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# Enrichment and Isolation of a Nitropropanol-Metabolizing Bacterium from the Rumen

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**A bacterium capable of metabolizing nitropropanol, nitropropionate, and nitrate has been isolated from a mixed ruminal population enriched for enhanced rates of nitropropanol metabolism. The numbers of nitropropanol-metabolizing bacteria in mixed populations increased >10,000-fold during enrichment; the rates of nitropropanol metabolism increased 8-fold. Hydrogen and phytone were important nutrients for nitropropanol metabolism.**

Enhanced ruminal metabolism of 3-nitro-1-propanol and 3-nitro-1-propionate, respiratory toxins (18, 19) contained in many forages (17, 21–23), is sought as a means of protecting ruminants from intoxication. Ruminal microbes are known to metabolize the nitrotoxins (10, 12–16) (reducing nitropropanol to aminopropanol and nitropropionate to β-alanine [1]), but the microbes primarily responsible for detoxification in vivo are not known. Cattle fed sublethal amounts of milk vetch, *Astragalus miser* var. *serotinus* (12), or diets supplemented with soybean meal or the innocuous nitroalkane nitroethane (12, 15) had increased rates of ruminal nitropropanol metabolism, presumably because of selection of competent nitropropanol-metabolizing bacteria. This report describes our efforts to enrich ruminal populations for nitropropanol-metabolizing bacteria and our isolation of a unique nitropropanol-metabolizing bacterium from an enriched population. Initial ruminal populations were from a fistulated cow maintained on an alfalfa-corn (9:1) diet. Initial enrichment was achieved during 24 h of consecutive batch culture (20) of mixed populations in medium supplemented either with milk vetch forage, which contained nitropropanol conjugated as 3-nitro-1-propyl-β-D-glucopyranoside (miserotoxin) (data not shown), or with alfalfa forage plus added nitropropanol (Table 1). Nitropropanol was needed to promote enrichment, as rates of nitropropanol metabolism remained below 0.1 μmol $ml^{-1}$ $h^{-1}$ when control populations, those cultured in medium plus alfalfa but lacking nitropropanol, were cultured for the first time in the same medium supplemented with 4.2 mM nitropropanol. Rates of nitropropanol metabolism increased more slowly and to a lesser extent when the enrichment medium lacked phytone peptone (BBL) (data not shown). Unless indicated otherwise, the medium used was medium A. Medium A contained $Na_2CO_3$, resazurin, L-cysteine-HCl, and vitamins at concentrations that were the same as in the complete medium of Bryant and Robinson (5). Medium A also contained (in 100 ml) phytone peptone (800 mg), lipoic acid (0.005 mg), vitamin $B_{12}$ (0.002 mg), clarified rumen fluid (11) (40% [vol/vol]), and the same minerals as in the nonrumen fluid medium of Dawson et al. (8). When supplemented, air-dried milk vetch (4.4% miserotoxin) or alfalfa, ground with a Wiley mill to a size that would pass through a 1.0-mm mesh-size screen, was added at 0.1% (wt/vol). Rates of nitropropanol metabolism were determined as before (1). Concentrations of bacteria were determined by using a three-tube most probable number test (3). Tubes were considered positive for nitropropanol-metabolizing bacteria if >75% of the 4.2 mM nitropropanol was metabolized within 96 h of incubation; tubes were scored (plus or minus) for growth by inspection for turbidity. Anaerobic techniques (4) were used, and unless otherwise stated, the gas phase was $H_2$-$CO_2$ (1:1). Cultures were incubated at 39°C.

After initial enrichment, further enrichment was achieved by progressively culturing in media designed to be more selective by deleting specific components. The various populations resulting from these progressive cultures were maintained by serial transfer (2 to 4% [vol/vol] inoculum) at 24- to 48-h intervals. The number of serial transfers between the steps varied, and higher concentrations of nitropropanol (to 33.6 mM) were added to the media in order to maintain the selective pressure of nitropropanol during incubation.

A population, designated as enriched population 1, was propagated from 0.1 ml of a culture of a population initially enriched in medium containing alfalfa and nitropropanol (Table 1). An attempt to isolate nitropropanol-metabolizing bacteria from enriched population 1 was unsuccessful, but another population (enriched population 2) was propagated from a $10^{-8}$ dilution of enriched population 1 $ml^{-1}$. The concentration of nitropropanol-metabolizing bacteria in enriched population 2 was substantially higher than that previously found after our initial enrichment (Table 1).

Culturing of enriched population 2 in medium B (medium A modified to contain 8% instead of 40% clarified rumen fluid) had little effect on the rate of nitropropanol metabolism or on concentrations of bacteria (Table 1). Confirming results obtained during preliminary studies, the mean rate of nitropropanol metabolism ± the standard deviation ($n = 2$) was 0.40 ± 0.01 μmol $ml^{-1}$ $h^{-1}$ when enriched population 2 was grown in medium B plus 33.6 mM nitropropanol with $H_2$-$CO_2$ (1:1) as the gas phase, but rates were 0.18 ± 0.01 and 0.14 ± 0.01 μmol $ml^{-1}$ $h^{-1}$ with $CO_2$ (100%) and $CO_2$-$CH_4$ (1:1), respectively. Thus, $H_2$ was a critical component for achieving higher rates of nitropropanol metabolism.

While rates of nitropropanol metabolism decreased (from 0.29 to 0.03 μmol $ml^{-1}$ $h^{-1}$) during four successive culturings

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TABLE 1. Rates of nitropropanol metabolism and estimates of most probable number of bacteria during enrichment of nitropropanol-metabolizing bacteria

| Stage in enrichment | Rate of nitropropanol metabolism ($\mu$mol ml$^{-1}$ h$^{-1}$) | Most probable number (organisms per ml) | |
|---|---|---|---|
| | | Nitropropanol-metabolizing organisms | Total culturable organisms |
| Ruminal fluid inoculum | 0.07 | $3.0 \times 10^4$ | Not done |
| Initial enrichment | 0.60 | $4.8 \times 10^6$ | Not done |
| Enriched population number 1 | 0.48 | Not done | Not done |
| Enriched population number 2 | | | |
| Basal medium A | 0.52 | $1.1 \times 10^8$ | $2.4 \times 10^9$ |
| Basal medium B | 0.48 | $1.1 \times 10^8$ | $>1.1 \times 10^9$ |
| Basal medium C | ND[a] | $1.5 \times 10^7$ | $1.5 \times 10^7$ |

[a] ND, not determined.

of enriched population 2 in medium C, the proportion of nitropropanol-metabolizing bacteria increased such that nitropropanol-metabolizing bacteria were among the most numerous members of the population (Table 1). Medium C was the same as medium A except energy-depleted rumen fluid (9) replaced the clarified rumen fluid and phytone was deleted. Upon further culturing of this population (enriched population 3) in medium B plus nitropropanol, enhanced rates of nitropropanol metabolism were again maintained, averaging $0.32 \pm 0.08$ $\mu$mol ml$^{-1}$ h$^{-1}$ after eight consecutive incubations. Upon microscopic evaluation, we observed a population consisting of nonmotile, gram-positive, irregular rods (0.5 to 1.0 by 0.5 to 1.5 $\mu$m). The longer gram-positive rods (0.5 to 1 by 1 to 2.5 $\mu$m) present in cultures prior to this step were not observed.

Of 27 isolated colonies subcultured from a roll tube inoculated with $10^{-4}$ ml of an enriched population 3 culture, only one had grown after 96 h of incubation, and this culture metabolized nitropropanol. This isolated bacterium, designated strain NPOH1 (previously designated strain NP1) (2), grew in medium B supplemented with nitropropanol, nitropropionate, or nitrate (each at 4.2 mM). Amounts of electron acceptor metabolized, as determined by colorimetric analysis (1, 6), by the cultures after 24 h of growth with $H_2$-$CO_2$ (1:1) exceeded 80%. The utilization of nitrate and nitropropionate by strain NPOH1 further describes the niche for this organism and is in agreement with the finding that rates of ruminal nitropropanol metabolism were enhanced when cattle were fed diets containing nitrate (7). Strain NPOH1 did not grow with the above-mentioned acceptors when 100% $CO_2$ was substituted for $H_2$-$CO_2$ (1:1). Thus, bacteria like strain NPOH1 may be in competition with methanogens and other $H_2$-utilizing microbes. Growth of strain NPOH1 was not observed in medium B supplemented with nitrite, fumarate, or sulfate (each at 4.2 mM) after 5 days of incubation. Rates of nitropropanol metabolism differed little during growth of strain NPOH1 in medium B whether supplemented with 4.2, 8.4, or 16.8 mM nitropropanol; the mean growth rate $\pm$ the standard deviation ($n = 2$) of cultures grown with 4.2 mM nitropropanol was $0.19 \pm 0.01$ $\mu$mol ml$^{-1}$ h$^{-1}$. Morphologically, cells of strain NPOH1 were indistinguishable from cells observed in enriched population 3.

A detailed characterization of strain NPOH1 is under way, as are studies toward development of better methods for isolating and enumerating these bacteria and for evaluating their role in the rumen.

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