) in culture medium samples. The lack of a red color reaction indicated the absence of NO₂⁻ because of its complete

reduction and, hence, a NirK⁺ phenotype of the mutant. Both assays were carried out after 3 days of incubation. The experiment was done twice with the same results.

Plant tests.

Spring wheat plant experiments were done as described by Pothier and associates (2007), under conditions where *nirK1* is not expressed if the plant is replaced with inert material (polyvinylidene fluoride filter membrane). Briefly, *Triticum aestivum* L. (cv. Fiorina) seedlings were prepared, inoculated with one of the *PnirK-egfp* transcriptional fusions, and grown axenically in 144-cm² petri dishes containing water agar. The dishes were placed standing for 7 days in a growth chamber (Sanyo MLR350, Loughborough, U.K.) at 75% relative humidity with 16 h of light (63 μ E m⁻² s⁻¹) at 26°C and 8 h of dark at 18°C. Root surface observations were performed using a confocal laser scanning microscope (CLSM) (510 Meta microscope; Carl Zeiss S.A.S.) equipped with an argon-krypton laser, detectors, and filter sets for green fluorescence (i.e., 488 nm for excitation and 510 to 531 nm for detection). After acquisition of transmitted light (in bright field mode), the two single-color images acquired were overlaid into a single image using LSM software release 3.5 (Carl Zeiss S.A.S.). Each fusion was studied using two 1-cm root pieces taken from the root tip area of two seedlings.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- National Center for Biotechnology Information BLAST database:
www.ncbi.nlm.nih.gov/BLAST/
- National Institute of Infectious Diseases CUPLOT Genus chooser database:
nocardia.nih.gov/jp/cuplot/
- Institut Pasteur FUZZNUC database:
mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=fuzznuc
- Berkeley Drosophila Genome Project Neural Network Promoter Prediction server: www.fruitfly.org/seq_tools/promoter.html
- Center for Biological Sequence (CBS) Analysis SignalP server:
www.cbs.dtu.dk/services/SignalP/
- CBS TMHMM server: www.cbs.dtu.dk/services/TMHMM/
- Molecular Evolutionary Genetics Analysis website:
www.megasoftware.net/
- A. brasilense* Sp245 genome sequencing data:
genomics.oml.gov/research/azo