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# Characterization of NADH Dehydrogenases of *Pseudomonas fluorescens* WCS365 and Their Role in Competitive Root Colonization

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**The excellent-root-colonizing *Pseudomonas fluorescens* WCS365 was selected previously as the parental strain for the isolation of mutants impaired in root colonization. Transposon mutagenesis of WCS365 and testing for root colonization resulted in the isolation of mutant strain PCL1201, which is approximately 100-fold impaired in competitive tomato root colonization. In this manuscript, we provide evidence that shows that the lack of NADH dehydrogenase I, an enzyme of the aerobic respiratory chain encoded by the *nuo* operon, is responsible for the impaired root-colonization ability of PCL1201. The complete sequence of the *nuo* operon (ranging from *nuoA* to *nuoN*) of *P. fluorescens* WCS365 was identified, including the promoter region and a transcriptional terminator consensus sequence downstream of nuoN. It was shown biochemically that PCL1201 is lacking NADH dehydrogenase I activity. In addition, the presence and activity of a second NADH dehydrogenase, encoded by the *ndh* gene, was identified to our knowledge for the first time in the genus *Pseudomonas*. Since it was assumed that low-oxygen conditions were present in the rhizosphere, we analyzed the activity of the *nuo* and the *ndh* promoters at different oxygen tensions. The results showed that both promoters are up-regulated by low concentrations of oxygen and that their levels of expression vary during growth. By using *lacZ* as a marker, it was shown that both the *nuo* operon and the *ndh* gene are expressed in the tomato rhizosphere. In contrast to the *nuo* mutant PCL1201, an *ndh* mutant of WCS365 appeared not to be impaired in competitive root tip colonization.**

*Additional keywords*: biological control, proton motive force.

Biological control of phytopathogenic fungi is an environmentally friendly alternative for the use of chemical pesticides in agriculture and horticulture. Biological control by *Pseudomonas* spp. is often based on the production of one or more antifungal factors (Thomashow and Weller 1996; Weller 1988; Weller and Thomashow 1994) and on an efficient root-colonizing ability as a delivery system of the antifungal factor or factors (Chin-A-Woeng 2000; Lugtenberg et al. 2000). Unsuccessful results in field trials with *Pseudomonas* biocontrol strains have been correlated with insufficient root colonization (Bull et al. 1991; Schippers et al. 1987). To elucidate genes and traits involved in root colonization, *Pseudomonas fluorescens* mutants were tested in our laboratory for their ability to colonize potato or tomato roots. It was shown that motility (de Weger et al. 1987), the synthesis of part of the O-antigen of the lipopolysaccharide (de Weger et al. 1989), and the synthesis of amino acids and vitamin B1 (Simons et al. 1997) are essential for competitive root tip colonization. *P. fluorescens* WCS365 was chosen as the parental strain for colonization mutants because of its excellent competitive root-colonizing ability (Simons et al. 1996). Screening a Tn*5lacZ* transposon library of WCS365 for mutants that are impaired in competitive root colonization resulted in the identification of novel colonization traits such as a site-specific recombinase (Dekkers et al. 1998a) and a two-component regulatory system (Dekkers et al. 1998b). Moreover, evidence was provided that NADH dehydrogenases could be important for competitive tomato root tip colonization (Dekkers et al. 1998c).

In this paper, we analyze the role of NADH dehydrogenases from *P. fluorescens* WCS365 in competitive root colonization. NADH dehydrogenases, also referred to as NADH/ubiquinone oxidoreductases, transfer electrons to one of the two terminal oxidase complexes (Anraku and Gennis 1987). In *Escherichia coli*, two distinct membrane-bound NADH dehydrogenases have been identified (Matsushita et al. 1987), NADH dehydrogenase I (NDH-1) encoded by the *nuo* operon, which contains 14 genes (Weidner et al. 1993), and NADH dehydrogenase II (NDH-2) encoded by the *ndh* gene (Jaworowski et al. 1981; Young et al. 1978, 1981). The two dehydrogenases can be distinguished based on their substrate specificity, sensitivity to inhibitors, and ability to generate proton motive force (PMF) (Matsushita et al. 1987). NDH-1 can oxidize both NADH and deamino-NADH (d-NADH) and can lead to the direct generation of a proton gradient (Matsushita et al. 1987). NDH-2 oxidizes only NADH, which is not directly coupled to the generation of a proton gradient (Matsushita et al. 1987; Zambrano and Kolter 1993). In *E. coli*, NADH dehydrogenases are located in the cytoplasmatic membrane and are part of the aerobic respiratory chain, the function of which is to generate a proton gradient or PMF across the cytoplasmatic membrane (Anraku and Gennis 1987; Calhoun and Gennis 1993). The PMF drives part of the cellular processes that require energy, such as ATP synthesis, active transport, and motility (Larsen et al. 1974), and plays a central role in sensory transduction in

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Nucleotide sequence data of the *nuo* operon and the *ndh* gene of *Pseudomonas fluorescens* WCS365 are available in the GenBank database under accession numbers AF281148 and AF281147, respectively.

response to oxygen, light, and other effectors (Taylor 1983). The proton gradient may also be required for the insertion of proteins into membranes and the translocation of proteins across membranes (Date et al. 1980; Gasser et al. 1982), as well as for regulating cellular processes such as cAMP synthesis by adenyl cyclase (Peterkofsky and Gazdar 1979).

In this paper, we describe (i) the isolation and sequencing of the complete *nuo* operon containing 14 genes and of the complete *ndh* gene of *P. fluorescens* WCS365; (ii) the construction of *nuo* and *ndh* mutant strains of WCS365, their biochemical properties, and their growth under conditions that are relevant to the rhizosphere and in a nonrhizosphere exudate component as the sole carbon source; and (iii) the roles of NDH-1 and NDH-2 in competitive root colonization.

## RESULTS

### Characterization of the *nuo* operon and of the *ndh* gene of *P. fluorescens* WCS365.

In a previous study, PCL1201 was selected for its impaired competitive root-colonizing ability, and sequencing of a small part of one of the flanking chromosomal regions of the Tn*5lacZ* transposon showed homology to *nuoD* from *E. coli* (Dekkers et al. 1998c). To identify the genes and the promoter region of the operon in which the Tn*5lacZ* transposon of PCL1201 is located, various cloning steps, library screening, and sequencing steps were performed as described below. Finally, this resulted in the identification of the complete sequences of 14 open reading frames with high homologies to the *nuoA* through *nuoN* genes of *E. coli*. The *nuoN* sequence was followed after 21 base pairs (bp) by a consensus terminator of transcription sequence (38 bp long). No consensus promoter sequences were recognized in the region upstream of the *nuoA* gene. Alignment studies showed that the deduced NuoA through NuoN proteins of WCS365 have high homology, ranging between 77 and 90% at amino acid level, to the corresponding *nuo* gene products of *E. coli* and that they are localized in the same order (Weidner et al. 1993; Yagi 1993).

In *E. coli*, a second NADH dehydrogenase encoded by the *ndh* gene is present (Young et al. 1978, 1981). A search in the *P. aeruginosa* genome sequence made available by the Pseudomonas Genome Project revealed the presence of a hypothetical *ndh* homologue. In order to isolate and subsequently analyze the function of a putative *ndh* homologue in *P. fluorescens* WCS365, polymerase chain reaction (PCR) primers were designed on conserved regions in the 3′ end obtained from the alignment of the hypothetical Ndh protein sequence of *P. aeruginosa* and the Ndh protein from *E. coli*. A PCR reaction with these primers and chromosomal DNA of *P. fluorescens* WCS365 as a template resulted in a 970-bp product, the sequence of which shows high homology to the 3′ end of *ndh*. Screening a genomic phage library of WCS365, with this *ndh* fragment as a probe, resulted in the isolation of a chromosomal clone containing the complete *ndh* homologous gene. Sequence analysis showed that the *ndh* gene of WCS365 consists of 1,296 bp and encodes a putative 47-kDa protein of 432 amino acid residues. The predicted protein has 51% identity at the amino acid level to NDH-2 from *E. coli* and 79% identity to the predicted hypothetical protein from *P. aeruginosa*.

### Oxidative ability of NDH-1 and NDH-2 mutants of WCS365.

A second NDH-1 mutant, strain PCL1090, was constructed to test whether the phenotype of PCL1201 is indeed due to the transposon insertion in the *nuoD* gene. PCL1090 was made by single homologous recombination in wild-type WCS365 with a suicide vector containing part of the *nuoD* gene (described in detail below). NDH-2 mutant PCL1091 was constructed by marker exchange with a kanamycin cassette flanked by 1-kilobase (kb) 5′ and 3′ fragments of the *ndh* gene present in a suicide vector (described in detail below). NDH-1 and NDH-2 mutants of *P. fluorescens* WCS365 were analyzed for their ability to oxidize NADH and d-NADH. Cell extracts of WCS365 grown overnight in liquid King's medium B contained an enzymatic activity, which oxidized both NADH and d-NADH at a rate of 40 and 50 nmol of substrate per min per mg of protein, respectively. Cell extracts of NDH-1 mutants PCL1201 and PCL1090 contained an enzymatic activity that oxidized NADH (17.5 ± 0.2 nmol of substrate per min per mg of protein) but very little d-NADH (7.5 ± 0.1 nmol of substrate per min per mg of protein). Cell extracts of NDH-2 mutant PCL1091 were able to oxidize both substrates (20 ± 0.7 nmol of NADH per min per mg of protein and 35 ± 0.6 nmol of d-NADH per min per mg of protein).

### Growth of NDH-1 and NDH-2 mutants in tomato root exudate.

Competition between WCS365 and its NADH dehydrogenase mutants was analyzed in mixed cultures grown in to-

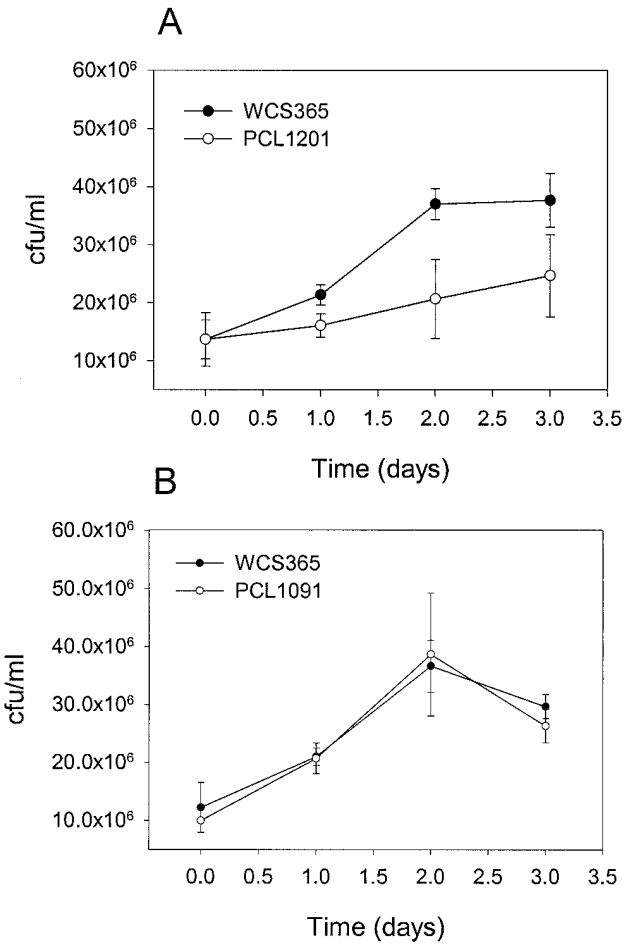


**Fig. 1.** Competition between *Pseudomonas fluorescens* WCS365 and NADH dehydrogenase mutants in tomato root exudate. Overnight bacterial cultures were washed and diluted to an optical density at 620 nm of 0.1 in phosphate buffer saline (PBS). Subsequently volumes of 125 µl were used to inoculate 2.5 ml of tomato root exudate (details described in text). Samples from the mixed cultures were taken each day during a period of 3 days and CFUs of the bacterial numbers determined. **A,** *nuo* Mutant PCL1201 in competition with the wild-type WCS365. **B,** *ndh* Mutant PCL1091 in competition with PCL1500 (a Tn*5lacZ* derivative of WCS365, which is not impaired in colonization). Shown are the average value of three measurements. Experiments were performed in triplicates.

mato root exudate. Competition between PCL1201 and WCS365 showed that PCL1201 grows significantly less than the wild type (Fig. 1A). It was calculated by analyzing CFU counts of the mixed cultures that, in competition, WCS365 made two to three generations and that PCL1201 made one to two generations in the first 48 h. In contrast, competition between PCL1091 and PCL1500 (*lacZ*-marked WCS365) showed that PCL1091 is able to compete in root exudate with its wild type (Fig. 1B). In contrast to root exudate, the competitive disadvantage of an *nuoD* mutation was not observed when the experiment was performed in a defined BM medium (Lugtenberg et al. 1999) containing 1% glycerol as a sole carbon source, since after 3 days of growth, the CFU values of WCS365 and PCL1201 were equal (data not shown).

**Expression of the *nuo* and *ndh* promoters during growth and at low concentration of oxygen.**

In order to analyze the expression of the *nuo* and *ndh* genes, their promoters were cloned in front of an e*gfp-lacZ* reporter gene (described in detail below). Promoter activities of *P. fluorescens* WCS365 wild type and its *nuo* and *ndh* mutant derivatives were analyzed during growth in liquid King's medium B by determining β-galactosidase activity (Fig. 2). Levels of P*nuo* and P*ndh* activities were comparable in the wild type and the mutants (Fig. 2). The activities of Pnuo and Pndh are growth phase dependent. The *nuo* promoter was most active during the mid logarithmic growth phase (Fig. 2A). When entering the stationary phase, its activity slightly decreased initially but increases again toward the late stationary phase. When 21% oxygen was bubbled through the culture, similar results were obtained (Fig. 3A). However, when 1% oxygen was bubbled through the culture, the *nuo* promoter was most active in the late logarithmic phase and its activity decreased as the culture entered the stationary phase (Fig. 3B). The level of expression of the *nuo* promoter under 1% oxygen in the late logarithmic phase was twice the level observed when 21% oxygen was pumped through the culture (Fig. 3A and B).

The *ndh* promoter was the least active during the early and mid logarithmic phases and its activity increased at the end of the log phase, reaching its maximum level in the stationary phase (Fig. 2B). Similar results were obtained when 21% oxygen was bubbled through the culture (Fig. 3A). The levels of expression under 1% oxygen conditions in the early log phase (Fig. 3B) were comparable to those observed when cells were grown under 21% oxygen. However, under 1% oxygen, activity of the *ndh* promoter strongly increased at the end of the log phase and at the beginning of the stationary phase and subsequently decreased during the stationary phase (Fig. 3B). The levels of expression of the *ndh* promoter under 1% oxygen in the late log phase and in the early stationary phase were twice the levels observed during the same growth phases under 21% oxygen (Fig. 3A and B). Under 1 and 21% oxygen, the *nuo* promoter is more active than the *ndh* promoter in the log phase, and in the stationary phase both promoters were active at comparable levels. Under 1% oxygen, both promoters were more active in the late logarithmic and the early stationary phases than under 21% oxygen.

**Tomato root tip-colonizing ability of NDH-1 and NDH-2 mutants.**

To analyze the influence of NADH dehydrogenases on root colonization, NDH-1 and NDH-2 mutants were tested for their root tip-colonizing ability on tomato after inoculation of germinated seeds and subsequent growth in the gnotobiotic system. When tested alone, both mutants were able to colonize the root tip in cell numbers comparable to those of the wild type (Table 1). NDH-1 mutants PCL1201 and PCL1090 were tested in competition with the wild-type WCS365 for tomato root tip colonization. Both mutants were significantly impaired in competitive rhizosphere colonization (Table 1; data not shown for PCL1090). In contrast, NDH-2 mutant PCL1091 was not impaired in competitive root colonization with its wild-type WCS365 (Table 1). When *nuo* and *ndh* mutants were tested against each other in competition for tomato root tip coloniza-

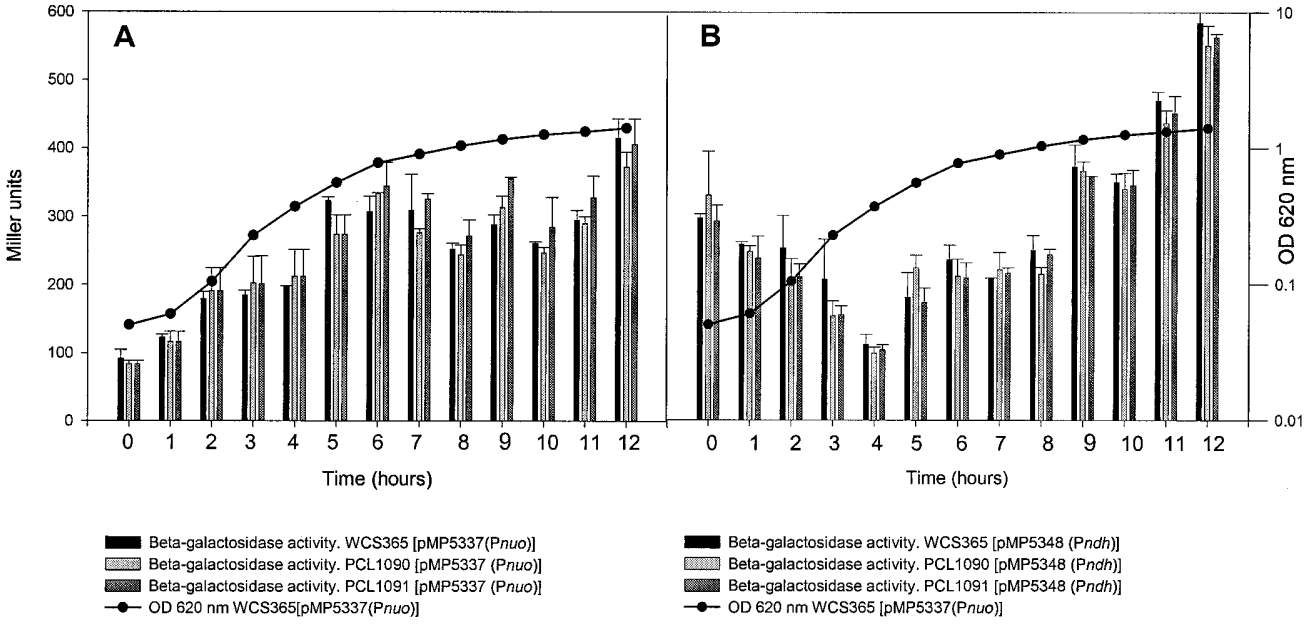


**Fig. 2.** Activity of the *nuo* and *ndh* promoters in *Pseudomonas fluorescens* WCS365 and its *nuo* and *ndh* mutant derivatives. *P. fluorescens* WCS365, PCL1090(*nuoD*::Km$^r$) and PCL1091(*ndh*::Km$^r$) containing the reporter constructs pMP5337 ($P_{nuo}$*lacZ*) and pMP5348 ($P_{ndh}$*lacZ*) were grown in liquid King's medium B. β-Galactosidase activity of cell lysates was determined during growth. Shown are the average values of three measurements. Experiments were performed in duplicate. The enzymatic activity (given in Miller units) was corrected for the amount of cells by calculating the activity per optical density at 660 nm. Shown is the growth curve of *P. fluorescens* WCS365 pMP5337. Growth curves of the other strains were similar and have been left out of the figure in order to keep it readable.

tion, the numbers of PCL1201 present on the root tip were significantly lower than those of PCL1091 (Table 1).

### Expression of the *nuo* and *ndh* promoters in the rhizosphere.

To analyze the expression of the *nuo* and *ndh* genes in the rhizosphere, tomato seedlings were inoculated with WCS365 cells containing the reporter constructs pMP5337 (*nuo* promoter in the correct orientation toward *lacZ*), pMP5338 (*nuo* promoter in reverse orientation), pMP5348 (*ndh* promoter in correct orientation), or pMP5346 (*ndh* promoter in reverse orientation) and grown in the gnotobiotic sand system. Roots were retrieved from the system 3, 5, and 7 days postinoculation, stained for β-galactosidase and examined by using light microscopy. At all three time points, light blue staining of the cells was observed along the whole root when seedlings had been inoculated with WCS365 containing pMP5337 or pMP5348. This staining was clearly visible in the microcolonies, which were formed in between the plant root epidermal cells (data not shown). In the case of WCS365 containing the negative control construct pMP5338 or pMP5346, no β-galactosidase staining was observed (data not shown).

## DISCUSSION

### Characterization of NDH-1.

Sequence analysis of the chromosomal region flanking the transposon insertion in PCL1201 revealed 14 genes, which form the *nuo* operon of *P. fluorescens* WCS365, the predicted protein sequences of which are highly homologous (between 77 and 90%) to the NuoA through NuoN proteins of *E. coli*. In *E. coli*, the *nuo* operon encodes 14 proteins, which form a complex named NADH dehydrogenase I. Our results show that *P. fluorescens* contains an *nuo* operon, which has the same organization as those of *E. coli* and other bacterial species (Weidner et al. 1993; Yagi 1993). In *E. coli*, the transcription of the *nuo* operon is dependent on $\sigma^{70}$ (Weidner et al. 1993). No recognizable consensus promoter sequences could be identified in the 1-kb base pair region upstream of *nuoA*. Downstream of *nuoN*, a consensus terminator of transcription sequence was detected, strongly indicating the end of the operon. Biochemical characterization of *nuo* mutant PCL1201 showed that cell extracts were reduced in their ability to oxidize NADH and even more reduced in oxidizing d-NADH in comparison to the wild-type WCS365. This shows that the gene products of the

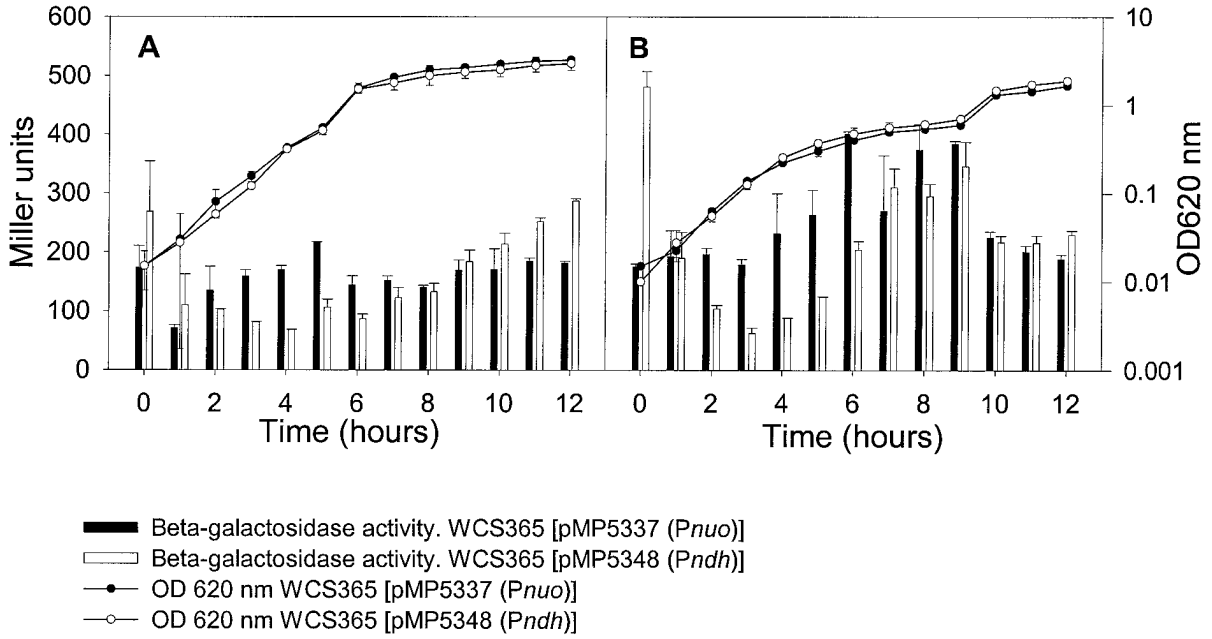


**Fig. 3.** Activity of the *nuo* and *ndh* promoters during growth under various oxygen concentrations. *Pseudomonas fluorescens* WCS365 containing the reporter constructs pMP5337($P_{nuo}$*lacZ*) and pMP5348 ($P_{ndh}$*lacZ*) were grown in liquid King's medium B. β-Galactosidase activity of cell lysates was determined during growth in the bubbling system with **A,** normal air (21% oxygen) or **B,** an oxygen/nitrogen gas mixture 1:99 (vol/vol) flowing through the system (details described in text). Shown are the average values of three measurements. Experiments were performed in duplicate. The enzymatic activity (given in Miller units) was corrected for the amount of cells by calculating the activity per optical density at 660 nm.

**Table 1.** Tomato root tip colonization by *Pseudomonas fluorescens* WCS365 and its *nuo* and *ndh* mutants

| Alone | | Competitive colonization[x] | | | |
|---|---|---|---|---|---|
| Strain | $\log_{10}$ (CFU + 1) per cm of root tip[y] | Strain | $\log_{10}$ (CFU + 1) per cm of root tip[y,z] | Strain | $\log_{10}$ (CFU + 1) per cm of root tip[y,z] |
| PCL1500 | 4.8 ± 0.3 | WCS365 | 4.4 a | PCL1201 | 2.8 b |
| PCL1201 | 4.3 ± 0.6 | PCL1500 | 4.4 a | PCL1091 | 4.5 a |
| PCL1091 | 4.7 ± 0.6 | PCL1091 | 4.4 a | PCL1201 | 2.6 b |

[x] *nuo* Mutant PCL1201 and *ndh* mutant PCL1091 were tested in competition with WCS365 or PCL1500 (a Tn*5lacZ* derivative of *P. fluorescens* WCS365, which is not impaired in colonization) and in competition with each other. For competition experiments, tomato seedlings were inoculated with 1:1 mixtures of bacterial strains and grown in the gnotobiotic system. Root tips were analyzed for the presence of bacteria after 7 days of growth.

[y] The values are expressed in mean $\log_{10}$ (CFU + 1) per cm of root tip for which 10 root tips of 10 independently grown plants were analyzed.

[z] When values are followed by a different letter, they are significantly different at $P = 0.05$ on nonparametric multiple comparisons by Wilcoxon-Mann-Whitney (Sokal and Rolhf 1981).

*nuo* operon of WCS365 have an enzymatic activity similar to that of NDH-1 in *E. coli* (Matsushita et al. 1987).

Although it is extremely difficult to measure oxygen tensions in a microenvironment such as the rhizosphere, there are indications that oxygen concentrations in the rhizosphere can locally be low (Hojberg et al. 1999). In addition, it has been reported that oxygen levels influence expression of NADH dehydrogenase genes in *E. coli* (Spiro et al. 1989). Therefore, we compared the growth rate and competitive ability of PCL1201 under conditions of low (1%) and high (21%) concentrations of oxygen. When 1 or 21% oxygen was pumped through the cultures, we observed that strain PCL1201 had growth rates similar to strain WCS365 (data not shown). However, a slight delay in the transition from the lag to the log phase was observed independent from the oxygen concentration used to grow the cells and independent from whether the *nuo* mutants were grown alone or in competition with the parental strain (data not shown). Promoter studies showed that the *P. fluorescens nuo* promoter is expressed at high levels during the early and mid logarithmic phases in King's medium B (Fig. 3), which can explain why an *nuo* mutant is slightly delayed in the transition from the lag into the logarithmic phase. In *E. coli*, a growth phase-dependent regulation of the *nuo* operon mediated by the growth responsive regulator Fis has been reported, in which maximal expression occurs in the early logarithmic phase (Wackwitz et al. 1999). The expression of the *E. coli* and the *P. fluorescens nuo* promoter are similar, which indicates that the regulation of the *nuo* promoter in *P. fluorescens* is also growth phase dependent.

**Characterization of NDH-2.**

Several NADH dehydrogenases with homology to NADH dehydrogenase II, encoded by the *ndh* gene, have been reported in different bacterial species (Yagi 1993) but not from the genus *Pseudomonas*. In this paper, we describe the isolation of the *ndh* gene of *P. fluorescens* WCS365, the predicted protein product of which shows a high homology to NADH dehydrogenase II from *E. coli* and to the predicted Ndh protein from *P. aeruginosa*, which we found in the recently sequenced genome. By using cell extracts of *ndh* mutant PCL1091 and the wild type, it was shown that these extracts were able to oxidize d-NADH but were reduced in their ability to oxidize NADH as compared with the wild type. This is similar to reports that indicate that NDH-1 from *E. coli* can oxidize both substrates, whereas NDH-2 oxidizes only NADH (Matsushita et al. 1987).

No difference in growth rate or delay in the transition from lag to log phase of *ndh* mutant PCL1091 was observed in liquid King's medium B (data not shown). In addition, we

**Table 2.** Bacterial strains, plasmids, and primers

| | Relevant characteristics/sequence[z] | Reference/source |
|---|---|---|
| Bacterial strains | | |
| *Pseudomonas fluorescens* | | |
| WCS365 | Efficient-root-colonizing *P. fluorescens* wild-type strain | Simons et al. 1996 |
| PCL1500 | Tn*5lacZ* derivative of *P. fluorescens* WCS365, not impaired in root colonization and growth rate; $Km^r$ | van der Bij et al. 1996 |
| PCL1201 | *P. fluorescens* WCS365 *nuoD*::Tn*5lacZ*; impaired in competitive root colonization; $Km^r$ | Dekkers et al. 1998c |
| PCL1090 | *nuo* Mutant derivative of *P. fluorescens* WCS365 constructed by single homologous recombination with pMP5301; *nuoD*::$Km^r$; $Km^r$ | This study |
| PCL1091 | *ndh* Mutant derivative of *P. fluorescens* WCS365 constructed by gene replacement; $Km^r$ | This study |
| *Escherichia coli* | | |
| DH5α | *endAl gyrSA96 hrdR17(rK-mK-)supE44 recAl* | Boyer and Roulland-Dussoix 1969 |
| XL1-Blue | *supE44 hsdR17 recAl endAi gyrA46 thi relAi lac- F'*[*proAB+laclq lacZM15 tn10(tetr)*] | Stratagene, La Jolla, CA, U.S.A. |
| XL1-Blue (MRA) | Δ(*mcrA*)*l83* Δ(*mcrCB-hsdSMR-mrr*)*l73 endAL supE44 thi-l gryA96 relAl lac* | Stratagene, La Jolla, CA, U.S.A. |
| XL1-Blue MRA (P2) | Δ(*mcrA*)*l83* Δ(*mcrCB-hsdSMR-mrr*)*l73 endAL supE44 thi-l gryA96 relAl lac* (P2 lysogen) | Stratagene, La Jolla, CA, U.S.A. |
| Plasmids | | |
| pBluescript SK(+/–) | Cloning vector; $Cb^r$ | Stratagene, La Jolla, CA, U.S.A. |
| pIC20H | Cloning vector; $Cb^r$ | Marsh et al. 1984 |
| pIC20R | Cloning vector; $Cb^r$ | Marsh et al. 1984 |
| pGEMT easy | Cloning vector for direct cloning of polymerase chain reaction (PCR) products; $Cb^r$ | Promega Benelux bv, Leiden, The Netherlands |
| pME3049 | ColE1 derivative; suicide vector in *P. fluorescens* developed for single homologous recombination; $Km^R$ $Hg^R$ | Schnider et al. 1995; Voisard et al. 1994 |
| pME6010 | Rhizosphere stable cloning vector; $Tc^R$ | Heeb et al. 2000 |
| pMP1008 | pACYC184 derivative containing a Tn*5*, the kanamycin resistance of which was used to construct pMP5358; $Cb^r$ $Km^r$ | H. Spaink, *personal communication* |
| pMP4439 | Reporter vector containing a promoterless *egfp-lacZ* gene fusion in pBluescript SK+; $Cb^R$ | Bakkers 2000 |
| pMP4606 | Modified pBluescript II SK(+) | Bakkers 2000 |
| pLAFR3 | Cloning vector; $Tc^R$ | Staskawicz et al. 1987 |
| pLAFR2 | pLAFR3 with a substituted multicloning site: *Eco*RI-*Cla*I-*Hin*dIII-*Xba*I-*Bgl*II-*Pst*I-*Bam*HI-*Eco*RI | Staskawicz et al. 1987 |
| pMP5245 | pIC20H containing a *Sal*I fragment of the chromosomal region of PCL1201 flanking the Tn*5lacZ*; $Cb^r$ $Km^R$ | Dekkers et al. 1998c |
| pMP5301 | pME3049 containing a 279-bp *Bam*HI-*Hin*dIII *nuoD* fragment of pMP5245; $Km^r$ | This study |
| pMP5304 | pIC20H containing the *Eco*RI fragment of the chromosomal region of PCL1201 flanking the Tn*5lacZ*; $Cb^r$ $Km^r$ | This study |
| pMP5310 | pME3049 plasmid derivative obtained after self-ligation of a *Hin*dIII chromosomal digest of PCL1090; $Km^R$ | This study |
| pMP5313 | pME3049 plasmid derivative obtained after self-ligation of a *Sac*I chromosomal digest of PCL1090; $Km^R$ | This study |
| pMP5320 | pMP4606 harboring a 970-bp PCR product containing the 3′ end of the *ndh* gene of WCS365 cloned in the *Sma*I site; $Cb^r$ | This study |
| pMP5324 | pBluescript SK+ containing an *Not*I insert (±10 kb) with *nuoA* through *nuoH* of WCS365; $Cb^R$ | This study |

(*continued on next page*)

[z] Cb = carbenicillin, Hg = mercury, Km = kanamycin, and Tc = tetracycline.

showed that PCL1091 was also able to compete with the wild type in tomato root exudate, in contrast to PCL1201, which loses from wild-type WCS365 (Fig. 1). These results show that, under these conditions, the lack of NDH-2 does not present the cells with a competitive disadvantage and indicates that NDH-1 is sufficient to provide electrons to the respiratory chain. Similar competition experiments in minimal medium containing glycerol (which has never been indicated to be a root exudate component) as the sole carbon source did not show a competitive disadvantage for PCL1201, thus indicating that this is a rhizosphere-specific effect. We studied the activity of the *ndh* promoter under low and high concentrations of oxygen. The activity of the *ndh* promoter was different when 1 or 21% oxygen (air) was pumped through cultures (Fig. 3A and B). At 1% oxygen, the *ndh* promoter was very active at the end of the log phase (Fig. 3B). The levels of expression of both the *nuo* and the *ndh* promoters were up-regulated when cultures were grown under low concentrations of oxygen (Fig. 3A and B) in the logarithmic phase. During the early stationary phase, the levels of expression of both promoters were higher under the 21% oxygen concentration. Our results show that the oxygen concentration, which presumably varies in the rhizosphere, plays a role in the expression of the *nuo* and *ndh* genes.

**Role of NADH dehydrogenases in rhizosphere colonization.**

Since we have shown the presence of a second NADH dehydrogenase encoded by the *ndh* gene in WCS365, we have analyzed its role in competitive root colonization. When tested alone, both *nuo* and *ndh* mutants were able to colonize the tomato rhizosphere to the same extend as the wild type (Table 1). When tested in competition with the parental strain, it was shown that only an *nuo* mutant has a root tip colonization defect. When the *nuo* and *ndh* mutants were tested in competition against each other, the *nuo* mutant showed a competitive root-colonizing defect (Table 1). These results show that NDH-1 is an important trait of *Pseudomonas* bacteria for competitive root tip colonization. In contrast, loss of NDH-2 does not result in a competitive disadvantage in the rhizosphere, although it is expressed in the rhizosphere, which indicates a function. We can speculate that an active NDH-1 can compensate for the loss of NDH-2 in the rhizosphere. It was indicated by growth experiments that the competitive disadvantage is caused by the presence or utilization of root exudate. Competition experiments in root exudate showed that the NDH-2 mutant can compete, but the NDH-1 mutant cannot compete, with the wild type (Fig. 1). This is in contrast to competition experiments in King's medium B (a rich medium) or a defined medium con-

**Table 2.** (*continued from preceding page*)

| | Relevant characteristics/sequence[z] | Reference/source |
|---|---|---|
| pMP5327 | pBluescript SK+ containing an *Not*I insert (±16 kb) with *nuoA* through *nuoH* of WCS365 and the region upstream of *nuoA*; $Cb^R$ | This study |
| pMP5331 | pMP4439 containing an *Nco*I-*Eco*RI PCR product (650 bp) with P*nuo* obtained with primers oMP472 and oMP473; $Cb^R$ | This study |
| pMP5332 | pMP4439 containing an *Nco*I-*Eco*RI PCR product (690 bp) with P*nuo* obtained with primers oMP474 and oMP473; P*nuo* in reverse orientation in respect to the reporter gene; $Cb^R$ | This study |
| pMP5337 | pME6010 containing a *Bam*HI-*Eco*RI insert with P*nuo-egfplacZ* from pMP5331; $Tc^R$ | This study |
| pMP5338 | pME6010 containing a *Bam*HI-*Eco*RI insert with P*nuo-egfplacZ* from pMP5332; $Tc^R$ | This study |
| pMP5346 | pMP5337 containing an *Eco*RI-*Nco*I PCR product (350 bp) with the P*ndh* region obtained with primers oMP478 and oMP479; P*ndh* in reverse orientation in respect to the fusion; $Tc^R$ | This study |
| pMP5347 | pMP4439 containing an *Nco*I PCR product (350 bp) with the P*ndh* region obtained with primers oMP476 and oMP477; $Cb^R$ | This study |
| pMP5348 | pME6010 harboring a *Bam*HI-*Eco*RI insert from pMP5347 containing P*ndh-egfp-lacZ* cloned into *Bgl*II-*Eco*RI; $Tc^R$ | This study |
| pMP5358 | pIC20R containing an *Hin*dIII-*Sal*I fragment from pMP1008 carrying a kanamycin resistance gene; $Cb^R$ $Km^R$ | This study |
| pMP5359 | pMP5358 harboring a *Cla*I-*Hin*dIII PCR product (950 bp), which contains the 3′ end of the *ndh* gene; $Km^R$ | This study |
| pMP5360 | pMP5359 harboring a *Bam*HI-*Sal*I PCR product (940 bp), which contains the 5′ end of the *ndh* gene; $Km^R$ | This study |
| pMP5361 | pLAFR2 harboring a *Bam*HI-*Cla*I 3.5-kb fragment from pMP5360; $Km^R$ $Tc^R$ | This study |
| pMP5374 | pBluescript SK+ containing an *Not*I insert (±7 kb) with *nuoH* through *nuoN* of WCS365; $Cb^R$ | This study |
| pMP5378 | pGEMT easy containing a ±1.5-kb chromosomal PCR containing the downstream sequence of the *nuo* operon starting at –43 bp from the 3′ end of *nuoN*; $Cb^R$ | This study |
| Primers | | |
| oMP468 | 5′ ctcagtgcagcttgaggcgtgg 3′ | This study |
| oMP469 | 5′ agcaccaccaacgacttcg 3′ | This study |
| oMP470 | 5′ cgtaaagcttggctcgatcgacatcaggtga 3′ | This study |
| oMP471 | 5′ cgtaggatcccagcgtatcgcagtcacca 3′ | This study |
| oMP472 | 5′ ggaattctggacattgatctgatctgacgaacctg 3′ | This study |
| oMP473 | 5′ ccttcggccatggtggaaatccttaac 3′ | This study |
| oMP474 | 5′ ggaattcggagatggtggaaatcc 3′ | This study |
| oMP475 | 5′ cttaaacgtgcaaaccccatgg 3′ | This study |
| oMP476 | 5′ cgcgagtgccatggaactcatag 3′ | This study |
| oMP477 | 5′ ggaattccatgatcgaacttggacg 3′ | This study |
| oMP478 | 5′ ggaattccatgcaactcatagcgtcag 3′ | This study |
| oMP479 | 5′ gcgcccatggtcgaacttgg 3′ | This study |
| oMP480 | 5′ ctatcgatattcaatgtcagtgctg 3′ | This study |
| oMP481 | 5′ catgaagcttagcactgcctgttcctcg 3′ | This study |
| oMP482 | 5′ gtcgggatccgccttgtgagcc 3′ | This study |
| oMP483 | 5′ gttcgcgtcgaccagcatcacactg 3′ | This study |
| oMP671 | 5′ cgcagccgttgctgaccctgg 3′ | This study |
| oMP672 | 5′ ggtacacaccgaggaagaacgcc 3′ | This study |

taining glycerol, a nonexudate component, as the sole carbon source, in which no competitive disadvantage was observed (data not shown). PMF is required for processes that require ATP such as motility and active transport across membranes. Although the conditions present in the rhizosphere are far from understood, we could hypothesize that, as the bacterial populations grow and colonize the rhizosphere, carbon sources that are secreted by the plant root (Lugtenberg et al. 1999) are consumed. The most efficiently growing bacteria will have a competitive advantage in the rhizosphere, and those cells with the ability to optimally use the scarcely available carbon sources will eventually take over the population. By using P*egfplacZ* reporters, we were able to show that the P*nuo* and P*ndh* promoters are expressed in the rhizosphere. The detected β-galactosidase staining in the rhizosphere as examined with light microscopy was light blue, suggesting that, although both the *nuo* and the *ndh* promoters were expressed, their levels of expression were relatively low. This notion was supported by the observation that the levels of expression of the reporter fusion under the control of the *nuo* or the *ndh* promoter were not high enough to detect *egfp* expression at the single-cell level by using epifluorescence confocal microscopy. Single cells in which *egfp* is expressed under the control of a strong constitutive promoter can easily be observed (Bloemberg et al. 1997).

From the fact that NDH-2 is expressed in the rhizosphere, it is indicated that NDH-2 has a role; however, we have shown that NDH-2 does not give a competitive advantage for colonization (Table 1). Our results show the specific importance of NDH-1 (Table 1) in the rhizosphere, giving a more detailed view of the bacterial physiology in the rhizosphere.

Summarizing, *P. fluorescens* possesses two NADH dehydrogenases, which are expressed in the tomato rhizosphere. Our results show that during the root colonization process only NDH-1 plays a crucial role in competitive colonization of the root tip. Apparently, the physiological state of the cells present in the rhizosphere requires the energy-coupled NDH-1 in order to be competitive. In addition, we have shown that only NDH-1, but not NDH-2, mutant numbers are significantly lower than those of the wild type when grown in root exudate (Fig. 1A and B). Therefore, we can conclude that under the conditions that occur in the rhizosphere, such as competition for nutrients and low oxygen tensions, the lack of the energy-coupling NADH dehydrogenase (NDH-1) leads to a competitive disadvantage in root tip colonization, whereas loss of NDH-2 has no influence or can be compensated for by the cell, showing that specific enzymatic activities are essential for an optimal functioning in the rhizosphere environment.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions.

Bacterial strains are listed in Table 2. *Pseudomonas* strains were grown overnight at 28°C in King's medium B (King et al. 1954) under vigorous aeration or for 2 days on solid King's agar medium B. *E. coli* strains were grown in Luria Bertani (LB) medium at 37°C under vigorous aeration or on LB agar plates. When necessary, kanamycin or tetracycline was added to a final concentration of 50 μg/ml or 80 μg/ml, respectively. Growth conditions for the screening of a chromosomal library were used as described by the manufacturer (Stratagene, La Jolla, CA, U.S.A.).

Competitive growth experiments in tomato root exudate were performed as follows: overnight King's medium B cultures were washed and resuspended in phosphate buffer saline, of which 125 μl was used to inoculate 2.5 ml of tomato root exudate (Lugtenberg et al. 1999) or BM medium (Lugtenberg et al. 1999) supplemented with 1% glycerol as the sole carbon source. Cultures were grown under vigorous aeration at 28°C for 3 days. To determine the CFUs in the cultures, samples were taken every 24 h, diluted, and plated on King's agar B supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml).

For growth under 1.0% oxygen, strains were cultured in 40 ml of liquid King's medium B, to which silicon antifoaming agent (BDH Limited, Poole, England) was added to a final concentration of 0.005% (vol/vol). A filter-sterilized mixture of oxygen and nitrogen (1:99, vol/vol) was pumped through a sterile glass filter immersed in the glass tube containing the culture. Composition of the gas mix was controlled with a gas flow controller and mixer (model 5878; Brooks Instruments B.V., Veenendaal, The Netherlands). We refer to this system as the bubbling system. As a control, air was pumped through the system during culturing.

### Isolation of NADH dehydrogenase I and II genes.

Several strategies were followed to identify genes and the organization of the *nuo* operon of WCS365. The opposite chromosomal flanking region of the Tn*5lacZ* in PCL1201 was isolated in the same way as the first one (Dekkers et al. 1998c), with *Eco*RI to digest the chromosomal DNA, and cloned into pIC20H, resulting in plasmid pMP5304. In order to isolate additional genes of the *nuo* operon of WCS365, chromosomal DNA of PCL1090 was isolated and digested with *Bam*HI or *Sac*I followed by a self-ligation. The ligation mixture was transformed to *E. coli* DH5α and colonies were selected on kanamycin, resulting in the isolation of plasmids pMP5310 and pMP5313, respectively. In addition, a 420-bp *nuoC* fragment obtained by PCR with primers oMP470 and oMP471 (Table 2) on pMP5310 was used to screen the custom-made genomic library of *P. fluorescens* WCS365. A ±10-kb *Not*I fragment hybridizing with the *nuoC* probe was cloned into pBluescript SK(+/–), resulting in plasmid pMP5324. Sequencing (performed at Baseclear, Leiden, The Netherlands) of the insert showed the presence of the 3′ end of *nuoA* through *nuoH*. To isolate the remaining 5′ end of *nuoA* and the promoter region of the *nuo* operon, pMP5324 was digested with *Hin*dIII and *Eco*RV to isolate a 690-bp fragment corresponding to the 3′ end of *nuoA* through the 5′ end of *nuoB*, which was used as a probe to rescreen the library. A ±16-kb *Not*I fragment was isolated and cloned into pBluescript SK(+/–). Sequencing showed that the resulting plasmid pMP5327 contains the promoter region of the *P. fluorescens* WCS365 *nuo* operon and the genes *nuoA* through *nuoN*, ending at 16 bp downstream of the stop codon of *nuoN*. To facilitate sequencing of the genes downstream of *nuoH*, a ±7-kb *Not*I insert with *nuoH* through *nuoN* was cloned into pBluescript SK+, resulting in pMP5374. In order to determine the nucleotide sequence downstream of *nuoN*, an inverted PCR was performed with *pfu*-polymerase after restriction of chromosomal DNA of WCS365 with *Nae*I with primers oMP671 and oMP674, the sequences of which are both located in the 3′ end of *nuoN* (oMP671 positions 15,114 to 15,134 and oMP672 positions 15,113 to 15,091 according to the nucleotide sequence as deposited in GenBank accession no. AF281148). The resulting ±1.5-kb PCR fragment was cloned in the pGEMT easy (Promega, Madison, WI, U.S.A.), resulting in pMP5378. Sequencing of this insert revealed a terminator of transcription sequence starting 29 bp downstream of the TGA stop codon of *nuoN*.

The most conserved regions of the Ndh protein based on the alignment of the Ndh from *E. coli* and the hypothetical Ndh from *P. aeruginosa* (Pseudomonas Genome Project) were used to design PCR primers oMP468 and oMP469 (Table 2). A PCR reaction with oMP468 and oMP469 on chromosomal DNA of *P. fluorescens* WCS365 resulted in a 970-bp product, which

was cloned into the *Sma*I site of pMP4606 (a modified pBluescript SK[+/–]), resulting in pMP5320. A custom-made genomic library of *P. fluorescens* WCS365 (Stratagene) in the Lambda FIX II vector was screened by using the protocol recommended by the manufacturer. The 970-bp PCR fragment mentioned above was marked by random-primed labeling (Memelink et al. 1994) and used as a probe for screening the genomic library. Subsequently, phage DNA was isolated, digested, and blotted according to standard methods (Sambrook et al. 1989). Hybridization and labeling of the 970-bp PCR fragment were performed with the AlkPhos Direct Kit (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands). Detection was performed with the CDP-*Star* detection reagent (Amersham Pharmacia Biotech Benelux).

**Construction of NADH dehydrogenase mutants.**

In order to construct an *nuoD* mutant in strain WCS365, a 279-bp *Bam*HI-*Hin*dIII fragment from pMP5245, containing part of *nuoD*, was cloned into pME3049, which resulted in pMP5301. This construct was electroporated into WCS365 and kanamycin-resistant homologous recombinants were selected and analyzed by Southern blot with the 279-bp fragment as a probe. This resulted in the isolation of the NDH-1 mutant strain PCL1090.

WCS365 NDH-2 mutant PCL1091 was constructed by gene replacement with pMP5361, which contains a kanamycin-resistant cassette flanked by two 1-kb PCR fragments containing the 5′ and 3′ ends of the *ndh* gene, respectively. Within the 5′ end of the gene, 203 bp had been deleted. A 950-bp *Hin*dIII-*Cla*I PCR fragment produced with primers oMP480 and oMP481 (Table 2) containing the 3′ end of the *ndh* gene of *P. fluorescens* WCS365, including the stop codon and 35 downstream bp, was cloned into pMP5358, resulting in pMP5359. A 940-bp *Bam*HI-*Sal*I PCR fragment produced with primers oMP482 and oMP483 (Table 2) containing the 5′ end on the *ndh* gene, including the start codon and 831 upstream bp, was cloned into pMP5359, resulting in pMP5360. The 3.4-kb *Cla*I-*Bam*HI fragment of pMP5360 containing the 5′ end of the *ndh* gene, the kanamycin cassette, and the 3′ end of the *ndh* gene was cloned into pLAFR2, resulting in pMP5361. This plasmid was electroporated to WCS365. Kanamycin-resistant, tetracycline-sensitive derivatives of WCS365 to which pMP5360 was electroporated were selected and checked with Southern blotting with the same probe used to screen the genomic library (970-bp PCR fragment on WCS365 chromosomal DNA with primers oMP468 and oMP469).

**Root colonization assay.**

Bacterial strains were tested for competitive colonization of tomato roots by using the gnotobiotic system and inoculation procedures as described previously (Simons et al. 1996). Briefly, sterile seedlings were inoculated with a bacterial suspension containing wild-type and mutant cells in a 1:1 ratio and grown in the gnotobiotic system. After 7 days of growth, root tips (1 cm) were isolated and bacteria were removed from the root tip by washing. CFUs were determined after diluting and plating.

**Biochemical characterization of NADH dehydrogenase activity.**

Volumes (25 ml) of overnight bacterial cultures grown in King's medium B were harvested by centrifugation and resuspended in 5 ml of buffer A consisting of potassium phosphate (pH 7.5)-5 mM $MgSO_4$ (Matsushita et al. 1987). Samples were passed through a French pressure cell (American Instrument Company, Silver Spring, MD, U.S.A.) at a shear force of 16,000 psi (Matsushita and Kaback 1986). Cell debris was removed by centrifugation for 15 min at 10,000 × *g*. Total protein content of the supernatants was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). Supernatants were diluted to 2.5 mg of total protein per ml in 50 mM potassium phosphate, pH 7.5, and were used to assay NADH dehydrogenase activity as described previously (Matsushita et al. 1987; Zambrano and Kolter 1993). Experiments were performed in triplicates.

**Construction of reporter plasmids and expression studies.**

The *nuoA* and *ndh* promoters were cloned upstream of the ATG start codon of a *gfp-lacZ* reporter fusion in the rhizosphere stable vector pME6010 (Heeb et al. 2000). As controls, promoter fragments were cloned in the reverse orientation. Promoter fragments contained regions starting immediately upstream of the ATG start codon of the analyzed genes.

A 650-bp *Eco*RI-*Nco*I PCR fragment produced with primers oMP472 and oMP473 (Table 2) was cloned into pMP4439, resulting in pMP5331 (*nuo* promoter in correct orientation). A 690-bp *Eco*RI-*Nco*I PCR fragment produced with primers oMP474 and oMP475 (Table 2) was cloned into pMP4439, resulting in pMP5332 (*nuo* promoter in reverse orientation). Both pMP5331 and pMP5332 were digested with *Eco*RI and *Bam*HI, and the resulting 4.5-kb fragments, containing the *nuo* promoter fragment in both orientations in front of the *egfp-lacZ* reporter fusion, were cloned into pME6010, which had been digested with *Eco*RI and *Bgl*II. Finally, this resulted in pMP5337 and pMP5338.

In order to construct a *ndh* promoter reporter fusion, a 660-bp PCR fragment produced with primers oMP477 and oMP476 (Table 2) was digested with *Nco*I and the resulting 350-bp *Nco*I-*Nco*I fragment was cloned into pMP4439, resulting in pMP5347. This plasmid was digested with *Eco*RI and *Bam*HI and the 4.1-kb fragment containing the promoter *egfp-lacZ* fusion was cloned into pME6010, which had been digested with *Eco*RI and *Bgl*II, resulting in pMP5348. In order to design a control construct with the *ndh* promoter in the reverse orientation in respect to the reporter gene fusion, a 660-bp PCR fragment was obtained with primers oMP478 and oMP479 (Table 2) and digested with *Nco*I and *Eco*RI and the resulting 300-bp *Eco*RI-*Nco*I fragment was cloned into pMP5337, which resulted in pMP5346.

Reporter constructs pMP5337 and pMP5338 were used to analyze the expression of the *nuo* promoter and pMP5346 and pMP5348 for the *ndh* promoter. Gene expression was determined during growth in liquid King's medium B under vigorous aeration or in the bubbling system. Culture samples were taken at various times and were assayed for β-galactosidase activity (Miller 1972). In order to assess gene expression in the rhizosphere, sterile germinated tomato seedlings were inoculated with WCS365 cells carrying reporter constructs and grown in the gnotobiotic system as described above. Plants were retrieved from the system after 3, 5, and 7 days. Plants were stained for β-galactosidase as described previously (Boivin et al. 1990) with minor modifications. After retrieval, plants were washed for 15 min in Z buffer and fixed for 30 min in a 1.25% (vol/vol) solution of glutaraldehyde in Z buffer. Subsequently, the plants were washed twice for 30 min in Z buffer and stained overnight at 28°C in a solution of X-Gal (0.8 mg/ml). Plants were rinsed three times with Z buffer prior to light microscopic analysis (Zeiss Axioplan; objectives 40×/1.3 oil immersion and 100×/1.3 oil immersion; Zeiss, Göttingen, Germany).

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## AUTHOR-RECOMMENDED INTERNET RESOURCE

Pseudomonas Genome Project: http://www.pseudomonas.com