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Nitrogen Oxide Reduction in *Wolinella succinogenes* and *Campylobacter* Species

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Wolinella succinogenes cells and extracts reduced nitric oxide, and cells, but not extracts, reduced nitrous oxide. Formate-reduced *W. succinogenes* extracts generated the 573-nm peak in difference spectra seen previously in response to nitric oxide in denitrifiers. The type strains of several *Campylobacter* species did not reduce either gaseous oxide. Cells, but not extracts, of *C. fetus* subspecies (*fetus* and *venerealis*) reduced nitrous oxide; acetylene inhibited reduction. Neither cells nor extracts reduced nitric oxide.

The nonfermenting anaerobe *Wolinella* (formerly *Vibrio*) *succinogenes* utilizes fumarate, malate, nitrate, nitrite, or nitrous oxide as terminal electron acceptors during growth. Considering only the nitrogen oxides, this bacterium was reported to reduce nitrate to nitrite, nitrite only to ammonia (8, 20), and nitrous oxide to dinitrogen (21). Acetylene selectively inhibits nitrous oxide reduction in this species as in ordinary denitrifiers. Nitric oxide provided over a range of concentrations in the absence of other oxidants fails to support growth (21).

Several denitrifiers lack the capacity for either nitrate reduction (15) or nitrous oxide reduction (5, 6), but only a strain of *Azospirillum brasilense* (17) and *W. succinogenes* (21) reportedly exhibit both those abilities while lacking the capability for denitrifying-type nitrite reduction. Denitrifiers employ either a *c-d* cytochrome (7, 9, 12) or a nonheme copper protein (8) for the dissimilatory reduction of nitrite. Nitric oxide appears as the first product of nitrite reduction, but either prolonging the incubation of reaction mixtures containing purified enzyme (19) or the provision of nitric oxide at the start also yields nitrous oxide (11). In addition, a separate *c*-type cytochrome seems involved in the reduction of nitric oxide but not nitrite (2, 3). The reduction of nitric oxide by isolated *c-d* cytochromes may be adventitious rather than physiological.

As a bacterium apparently unable to form any gas-producing *c-d* cytochrome or copper-containing nitrite reductase, *W. succinogenes* may be an ideal organism in which to study nitric oxide reduction unrelated to nitrite reduction. The inability to grow at the expense of nitric oxide should not preclude assaying for its reduction by *W. succinogenes*. Of all denitrifiers, only a nonfermenting *Bacillus* species reportedly grows with nitric oxide as the sole oxidant (16), whereas cells or extracts of all of them reduce the gas. We therefore tested for nitric oxide reduction in *W. succinogenes*. Further, because of their metabolic relatedness, we also tested several nitrate-reducing *Campylobacter* species for the ability to reduce both nitric and nitrous oxides.

We cultured *W. succinogenes* (VPI 10659) and several strains of *Campylobacter* species (Table 1) anaerobically in 1.5-liter lots of appropriate medium in 2-liter flasks incubated at 37°C under oxygen-stripped argon or nitrous oxide. *W. succinogenes* cells were obtained after growth for 48 h on VSY-4 medium (20) supplemented with 22 mM fumarate, 100 mM nitrate, or 500 ml of N_2O. We grew *Campylobacter* species for 24 h on TF medium (13) buffered at pH 7.0 and supplemented with 0.05% thioglycolate (autoclaved separately and added aseptically) and with either 12 mM KNO_3 or 500 ml of N_2O. Inoculum for each test culture was 10 ml of cell suspension from cultures in homologous medium or in nitrate medium for strains not growing as nitrous oxide reducers. We harvested cells by centrifugation and held them in 0.1 M phosphate buffer (pH 7.0) under argon until assayed. We froze several lots in identical buffer at −20°C under argon and stored them for several days before rupturing under argon by two passages through a chilled French press. Extracts of these cells were clarified by centrifugation in the cold under argon and assayed for the reduction of nitrate, nitrite, nitric oxide, and nitrous oxide as previously described (1, 14). In addition, we examined oxidation-reduction-dependent changes in the visible light-absorption spectra of the cell extracts as described earlier (14, 23). When appropriate, acetylene was added to the atmosphere of reaction mixtures or cultures to inhibit nitrous oxide reduction (1, 4, 22).

TABLE 1. Responses of several *Campylobacter* species to nitrogen oxides

Species	Growth on:		Reduction[a] of:	
	NO_3^-	N_2O	NO	N_2O
C. coli (H550 and type strain 7080 from the collection of the Pasteur Institute obtained from CDC)	+	−	−	−
C. jejuni (type strain, ATCC 33560)	+	−	−	−
C. sputorum subsp. *mucosalis* (CDC)	+	−	−	−
C. sputorum subsp. *bubulus* (CDC)	+	−	−	−
C. fetus subsp. *fetus* (type strain, ATCC 27374)	+	+	−	+[b]
C. fetus subsp. *venerealis* (type strain, ATCC 19438)	+	+	−	+[c]

[a] Reaction mixtures contained: cells (approximately 15 mg of protein); sodium formate, 15 mmol; chloramphenicol, 10 μg; and nitric oxide, 500 μl, or nitrous oxide, 100 μl, in 1 ml of degassed 0.1 M phosphate buffer (pH 7.0) under helium in 5.5-ml stoppered vials.

[b] Cells active when grown at the expense of either nitrate or nitrous oxide. Activity (NO_3^- grown) = 0.525 nmol/min per mg of protein; (N_2O grown) = 1.47 nmol/min per mg of protein.

[c] Cells active when grown at the expense of nitrous oxide but not nitrate. Activity = 1.04 nmol/min per mg of protein. Neither boiled cells of *C. fetus* subspecies nor cell extracts reduced nitrous oxide. Nitrous oxide was not reduced in mixtures without cells.

Whole *W. succinogenes* cells from any of the test media reduced both nitric and nitrous oxides. This is the first report of nitric oxide reduction by *W. succinogenes*. Gas chromatography revealed carbon dioxide and nitrous oxide as the products in acetylene-containing, anaerobic reaction mixtures supplied with cells, chloramphenicol, formate, and nitric oxide. Identical reaction mixtures devoid of electron acceptor formed carbon dioxide, as did those supplied with nitrate or nitrite instead of nitric oxide. Although both nitrate and nitrite were reduced when provided, no nitric or nitrous oxide appeared in any of these mixtures even after 18-h incubations. The quantity of nitrous oxide produced by the nitric oxide reduction increased with time in the presence of acetylene (Fig. 1A). In the absence of acetylene, the cells rapidly reduced nitrous oxide to dinitrogen, as expected (21). Extracts reduced nitric oxide only to nitrous oxide (Fig. 1B), irrespective of the presence or absence of acetylene, and nitrous oxide accumulated unchanged for as long as 18 h. Like whole cells, the extracts failed to reduce nitrate or nitrite to gaseous products, and neither boiled (5 min) nor enzymeless control mixtures reduced either nitric or nitrous oxide.

Spectrophotometric assays of reaction mixtures containing clarified *W. succinogenes* cell extracts reduced with formate revealed small, *c*-type cytochrome peaks (552, 522, and 415 nm) resembling the spectrum of the c_{552} cytochrome from "*Pseudomonas perfectomarinus*" when grown as a denitrifier (10). The injection of nitrate or nitrite into the reduced reaction mixture affected peak heights but little and nitrous oxide not at all. In contrast, the injection of 500 μl of nitric oxide resulted in the inversion of peaks, with a deep trough at 552 to 554 nm and a peak at 573 nm. The introduction of more formate restored the reduced spectrum, as expected (14). These responses were observed before in *P. perfectomarinus* and other *c-d* cytochrome-producing denitrifiers but not in nitrate-respiring bacteria or nitrate-assimilating fungi (14, 23). This is our first demonstration of the responses in extracts of a bacterium not producing *c-d* cytochrome.

Except for the disjunction in the usual denitrifying pathway, in which *W. succinogenes* cells and extracts reduce nitrite to ammonia rather than a gaseous product (20), this bacterium displays all of the other reactions characteristic of ordinary denitrifiers.

Campylobacter species responded variously to nitrogen oxides. *C. coli* (H550 and the type strain 7080 from the collection of the Pasteur Institute obtained from the Centers for Disease Control (CDC) Atlanta, Ga.), the type strain of *C. jejuni* (ATCC 33560), and both *C. sputorum* subspecies *mucosalis* and subspecies *bubulus* (CDC) failed to grow at the expense of nitrous oxide reduction. Cells of these species grown as nitrate reducers neither produced nor reduced nitric or nitrous oxide.

The type strains of both *C. fetus* subspecies also reduced nitrate to nitrite while growing but formed no gaseous nitrogen oxides. From that point on, these organisms diverged both from the other species and from each other. Cells of *C. fetus* subsp. *fetus* grown on either nitrate- or nitrous oxide-enriched medium reduced nitrous oxide (Table 1). The rate of reduction by the nitrate-grown cells was the slower, and cells from neither type of culture reduced nitric oxide. Cells of *C. fetus* subsp. *venerealis* grown on nitrate medium also failed to reduce nitric oxide and unexpectedly failed to reduce nitrous oxide as well. Despite that incapacity, growth at the expense of nitrous oxide reduction yielded *C. fetus* subsp. *venerealis* cells that did reduce nitrous oxide, but still not nitric oxide. The addition of nitric oxide to reduced *C. fetus* cell extracts did not produce the 573-nm peak response.

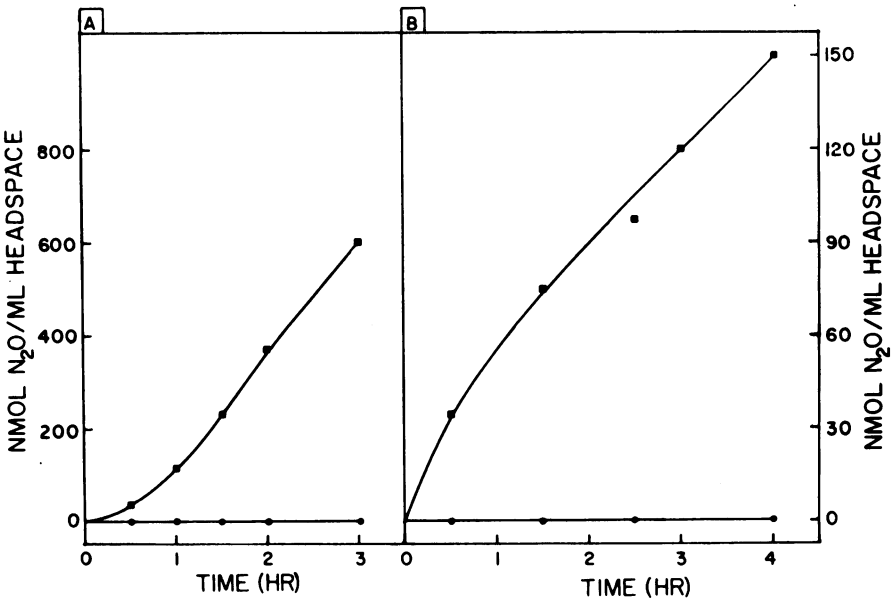

FIG. 1. Reduction of nitric oxide to nitrous oxide by whole cells and extracts of *W. succinogenes* grown on nitrous oxide, fumarate, or nitrate. (A) The reaction mixture contained approximately 1.5×10^{10} cells, sodium formate (15 mmol), and chloramphenicol (10 μg) in 1 ml of degassed 0.1 M phosphate buffer (pH 7.0) under helium in a 7-ml serum bottle-stoppered vial. (B) As in (A) except that 15 mg of extract protein replaced the whole cells. To initiate test reactions, each vial was charged with 300 μl of acetylene and 500 μl of nitric oxide and incubated at 37°C. In control experiments, boiled cells or buffer replaced fresh cells or extract; alternatively, either nitrate (12 mmol), nitrite (15 mmol), or nitrous oxide (50 μl) replaced nitric oxide. Symbols: ■, nitric oxide-containing reaction mixtures; ●, controls.

Acetylene inhibited the growth of both *C. fetus* subspecies on nitrous oxide but not nitrate. Extracts of neither of the *C. fetus* subspecies reduced nitric or nitrous oxide.

This is the first report of nitrous oxide reduction in the genus *Campylobacter*. Extensive testing of various strains and isolates will be required to determine whether the property has useful prospects for taxonomy. However, our observations permit conjecture that other nitrate-reducing but non-denitrifying bacteria may also reduce one or both of the gaseous oxides. Organisms such as *W. succinogenes* and *C. fetus* subsp. *fetus* and *venerealis* appear able to make ideal contributions to the cycling of nitrogen. They conserve nitrogen in an assimilable form for plants and other microorganisms by reducing nitrate through nitrite to ammonia rather than gaseous compounds. They also have the potential for useful contributions by ridding systems of two harmfully reactive or toxic gaseous nitrogen oxides. We would prefer to see these reduced rather than released intact into the earth's atmosphere.

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