Denitrification by *Chromobacterium violaceum*

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One host (*Rana catesbiana*)-associated and two free-living mesophilic strains of bacteria with violet pigmentation and biochemical characteristics of *Chromobacterium violaceum* were isolated from freshwater habitats. Cells of each freshly isolated strain and of strain ATCC 12472 (the neotype strain) grew anaerobically with glucose as the sole carbon and energy source. The major fermentation products of cells grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with glucose included acetate, small amounts of propionate, lactate, and pyruvate. The final cell yield and culture growth rate of each strain cultured anaerobically in this medium increased approximately twofold with the addition of 2 mM NaNO₃. Final growth yields increased in direct proportion to the quantity of added NaNO₃ over the range of 0.5 to 5 mM. Each strain reduced NO₃⁻, producing NO₂⁻, NO, and N₂O. NO₃⁻ accumulated transiently. With 2 mM NaNO₃ in the medium, N₂O made up 85 to 98% of the N product recovered with each strain. N-oxides were recovered in the same quantity and distribution whether 0.01 atm (ca. 1 kPa) of C₂H₂ (added to block N₂O reduction) was present or not. Neither N₂ production nor gas accumulation was detected during NO₃⁻ reduction by growing cells. Cell growth in media containing 0.5 to 5 mM NaNO₃ in lieu of NaNO₃ was delayed, and although N₂O was produced by the end of growth, NO₂⁻-containing media did not support growth to an extent greater than did medium lacking NO₃⁻ or NO₂⁻. The data indicate that *C. violaceum* cells ferment glucose or denitrify, terminating denitrification with the production of N₂O, and that NO₃⁻ reduction to N₂O is not coupled to growth but may serve as a detoxification mechanism. No strain detectably fixed N₂ (reduced C₂H₂).

The freshwater violacein-producing chromobacteria are widespread in their distribution (7, 17, 19). Species associated with animals or plants appear to be either commensals (4, 19, 30) or virulent parasites (15), although relatively little is known of the ecological niche(s) that they occupy in natural ecosystems. Knowledge of the overall physiology and metabolism of these interesting bacteria, particularly of their nitrogen metabolism and denitrifying potential, for instance, is meager (20).

The mesophilic (*Chromobacterium violaceum*) and psychrophilic (former *C. lividum*) species were reported to differ in their pattern of nitrate dissimilation (20, 25). Molecular taxonomy studies have resulted in the assignment of *C. lividum* species to the genus Janthinobacterium (10, 22, 27). Most *J. lividum* isolates reduce nitrate with the evolution of gas into the culture medium, whereas most *C. violaceum* isolates reduce NO₃⁻ to NO₂⁻ and also deplete NO₂⁻ but appear variable in respect to gas evolution (11, 18, 25, 26). We investigated the nitrogen metabolism of several freshly isolated free-living and host-associated *Chromobacterium* strains to better understand the potential of this species in nitrogen cycling with particular attention to denitrification and nitrogen fixation.

MATERIALS AND METHODS

**Bacterial strains.** *C. violaceum* ATCC 12472, the neotype strain, was obtained from W. J. Payne and from the American Type Culture Collection, Rockville, Md. Three recently isolated strains of *C. violaceum* were also used in this study. Strain CS-1 was isolated from the water treatment plant, Durham, N.H., in agar shake dilution tubes containing a succinate-nitrate-salts growth medium suitable for culturing *Aquaspirillum magnetotacticum* (5). Strain CF-1 was isolated on nutrient agar (Difco Laboratories, Detroit, Mich.) as a component of the cloacal flora of a bullfrog, *Rana catesbiana*, obtained from an abandoned reservoir in Durham, N.H. Strain CR-1 was isolated on rice grains (from mud from the site at which the frog was collected) by using the method of Corpe (7). All isolations were at 25°C. Isolates were cloned three times on nutrient agar and stored on nutrient agar slants at 4°C. Stock cultures were frozen in 8% (vol/vol) dimethyl sulfoxide and stored at −70°C.

**Culture conditions and biochemical methods.** All *C. violaceum* strains were routinely cultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 0.1% Difco yeast extract (TSBYE). Cells were cultured anaerobically at 28 or 37°C in anaerobic culture tubes (18 by 150 mm) (catalog no. 2048; Belco Glass, Inc., Vineland, N.J.) or in stoppered 155-ml serum vials each containing 55 ml of medium. Anaerobic and microaerobic cultures were obtained by using nitrogen or helium gas delivered by means of a vacuum manifold as previously described (1). Absence of O₂ in anaerobic cultures was easily confirmed by the fact that cells did not produce the purple pigment violacein under anaerobic conditions. To allow NO₃⁻ reduction or fermentation, NaNO₃ (2 mM unless otherwise stated) or glucose (0.05 or 0.25%), respectively, was added to culture media. C₂H₂ reduction was investigated in cultures grown in the chemically defined succinate-salts growth medium (5) lacking the combined nitrogen source, with and without 0.01% Difco yeast extract.

Biochemical characteristics of strains were determined with the API 20E System (Analytab Products, Plainview, N.Y.). Esulin hydrolysis was determined as described by Smibert and Krieg (23).

**Analysis of fermentation products.** Volatile fatty acids and alcohols were extracted from acidified growth medium with ethyl ether (14). Methyl esters of nonvolatile fatty acids were extracted with chloroform (14). Extracted fermentation products were resolved using capillary gas chromatography (23).
### Table 1. Characteristics of *C. violaceum* isolates

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Reaction</th>
<th>CS-1</th>
<th>CR-1</th>
<th>CF-1</th>
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<tbody>
<tr>
<td>Violacein production</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Motility</td>
<td>+</td>
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<tr>
<td>Growth</td>
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<td>4°C</td>
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<td>25°C</td>
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<td>37°C</td>
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<tr>
<td>Oxidase</td>
<td>+</td>
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<td>Catalase</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NO₂⁻ from NO₃⁻</td>
<td>+</td>
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<tr>
<td>Gas evolution from NO₃⁻</td>
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<td>Hydrolysis</td>
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<td>Esculin</td>
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<td>Gelatin</td>
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<td>Indole production</td>
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<td>Voges-Proskauer</td>
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<td>H₂S production</td>
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<tr>
<td>Citrate utilization</td>
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<tr>
<td>Arginine iminohydrolase</td>
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<tr>
<td>Lysine decarboxylase</td>
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<td>Ornithine decarboxylase</td>
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<tr>
<td>Tryptophan deaminase</td>
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<tr>
<td>Acid from</td>
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<tr>
<td>D-Glucose</td>
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<tr>
<td>Rhamnose</td>
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<td>Sucrose</td>
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<td>Melibiose</td>
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<td>Arabinose</td>
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<td>Amygdalin</td>
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<td>Mannitol</td>
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<td>Inositol</td>
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<td>Sorbitol</td>
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* Determined with Durham tubes in TSBYE. Each isolate produced N₂O, which remained dissolved in the medium.

products were analyzed by gas chromatography with a gas chromatograph equipped with a thermal conductivity detector (model 3920A; The Perkin-Elmer Corp., Norwalk, Conn.). The stationary phase was GP 10% SP-1000-1% H₃PO₄ on 100/120 mesh Chromosorb W AW (Supelco, Inc., Bellefonte, Pa.) in a stainless steel column (3.0 m by 3 mm). The bridge current was 175 mA, and the detector, injector, and column oven were each at 150°C. O₂-free He at a flow rate of 60 ml min⁻¹ was the carrier gas.

**Estimation of culture growth.** Cell numbers were estimated by means of direct cell counts by using a Petroff-Hauser counting chamber with a Zeiss standard research phase-contrast microscope. Samples removed by syringe were diluted into an equal volume of 0.1% Formalin to arrest cell motility before counting. To evaluate the effects of added NO₃⁻ and NO₂⁻ in some experiments, culture growth in sealed tubes was measured as a change in optical density at 660 nm.

### C₂H₂ reduction.

C₂H₂, generated from distilled H₂O and CaC₂ (granular; Fisher Scientific Co., Medford, Mass.), was added to cultures to a final headspace concentration of 0.1 atm (ca. 10 kPa). All cultures containing C₂H₂ were incubated anaerobically or microaerobically (initial P₂O₅ = 1 kPa) in a shaking water bath (20 oscillations min⁻¹) at 37°C. Ethylene (C₂H₂) was determined by using gas chromatography as previously described (3).

**Nitrate reduction.** NO₃⁻ was measured with a Beckman Selection 2000 ion analyzer (Beckman Instruments, Inc., Irvine, Calif.). NO₃⁻ was determined colorimetrically by the method of Snell and Snell (28). NO₃⁻ and NO₂⁻ were also determined with a Technicon computerized AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N.Y.) by using Cd reduction to convert NO₃⁻ to NO₂⁻. NO₂⁻ was determined from the difference between unreduced (NO₃⁻) and Cd-reduced (NO₃⁻ and NO₂⁻) samples. N₂O, NO, and N₂ were analyzed by gas chromatography as previously described (2). For each gas, standards were run at the time of each analysis and when necessary to avoid saturating the detector; gas samples were quantitatively diluted into previously evacuated serum vials containing 1 atm (ca. 100 kPa) of N₂ or He. Samples were then removed from these for injection into the gas chromatograph. Culture gas production at 28 and 37°C was evaluated with Durham tubes filled with TSBYE containing 2 or 20 mM NaNO₃. A sterile mineral oil overlay provided anaerobic conditions soon after growth commenced.

**Figure 1.** *C. violaceum* CS-1 growth and denitrification in anaerobic TSBYE containing 2 mM NaNO₃. Symbols: ⬤, cells per milliliter; □, culture nitrate; ▲, culture nitrite; △, headspace nitrous oxide.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Final cell yield/culture doubling time (10^8 cells mL⁻¹)/divisions h⁻¹)</th>
<th>With NO₃⁻</th>
<th>Without NO₃⁻</th>
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<tbody>
<tr>
<td>CS-1</td>
<td>9.0/1.20</td>
<td>3.0/0.56</td>
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</tr>
<tr>
<td>CR-1</td>
<td>9.0/0.83</td>
<td>3.5/0.56</td>
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<tr>
<td>CF-1</td>
<td>4.5/0.95</td>
<td>2.6/0.61</td>
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</tbody>
</table>

* Sealed anaerobic cultures in TSBYE with 2 mM NO₃⁻ and 0.05% glucose.
* Sealed anaerobic cultures in TSBYE with 2.5% glucose.

**RESULTS**

Cultural and biochemical characteristics. Relevant biochemical and cultural characteristics of the three freshly isolated strains used in this study are shown in Table 1. All strains appeared to be identical and exhibited morphological, cultural, and biochemical characteristics of the mesophilic, motile, gram-negative, rod-shaped, violacein-producing species *C. violaceum* (8, 18, 19, 25–27).

Fermentation products. Each isolate produced acetate and traces of propionate, pyruvate, and lactate when grown anaerobically on TSBYE. Strain CF-1 also produced a small but measurable amount of oxalacetate.

NO₃⁻ reduction and denitrification. Each strain reduced NO₃⁻ and produced NO₂⁻, NO, and N₂O. Figure 1 shows the products of NO₃⁻ reduction detected during growth of strain CS-1 in TSBYE with 2 mM NO₃⁻. Data for the other freshly isolated strains were very similar and are not shown. Cell lysis became evident after 19 h in TSBYE with and without 0.5 to 5 mM NO₃⁻. NO₃⁻ accumulated transiently during cell growth to a maximum at 6 h of approximately three-fourths of the NO₃⁻ supply. After 6 h, as cells entered stationary growth, the net quantity of NO₃⁻ decreased, and N₂O appeared in detectable quantities. During the interval 6 to 13 h, less than 75% of the N supplied as NO₃⁻ was recovered in measured products. By the end of the sampling period (15 h) NO₃⁻ and NO₂⁻ were completely depleted and 85 to 98% of the NO₃⁻ supply was recovered as N₂O. NO₂⁻ did not disappear from the headspace even after 5 days. The N recovery was somewhat higher for the neotype strain than for the new isolates. The time course and amount of N₂O recovered was the same whether the acetylene block technique (31) to inhibit N₂O reductase was used or not. Neither gas (bubbles) nor N₂ (gas chromatography) were detected as products of NO₃⁻ reduction by any of the *Chromobacterium* strains tested, despite the use of various culture media, two incubation temperatures (28 and 37°C), or two NO₃⁻ concentrations (2 and 20 mM).

The final cell yield and culture growth rate of each strain grown anaerobically in TSBYE with 2 mM NO₃⁻ and 0.05% glucose were consistently twofold higher than those of cultures with 0.25% glucose but without NO₃⁻ (Table 2). Culture optical density values by the end of growth (19 h) had increased in direct proportion to the quantity of added NO₃⁻ over the range of 0.5 to 5 mM. NaNO₃ at 0.5, 1, 2, or 5 mM added to TSBYE without NO₃⁻ was initially toxic for cells of each strain tested (CS-1, ATCC 12472, and CF-1) as evidenced by delayed growth as compared to that observed with unsupplemented control medium. Cells of each strain eventually grew with NO₃⁻ and produced N₂O in direct proportion to the quantity added. However, the final cell yield was no higher with NO₃⁻ than at any concentration tested than those of control cultures lacking NO₃⁻ or NO₂⁻.

**DISCUSSION**

Denitrifiers convert 90% or more of the N-oxide (NO₃⁻ or NO₂⁻) which they reduce to an N gas (usually N₂) and couple this reduction to electron transport phosphorylation (6, 20). The *Chromobacterium* strains used in this study did not produce N₂. However, they did convert more than 85% of the added NO₃⁻ to N₂O by the end of growth. Thus, the amount of NO₃⁻ reduced to N₂O by the strains used was significantly higher than that (usually 30% or less) reported for nondenitrifying strains of bacteria that also accumulate N₂O (6). We were unable to obtain a satisfactory mass balance for N between 6 and 13 h. Small amounts of NO were detected, and growing cells also produced NH₄⁺ which might have resulted from NO₃⁻ reduction (24). Since we used TSBYE culture medium we could not easily determine the extent of NO₃⁻ incorporation into cell material. However, it is unlikely that these potential N products would collectively represent the missing N, and we attribute the low N recovery during the interval 6 to 13 h to failure of N₂O to establish a true equilibrium with the culture headspace until after 13 h of culture growth. Nitrous oxide was actively produced well into the stationary period of growth, although NO₂⁻ depletion during this period did not stimulate further growth of the culture.

Cell growth rates and yields of anaerobic cultures were significantly higher with NO₃⁻ present than without it. Culture final yields and N₂O increased in direct proportion to the quantity of added NO₃⁻. This is consistent with the higher energy yield expected from a respiratory process compared with fermentation, suggesting that NO₃⁻ reduction in this species is coupled to energy conservation. The stimulatory effect of NO₃⁻ was probably not due to nitrogen enrichment because this organism can use as sole sources of N many of the complex nitrogenous compounds found in TSBYE. The failure of N₂O to provide for increased growth over that obtained in unsupplemented control cultures, together with its initially toxic effects over the range 0.5 to 5 mM, suggests that its reduction to N₂O by cells of this species may be another instance of NO₃⁻ detoxification as has been described for propionibacteria by Kaspar (16).

Our results indicate that *C. violaceum* is a fermenter and a denitrifier that terminates denitrification at N₂O. The terminal product of denitrification by most denitrifying species is N₂, which due to its low solubility accumulates relatively in the medium (20). Neither N₂ formation specifically nor gas evolution was ever detected in denitrifying cultures of the four *C. violaceum* strains we tested. Our inability to detect gas is consistent with reported results (20, 21, 26), although in one study (12), the neotype strain (ATCC 12472) was reported to produce N₂ with NO₃⁻. In the light of these apparently conflicting results we were interested to find that each strain used in our work accumulated N₂O which did not thereafter decrease whether CFH₂ was present or not. Moreover, cells grew aerobically but not anaerobically in the simple succinate-salts defined medium (5) with N₂O in lieu of NO₃⁻ as the acceptor. We conclude that under the conditions of our experiments, all four strains terminated denitrification with N₂O formation. Termination of denitrification at N₂O is not unique and is due to the absence of a single enzyme, N₂O reductase as in "Achromobacter (Corynebacterium) nephridii" (21), certain strains of Pseudomonas, and a number of other nitrogen-oxidizing species.
domonas fluorescens (13), Aquaspirillum itersiorii (20), Rhizobia
dum japonicum (9), and propionibacteria (16).
The isolates used in this study showed no evidence of N₂
fixation. This could be of taxonomic importance especially
since at least some strains of J. lividum have been found to
do so, particularly the host-associated ones (4).

At present, only certain strains of Bacillus licheniformis
(20) and Propionibacterium acidipropionici ("P. pento-
sacceum" [20, 29]) are also known to be fermentative denitri-
fiers. The fact that these species possess diverse physiolog-
ical capabilities gives them interesting questions of regulation and
control but could be of advantage to survival in habitats
containing low or variable concentrations of oxygen and
combined forms of nitrogen.

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