Denitrification by Strains of *Neisseria*, *Kingella*, and *Chromobacterium*

MICHAEL A. GRANT AND W. J. PAYNE

Department of Microbiology, University of Georgia, Athens, Georgia 30602

The information in the existing literature concerning the denitrifying capacities of certain species of *Kingella*, *Chromobacterium*, and *Neisseria* is ambiguous. Therefore, we used gas chromatography to obtain a better understanding of the capacities of strains of several species in these genera for dissimilatory nitrate and nitrite reduction under anoxic conditions. A strain of *Kingella denitrificans* used both nitrate and nitrite as electron acceptors for denitrification, as did strains of "*Chromobacterium lividum*" and *Chromobacterium violaceum*. In contrast, strains of four of the *Neisseria* species tested denitrified at the expense of nitrite, but strains of three species did not reduce nitrite. Only a strain of *Neisseria mucosa* used nitrate as an electron acceptor. Therefore, all strains tested were capable of denitrification. Where lapses occurred, it was the capacity for nitrate reduction that was missing, as in certain species of *Alcaligenes*. The lack of the ability to reduce nitrous oxide that is found in some *Pseudomonas* species was not observed in this study.

Information concerning the nitrate- and nitrite-reducing properties of species of *Kingella*, *Chromobacterium*, and *Neisseria* is limited to data obtained by standard nitrate and nitrite broth tests (2, 7, 10, 11, 13). The results of such tests do not necessarily reveal whether a nitrate-reducing bacterium uses assimilatory or dissimilatory reduction or nitrate fermentation (4, 5). Dissimilatory reduction of nitrate can produce nitrite and stop or can produce ammonia and stop, or it can go on in denitrifiers and release nitric oxide and nitrous oxide as intermediates and dinitrogen as the final product (6). Assimilatory reduction of nitrate and nitrate fermentation produces nitrite that can then be further reduced to ammonia.

The taxonomy of bacteria would be served if the capabilities of all nitrate and nitrite reducers were determined more precisely. To that end, we examined strains of *Kingella denitrificans*, "*Chromobacterium lividum*" (not on the Approved Lists of Bacterial Names [8]), *Chromobacterium violaceum*, *Neisseria mucosa*, *Neisseria sicca*, *Neisseria flavescens*, and *Neisseria subflava* to determine whether they are capable of denitrification, as suspected.

MATERIALS AND METHODS

Bacterial strains. *C. violaceum* ATCC 12472 and "*C. lividum*" ATCC 12473 were obtained from the American Type Culture Collection, Rockville, Md. A culture of *K. denitrificans* was kindly provided by Richard George, and the *N. mucosa*, *N. sicca*, *N. subflava*, and *N. flavescens* strains were obtained from Douglas Kellog, Centers for Disease Control, Atlanta,

Ga. It should be noted that since the strains which we used are not the type strains of the species, it would be taxonomically improper to extrapolate the results obtained with these strains to the whole species.

Culture conditions. Cultures of the *Neisseria* and *Kingella* strains were grown at 37°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 1.0% defibrinated sheep blood and either 0.1% (wt/vol) NaNO_3 or 0.01% (wt/vol) NaNO_2 . Cultures of the *Chromobacterium* strains were incubated at 22°C in tryptic soy broth containing 0.1% yeast extract and either 0.1% NaNO_3 or 0.01% NaNO_2 .

The media were dispensed in 50-ml portions into 120-ml serum bottles and sterilized. Immediately after autoclaving, the containers were sealed with rubber serum bottle stoppers. After the media cooled to approximately 70°C, blood was injected aseptically into the appropriate media, thus rupturing the blood cells. Needles were inserted through the stoppers, and all of the test flasks were flushed aseptically for approximately 5 min with either helium or argon (made oxygen-free by passage over hot copper filings). At periodic intervals, samples were withdrawn from the headspace of each culture with a Hamilton gas-tight syringe or a disposable tuberculin syringe. Samples of medium were also withdrawn periodically and diluted into a Petroff-Hausser counting chamber for direct cell counts with a phase-contrast microscope.

Gas chromatography. Gas samples were analyzed with either a Carle III analytical gas chromatograph or a Perkin-Elmer model 900 gas chromatograph. The Carle instrument was equipped with a Poropak Q column (approximately 304 by 0.32 cm), which was operated at 70°C. This instrument was also equipped with a thermal conductivity microdetector, and helium was used as the carrier gas (flow rate, approximately 31 ml/min). The Perkin-Elmer model 900 chromatograph was equipped with a Poropak Q column (ap-

proximately 183 by 0.34 cm), which was operated at ambient temperature. This apparatus contained a thermal conductivity detector which was operated at 100°C and at a current setting of 15 mA. Argon was used as the carrier gas (flow rate, approximately 40 ml/min).

RESULTS AND DISCUSSION

The strain of *K. denitrificans* examined was clearly capable of denitrifying metabolism when it was grown with nitrate as the electron acceptor (Table 1). We sought qualitative rather than quantitative indications of gas release. Thus, we made our final observations before complete conversion of nitrate or nitrite nitrogen. The quantities of dinitrogen being released were still increasing when our observations were concluded. Nitrous oxide, which accumulated transiently during the dissimilatory process, was observed first at 48 h and appeared maximally at 72 h. Smaller quantities of typical denitrifying products accumulated when nitrite was the electron acceptor. Dinitrogen production with nitrite was only one-half that observed with nitrate, and a small quantity of nitrous oxide was de-

tected only at 24 h. Nonetheless, nitrite apparently supported true denitrification since dinitrogen concentrations increased steadily and ammonia concentrations decreased from 27 to 20 μg of NH_3 -nitrogen per ml during the incubation period. Such a result was not unexpected, for certain denitrifying bacteria do not liberate nitrous oxide during normal denitrifying growth (1).

Snell and Lapage (13) reported gas production during growth of *K. denitrificans* on nitrite broth but not during growth on nitrate broth. In our experiments, the production of gas at the expense of nitrate may have been attributable to the nutritionally richer medium which we used or to the lower oxygen tension which was likely to be achieved in our sealed vessels than in the standard tube test.

C. violaceum ATCC 12472 exhibited denitrifying metabolism with either nitrate or nitrite as the electron acceptor (Table 2). As expected, smaller quantities of dinitrogen and carbon dioxide (the two major gaseous metabolic end products) were released from the nitrite-containing medium than from the nitrate-enriched medium. The benefit of oxidative phosphorylation coupled to nitrate reduction was not available when nitrite was the electron acceptor. In addition, a concentration of nitrite only one-tenth the concentration of nitrate is likely to be tolerated by these bacteria, which are sensitive to elevated quantities of nitrite (6) even if they can reduce it.

"*C. lividum*" ATCC 12473 also exhibited denitrifying metabolism with nitrate or nitrite. Chromobacteria currently are described (12) as organisms that reduce both nitrate and nitrite, sometimes with visible gas production. Our study indicates that total gas production in standard test tubes was probably greater on nitrate-containing media than on nitrite-containing media for both *C. violaceum* and "*C. lividum*." Nitrous oxide should have contributed

TABLE 1. Gas production by a *K. denitrificans* strain with nitrate or nitrite as electron acceptor

Duration of incubation (h)	Amt (nmol/ml of headspace) of gases produced by cells grown with:					
	Nitrate ^a			Nitrite ^b		
	CO ₂	N ₂ O	N ₂	CO ₂	N ₂ O	N ₂
0	0	0	0	0	0	0
12	162	0	20	72	0	46
24	269	0	162	150	4	111
48	440	47	699	539	0	1,208
67	4,989	833	1,758	620	0	2,164
72	5,012	1,041	1,825	648	0	2,295
100	5,338	667	2,610	851	0	2,900

^a Initially, the cultures contained 11.8 mmol of NO_3^- per ml (167.9 μg of NO_3^- nitrogen per ml).

^b Initially, the cultures contained 1.4 mmol of NO_2^- per ml (20.46 μg NO_2^- nitrogen per ml).

TABLE 2. Gas production by chromobacteria grown on media supplemented with nitrate or nitrite

Duration of incubation (h)	Amt (nmol/ml of headspace) of gases produced by " <i>C. lividum</i> " ATCC 12473 grown with:						Amt (nmol/ml) of gases produced by <i>C. violaceum</i> ATCC 12472 grown with:					
	Nitrate ^a			Nitrite ^b			Nitrate ^a			Nitrite ^b		
	CO ₂	N ₂ O	N ₂	CO ₂	N ₂ O	N ₂	CO ₂	N ₂ O	N ₂	CO ₂	N ₂ O	N ₂
0	0	0	0	0	0	0	0	0	0	0	0	0
14	252	60	192	158	152	227	330	12	160	108	0	251
22	328	51	507	348	277	501	1,116	66	1,120	201	0	541
48	519	59	1,014	413	312	773	483	1,325	1,690	413	161	687
72	1,204	73	1,332	822	259	924	594	1,191	2,000	662	572	738

^a Initial quantity as described in Table 1, footnote a.

^b Initial quantity as described in Table 1, footnote b.

TABLE 3. Gas production by strains of four species of *Neisseria*

Duration of incubation (h)	Amt (nmol/ml of headspace) of gases produced														
	<i>N. sicca</i> : nitrite medium ^a			<i>N. flavescens</i> : nitrite medium ^a			<i>N. subflava</i> : nitrite medium ^a			<i>N. mucosa</i>					
										Nitrite medium ^a			Nitrate medium ^b		
	CO ₂	N ₂ O	N ₂	CO ₂	N ₂ O	N ₂	CO ₂	N ₂ O	N ₂	CO ₂	N ₂ O	N ₂	CO ₂	N ₂ O	N ₂
0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	208
16	158	43	223	56	9	135	55	5	122	1,189	.97	722	748	11	365
24	265	17	311	69	38	183	119	4	312	2,236	0	1,086	1,515	0	487
48	420	0	400	214	166	863	396	0	479	3,006	0	1,139	4,351	0	1,679
67	437	0	501	500	0	1,138	478	0	872	3,290	0	1,275	5,609	0	2,342

^a Initial quantity as described in Table 1, footnote a.

^b Initial quantity as described in Table 1, footnote b. The strain of *N. mucosa* was the only *Neisseria* strain tested that reduced nitrate.

only minimally to the final volume of gas, as it accumulates only transiently and is more soluble than dinitrogen in water.

It should be noted that carbon dioxide production may not be due solely to anaerobic respiration in any of the organisms used in this study. All seven strains which we examined are capable of fermentation, and it is not known whether this process is repressed during respiratory reduction of nitrate or nitrite. Fermentation may be responsible, in part, for fluctuating CO₂/N₂ ratios and CO₂ disappearance (Table 2). *Propionibacterium pentosaceum* reportedly exhibits different patterns of organic acid release in the presence of nitrate and in its absence, but none of the denitrifying bacteria has been studied extensively with regard to fermentative versus denitrifying metabolism (3, 14).

The four *Neisseria* strains tested used nitrite as the terminal electron acceptor for denitrifying metabolism (Table 3). All of the nitrite which was provided was consumed by each strain within the first 24 h of incubation. Currently, these four strains are described as nitrite reducers (7). After 3 days, only a very small quantity of nitrous oxide was produced by the strain of *N. subflava*, and the ammonia concentration in the *N. subflava* cultures increased from 22 to 31.5 µg of NH₃ nitrogen per ml. The other *Neisseria* strains did not produce increased concentrations of ammonia. Consequently, even though the strain of *N. subflava* clearly denitrified (as shown by nitrous oxide production and dinitrogen production), assimilatory nitrite reduction or nitrite fermentation may have been competing simultaneously for the electron acceptor. The strain of *N. mucosa* was the only *Neisseria* strain which was able to use nitrate as an electron acceptor for denitrification.

Our results show that although nitrous oxide is an indicator of denitrification, it is present only transiently. This observation raises the pos-

sibility that a single analysis of evolved gases after incubation of an unknown bacterium for a specified length of time might not reveal this characteristic gas. Alternatively, a procedure such as that described by Tiedje (J. M. Tiedje, in *Methods of Soil Analysis*, 2nd ed., in press) may be preferred for detecting denitrifying metabolism. This procedure involves incubating the test bacterium in nitrate broth in a sealed, Hungate-type tube closed with a screw cap that exposes a butyl rubber septum. The culture is flushed with inert gas, and 1 ml of acetylene is injected through the septum into the headspace over the liquid medium immediately after inoculation. Acetylene prevents reduction of nitrous oxide to dinitrogen but does not otherwise affect denitrification (1, 9, 15). Consequently, nitrous oxide accumulates and may be withdrawn and identified by gas chromatography as a certain indicator of denitrification (6).

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REPRINT REQUESTS

Address reprint requests to: M. A. Grant, Department of Microbiology, University of Georgia, Athens, GA 30602.

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