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Novel *nirK* Cluster Genes in *Nitrosomonas europaea* Are Required for NirK-Dependent Tolerance to Nitrite

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Nitrite reductase (NirK) of *Nitrosomonas europaea* confers tolerance to nitrite (NO_2^-). The *nirK* gene is clustered with three genes of unknown physiological function: *ncgABC*. At present, this organization is unique to nitrifying bacteria. Here we report that the *ncgABC* gene products facilitate NirK-dependent NO_2^- tolerance by reversing the negative physiological effect that is associated with the activity of NirK in their absence. We hypothesize that the *ncg* gene products are involved in the detoxification of nitric oxide that is produced by NirK.

Nitrosomonas europaea is a nitrifying bacterium that makes free energy available by the aerobic oxidation of ammonia (NH_3) to NO_2^- (12). During nitrification, this organism expresses NirK and nitric oxide reductase, both enzymes that are classically associated with anaerobic respiration in denitrifying bacteria (1, 3). Inactivation of the *nirK* gene of *N. europaea* rendered the cells more sensitive to the toxic effects of NO_2^- produced during nitrification (1). Recently, we demonstrated that this bacterium expresses increasing levels of NirK in response to the accumulation of NO_2^- in its environment (2). This NirK-dependent tolerance to NO_2^- might constitute a defense mechanism that protects the cell against the toxic product of aerobic NH_3 oxidation (2, 10).

The *nirK* gene of *N. europaea* is clustered with three other genes (NE0927, NE0926, and NE0925) (1, 5). We have designated these *nirK* cluster genes *ncgA*, *ncgB*, and *ncgC*, respectively (Fig. 1a). Inspection of the preliminary genome sequence of the NO_2^--oxidizing nitrifying bacterium *Nitrobacter hamburgensis* revealed the presence of a similar *nirK* gene cluster (U.S. Department of Energy Joint Genome Institute [http://www.jgi.doe.gov/]). Other bacterial *nirK* genes characterized thus far are transcribed either monocistronically or polycistronically in conjunction with *nirV* (8). The physiological role of the latter gene is not known; *nirV* of *Rhodobacter sphaeroides* is required neither for the synthesis of active NirK nor for wild-type growth (8). The *ncg* genes of *N. europaea* do not bear significant homology to *nirV*.

The close association of *ncgABC* and *nirK*, along with the correlation between NirK expression and the activity of a promoter located upstream of *ncgA* (1, 2), suggests that their gene products might also engage in functional interactions. To test this hypothesis, we constructed mutants of *N. europaea* in which the *ncg* genes were disrupted and determined the effects on (i) NirK expression and activity, (ii) cell growth, (iii) NO_2^- tolerance, and (iv) the physiological consequences of NirK expression.

***nirK* cluster genes of *N. europaea*.** *ncgA* encodes a periplasmic "blue" copper oxidase that has oxidase activity with a range of electron donors and a minor nitrite reductase activity with reduced *N. europaea* cytochrome *c* in vitro (1, 6). The N terminus of NcgA has some sequence similarity with the copper resistance protein (CopA) of *Pseudomonas syringae* (4). A full-length *copA* homologue is present at another location in the genome of *N. europaea* (5). *ncgB* and *ncgC* encode *c*-heme containing polypeptides that copurified upon isolation from the soluble protein fraction (11). The number of *c*-heme binding motifs (Cys-X-Y-Cys-His) present in NcgB and NcgC suggests that they are di- and monoheme *c*-type cytochromes, respectively. An N-terminal leader peptide was predicted for both proteins, suggesting that they reside in the periplasm, as has been established experimentally for NirK and NcgA (1, 9).

***ncgABC* are not required for synthesis of active NirK.** Cells of *Nitrosomonas europaea* ATCC 19718 and mutants thereof were cultured in liquid mineral medium containing 25 mM $(NH_4)_2SO_4$ as a nitrogen and free-energy source at 30°C in shaken (175 rpm) batch cultures (150-ml culture in a 500-ml bottle with a loose cap) as described by Hyman and Arp (7). Mutants in which one of the *ncg* genes was disrupted were constructed by the insertion of a suicide vector, containing a transcriptional terminator, via homologous recombination and were confirmed by PCR as described previously (2). The expression level of NirK in cells that were in the early stationary growth phase was measured by Western blot analysis of NirK protein in lysates with polyclonal NirK antibodies as described in detail elsewhere (2). Disruption of *ncgA*, *ncgB*, and *ncgC* all resulted in a diminished expression of NirK (Fig. 1b). This was most likely due to polar effects and suggests that *ncgABC* and *nirK* are expressed as an operon. A residual amount of NirK was still present in each of these mutants. Measurement of the specific NirK activity in these protein preparations with hydroxylamine as an electron donor, performed as described previously (2), revealed that the residual NirK protein in these mutants was active. Moreover, introduction of the NirK expression vector pHOP, which contained *nirK* under the control

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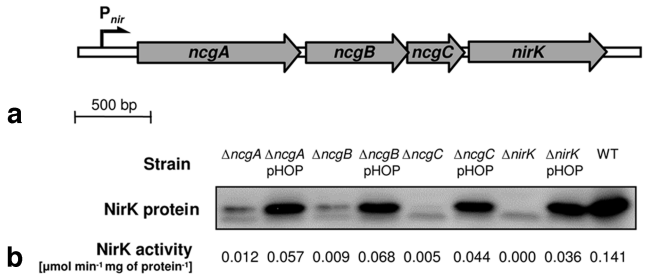

FIG. 1. (a) *nirK* gene cluster of *N. europaea*. The small arrow indicates the *nirK* gene cluster promoter (P_{nir}) that was described previously (2). (b) Western blot detection of NirK protein and NirK activity in wild-type cells (WT) and in *ncgA*, *ncgB*, *ncgC*, and *nirK* mutant cells with and without the NirK expression vector pHOP. The second (lower) band is not related to NirK protein.

of a constitutive promoter and is described elsewhere (1), in these *ncg* mutants resulted in an increase of the specific NirK activity that corresponded to that observed in the *nirK* mutant upon insertion of the same vector. Taken together, these observations show that *ncgABC* are not required for the synthesis of active NirK in *N. europaea*.

The beneficial physiological effect of NirK requires *ncgABC*. Physiologically, interruption of the *ncg* genes had negative effects on growth that were similar to those of the inactivation of *nirK*: both mutations caused cells to reach a lower maximal biomass concentration than wild-type cells (Fig. 2a through d). The negative consequences of the inactivation of *nirK* were partially compensated for by the introduction of the NirK expression vector, which increased the maximal biomass concentration that was reached by this mutant toward that of wild-type cells (Fig. 2a). To test whether the debilitated growth of the *ncg* mutants could also be reversed by increasing the level of NirK expression, we determined the growth characteristics of *ncg* mutant cells that harbored the NirK expression vector (Fig. 2b through d). In contrast to the *nirK* mutant, none of the *ncg* mutants reached higher maximal biomass concentrations as a result of the elevated NirK expression level (Fig. 2b through d). Strikingly, the expression of NirK in the *ncgA* mutant, and to a much larger extent in the *ncgB* and *ncgC* mutants, even decreased the maximal cell density that

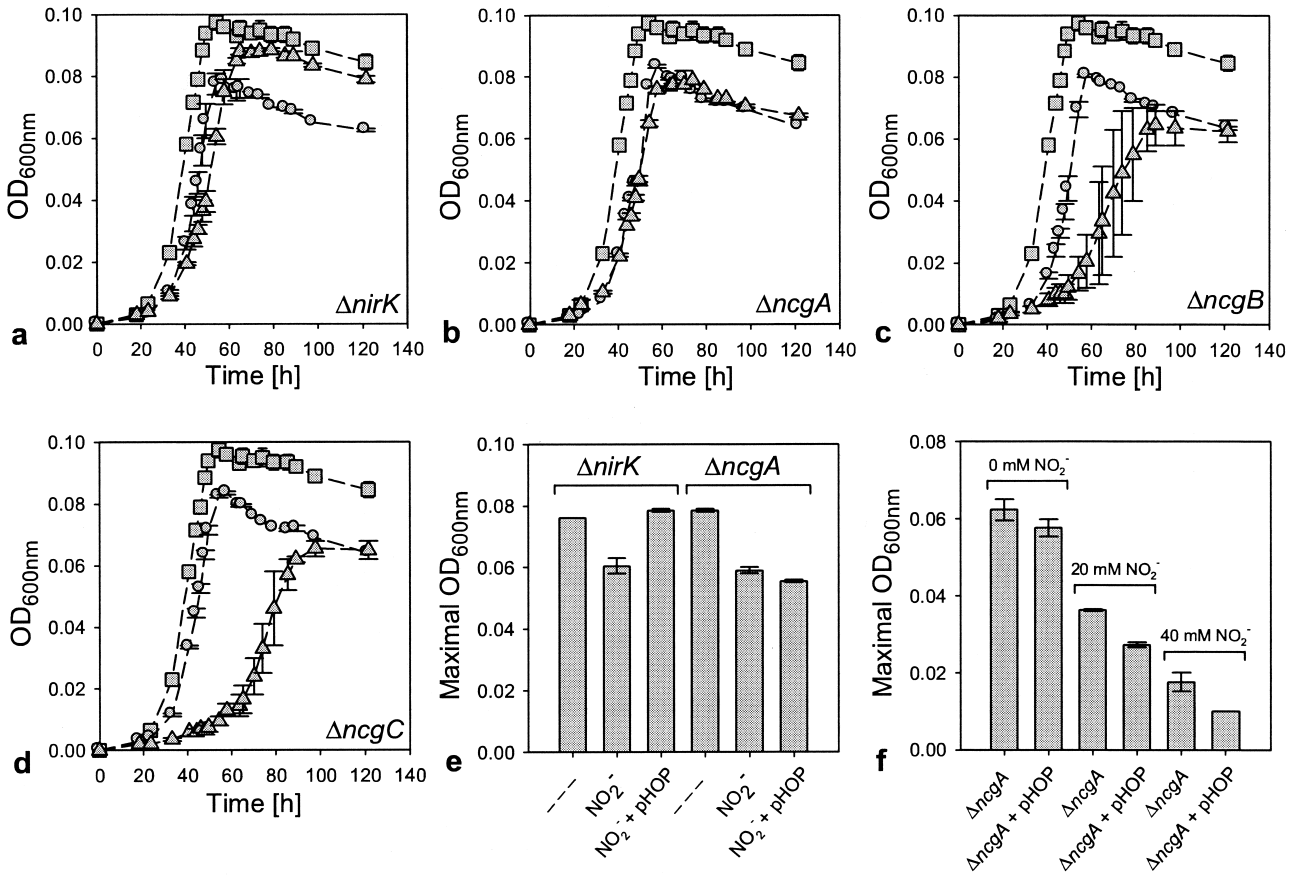

FIG. 2. Growth curves and maximal biomass concentrations of wild-type cells of *N. europaea* and of *ncg* and *nirK* mutants with and without the NirK expression vector pHOP. OD_{600nm}, optical density at 600 nm. (a) Squares, wild-type cells; circles, *nirK* mutant cells; triangles, pHOP-harboring *nirK* mutant cells. (b) Squares, wild-type cells; circles, *ncgA* mutant cells; triangles, pHOP-harboring *ncgA* mutant cells. (c) Squares, wild-type cells; circles, *ncgB* mutant cells; triangles, pHOP-harboring *ncgB* mutant cells. (d) Squares, wild-type cells; circles, *ncgC* mutant cells; triangles, pHOP-harboring *ncgC* mutant cells. Error bars indicate the standard errors of the means (SEM) ($n = 3$). (e) Maximal cell densities reached by the *nirK* and *ncgA* mutants with and without pHOP and in the presence of 10 mM of $NaNO_2$. Dashes indicate that cells did not contain pHOP and that incubation was carried out in the absence of added $NaNO_2$. Error bars indicate the SEM ($n = 2$). (f) Maximal cell densities reached by *ncgA* mutant cells with and without pHOP in the presence of 0, 20, and 40 mM of $NaNO_2$. Error bars indicate the SEM ($n = 3$).

was reached. In addition, the growth rates of the *ncgB* and *ncgC* mutants were approximately halved by the introduction of the NirK expression vector.

Expression of NirK in the *ncgA* mutant decreases NO_2^- tolerance. Batch cultures of *N. europaea* typically produce NO_2^- to a concentration of ≈20 mM at the onset of the stationary growth phase. To assess whether the reversed phenotypic consequences of NirK in the *ncg* mutants involved changes in the NO_2^- tolerance of these cells, *nirK* and *ncgA* mutants, both with and without the NirK expression vector, were cultured in the presence of added sodium nitrite ($NaNO_2$) (Fig. 2e). The addition of 10 mM $NaNO_2$ at the start of cultivation reduced the maximal cell densities that were reached by cultures of the *nirK* and *ncgA* mutants to the same extent. In *nirK* mutants, this negative effect was fully alleviated by the expression of NirK from the NirK expression vector. In contrast, the negative effect of $NaNO_2$ on the *ncgA* mutant was not reduced by the introduction of this vector but rather appeared to be aggravated. To substantiate the latter observation, the effects of increasing concentrations of $NaNO_2$ (i.e., 0, 20, and 40 mM) on the maximal cell density were examined (Fig. 2f). In the presence of 20 and 40 mM $NaNO_2$, *ncgA* mutants that harbored the NirK expression vector reached a lower maximal density than those without this vector.

Conclusion. We have characterized three novel *nirK* cluster genes that are required for the NirK-dependent tolerance to NO_2^- observed previously in *N. europaea* (1). Expression of NirK in mutants in which the *ncg* genes were disrupted reduced rather than increased the tolerance of the cells to NO_2^-. The data suggest that the *ncgABC* gene products interact with NirK, reversing a negative effect of the activity of NirK, which is otherwise essential for tolerance to NO_2^-. The polar effects observed in this study hamper inference of the roles of the individual *ncg* genes. At present, the most parsimonious hypothesis regarding the underlying mechanism is that the *ncgABC* gene products facilitate scavenging of the toxic NO produced by NirK.

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