Effect of Chloramphenicol on Denitrification in *Flexibacter canadensis* and "*Pseudomonas denitrificans*"

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It was recently reported that chloramphenicol inhibits existing denitrification enzyme activity in sediments and carbon-starved cultures of "*Pseudomonas denitrificans*." Therefore, we studied the effect of chloramphenicol on denitrification by *Flexibacter canadensis* and "*P. denitrificans*." Production of N_2O from nitrate by *F. canadensis* cells decreased as the concentration of chloramphenicol was increased, and 10.0 mM chloramphenicol completely inhibited N_2O production. "*P. denitrificans*" was less sensitive to chloramphenicol, and production of N_2O from nitrate was inhibited by only about 50% even in the presence of 10.0 mM chloramphenicol. These results suggested that inhibition of denitrification enzyme activity depended on the concentration of chloramphenicol. Increasing the concentration of chloramphenicol decreased the rate of production of nitrite from nitrate by *F. canadensis* cells, and the concentration of chloramphenicol which resulted in 50% inhibition of production of nitrite from nitrate was 2.5 mM. In contrast, the rates of production of nitrite from nitrate by intact cells and cell extracts of "*P. denitrificans*" were inhibited by only 58 and 54%, respectively, at a chloramphenicol concentration of 10.0 mM. Chloramphenicol caused accumulation of NO from nitrite but not from nitrate and inhibited NO consumption in *F. canadensis*; however, it had neither effect in "*P. denitrificans*." Chloramphenicol did not affect N_2O consumption by these organisms. We concluded that chloramphenicol inhibits denitrification at the level of nitrate reduction and, in *F. canadensis*, also at the level of NO reduction.

Denitrification is defined as the dissimilatory reduction of nitrate to nitrogen gases by denitrifiers under anaerobic conditions. This reduction occurs as follows: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (10, 28). The enzymes associated with these processes are nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (8). Denitrification is often assayed by measuring the accumulation of N_2O , using the C_2H_2 inhibition technique (9, 27).

Chloramphenicol is a broad-spectrum antibiotic which is widely used to inhibit cell growth and new enzyme synthesis; this antibiotic acts by binding to ribosomes in prokaryotic organisms (1). Smith and Tiedje (19) first used chloramphenicol to measure denitrifying enzyme activity (DEA) in soils and suggested that addition of this compound to environmental samples should be part of the recommended protocol for DEA assays based on the acetylene inhibition method but only if chloramphenicol does not affect the functioning of existing enzymes (21). The chloramphenicol concentrations used in DEA assays (6, 16, 18) range from 0.3 to 15.5 mM since the effective concentration of chloramphenicol changes with the type of sample. However, such concentrations may be twice the concentration of a saturated solution of chloramphenicol and may cause effects other than inhibition of protein synthesis. Brooks et al. (2) found that 0.92 mM chloramphenicol inhibits nitrate disappearance and production of N_2O from nitrate in sediments and in carbon-starved cultures of "*Pseudomonas denitrificans*" ATCC 13867, although such inhibition by chloramphenicol was not detected in DEA assays of soils (6, 7, 18). This inhibition may be due to a specific interaction of chloramphenicol with denitrifying reductases or other components

(2), but Brooks et al. did not identify the actual steps of denitrification that were affected by chloramphenicol. Dendooven et al. (4) reported that addition of chloramphenicol to a soil results in an increase in the production of CO_2 and N_2O that may be due to biological degradation of chloramphenicol and/or diversion of carbon from protein synthesis to respiratory processes. To elucidate the mechanism(s) of chloramphenicol inhibition of DEA, we studied the effect of chloramphenicol on denitrification in *Flexibacter canadensis* and "*P. denitrificans*."

MATERIALS AND METHODS

Organisms and culture conditions. *F. canadensis* Is-11 (= ATCC 49765), and "*P. denitrificans*" ATCC 13867 were the strains used in this study. These organisms were stored in 20% glycerol at $-70^\circ C$. The culture media and conditions used have been described previously (23). Late-exponential-phase cells were harvested by centrifugation at $12,500 \times g$ for 10 min at $4^\circ C$. The resulting pellets were washed twice with 10 mM potassium phosphate buffer (pH 7.4) and resuspended with the same buffer to give suspensions that had an optical density at 660 nm (OD_{660}) of 0.5.

Cell extracts. A 10-ml portion of washed cells (OD_{660} 40 to 50) was added to 10 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 5 mM EDTA (disodium salt) and 5 μM phenylmethylsulfonyl fluoride. A cell extract was then prepared by the procedure of Wu and Knowles (23).

Denitrifying reductase assays. The nitrate reductase assay which we used was based on the assays of MacGregor (15) and Wu and Knowles (23). The assays were performed in 14-ml serum bottles fitted with silicone-reinforced butyl rubber stoppers (Wheaton) and aluminum seals (ColorSeal; Pierce Chemical Co.). To study production of nitrite from nitrate by whole cells, the serum bottles, which contained 5.0 mM glucose, 0.2 mg of catalase (ICN Biochemicals) per ml of mixture, and cell suspensions (0.28 and 0.20 mg of protein per ml of mixture for *F. canadensis* and "*P. denitrificans*," respectively) in 2.0 ml of 10 mM potassium phosphate buffer (pH 7.4), were evacuated and backfilled with helium three times to obtain anaerobic conditions. Chloramphenicol was obtained from ICN Biochemicals and was prepared fresh as ethanolic solutions; this antibiotic was added to the preparations to give the concentrations indicated below. Control experiments performed with ethanol alone at final concentrations of 0.5 to 2.0% (vol/vol), which were equivalent to the final concentrations of ethanol in chloramphenicol-treated preparations, showed that the ethanol did not affect any of the processes studied. The reactions were started by injecting 10 μl of 100 mM potassium nitrate. The serum bottles were incubated at $25^\circ C$ for 5 min on a

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gyratory water bath shaker (220 rpm). Then 10 μ l of 10% hydrogen peroxide was injected into each bottle to stop the reaction. A 1-ml portion of each mixture was immediately removed and centrifuged for 5 min in a Hermle microcentrifuge (12,000 rpm) at room temperature to remove the cells. The supernatant fraction was then subjected to nitrite analysis. To study production of nitrite from nitrate by cell extracts, the procedure was performed as described above except that NADH (Sigma) was the reductant used and the mixture contained 0.6 and 1.3 mg of cell extract protein per ml for *F. canadensis* and "*P. denitrificans*," respectively. The rates were expressed in nanomoles of nitrite produced from nitrate per minute per milligram of protein.

To study production of N_2O from nitrate, acetylene was added to the serum bottles at a final concentration of 4 kPa to inhibit further reduction of N_2O , and the reaction was started by injecting 10 μ l of 100 mM KNO_3 . To study NO accumulation during reduction of nitrate or nitrite, the pH of the potassium phosphate buffer was adjusted to 6.5 since this pH is the optimum pH for net NO production (22, 26). The reactions were begun by injecting 10 μ l of 100 mM potassium nitrate or sodium nitrite. To study the consumption of NO or N_2O , 20 nmol of NO per bottle or 600 nmol of N_2O per bottle was injected to begin the reaction. In each case, 0.2 ml of the gas phase was removed after a known time interval for NO and/or N_2O analysis with a 1.0-ml syringe fitted with a Mininert valve.

Analyses. Nitrite contents were determined by the method of Strickland and Parsons (20) by using microplates and a Bio Tek microplate reader (model EL-390). NO contents were determined by using a NO chemiluminescence analyzer (model 270B NOA; Sievers). The model 270B NOA analyzer is based on a gas phase chemiluminescence reaction between NO and ozone. Emission from electronically excited nitrogen dioxide is in the red and near-infrared regions of the spectrum and is detected by a thermoelectrically cooled, red-sensitive photomultiplier tube. The rate of oxygen flow through the ozone generator was 60 ml/min at 6 lb/in². The NO concentrations in gas phase samples were determined by comparison with the peak height responses of standard NO mixtures. The detection limit was 2 pmol/ml of headspace gas (50 nl/liter). N_2O contents were determined by using gas chromatography and an electron capture detector as previously described (3). Protein contents were determined by the method of Lowry et al. (14); intact cells (but not cell extracts) were first boiled for 5 min in the presence of 1 M NaOH, and bovine serum albumin was used as the standard.

RESULTS

Short-term effect of chloramphenicol on denitrification. To see if existing DEA was affected by chloramphenicol, production of N_2O from nitrate was studied by using the acetylene inhibition technique. The rates of production of N_2O from nitrate by whole cells (OD_{660} , 0.5) of *F. canadensis* and "*P. denitrificans*" in both the presence and the absence of 1.0 mM chloramphenicol were relatively constant for the first 30 min (data not shown), indicating that N_2O production could be attributed to active preexisting denitrifying enzymes and not to significant new enzyme synthesis. Therefore, in subsequent experiments, the existing DEA was determined by measuring the production of N_2O from nitrate during the first 10 min of incubation.

To determine whether chloramphenicol inhibition of existing DEA was related to chloramphenicol concentration, production of N_2O from nitrate by the same two organisms was studied (Fig. 1). N_2O production by *F. canadensis* cells decreased as the concentration of chloramphenicol was increased, and 10.0 mM chloramphenicol completely inhibited N_2O production. "*P. denitrificans*" was much less sensitive to chloramphenicol, and production of N_2O from nitrate was inhibited by only about 50% even in the presence of 10.0 mM chloramphenicol. Thus, chloramphenicol inhibition of DEA depended on the chloramphenicol concentration and on the sensitivity of the denitrifiers to chloramphenicol. In addition, the rate of N_2O production by "*P. denitrificans*" was greater than the rate of N_2O production by *F. canadensis*.

Effect of chloramphenicol on the reduction of nitrate to nitrite. To investigate whether the inhibition of DEA could be attributed to chloramphenicol acting specifically on nitrate reductase and/or nitrate transport, production of nitrite from nitrate by *F. canadensis* cells and by intact cells and cell extracts of "*P. denitrificans*" was studied (Table 1). The rate of produc-

FIG. 1. Effect of chloramphenicol (Cm) on production of N_2O from nitrate by intact cells (OD_{660} , 0.5) of *F. canadensis* (●) and "*P. denitrificans*" (▼) during the first 10 min under anaerobic conditions. The data are the means of triplicate determinations, and the bars indicate ± 1 standard error of the mean. Where not shown, the error bars are within the symbols.

tion of nitrite from nitrate by *F. canadensis* cells decreased as the concentration of chloramphenicol was increased. The concentration of chloramphenicol that resulted in 50% inhibition of production of nitrite from nitrate was 2.5 mM. In contrast, the production of nitrite from nitrate by intact cells and cell extracts of "*P. denitrificans*" was inhibited by only 58 and 54%, respectively, at a chloramphenicol concentration of 10.0 mM. These results are consistent with the data for chloramphenicol inhibition of production of N_2O from nitrate described above. Moreover, the levels of chloramphenicol inhibition of production of nitrite from nitrate by intact cells and cell extracts of "*P. denitrificans*" were very similar, suggesting that chloramphenicol inhibited the reduction of nitrate to nitrite by nitrate reductase, but not nitrate transport. Unfortunately, we failed to measure production of nitrite from nitrate by cell extracts of *F. canadensis*, since NADH is not an effective electron donor in this organism (23; see below).

Effect of chloramphenicol on accumulation of NO during reduction of nitrate or nitrite. To determine whether inhibition of N_2O production by chloramphenicol was related to the NO released as a free intermediate, production of NO from nitrate and nitrite by the same two organisms was measured in the presence and in the absence of 5.0 mM chloramphenicol. In *F. canadensis*, NO accumulated from nitrite but not from nitrate in the presence of chloramphenicol (Fig. 2). The rate of NO accumulation was constant for the first 30 min, and the

TABLE 1. Effects of chloramphenicol on production of nitrite from nitrate by *F. canadensis* cells (OD_{660} , 0.5) and intact cells (OD_{660} , 0.5) and cell extracts of "*P. denitrificans*"

Chloramphenicol concn (mM)	Nitrite production (nmol of NO_2^- min ⁻¹ mg of protein ⁻¹) ^a		
	<i>F. canadensis</i> intact cells	<i>"P. denitrificans"</i>	
		Intact cells	Cell extracts
0	115.9 $\pm$ 1.3 ^b	164.8 $\pm$ 1.0	101.1 $\pm$ 5.6
1	97.1 $\pm$ 4.8	160.8 $\pm$ 1.4	99.4 $\pm$ 2.4
2	62.3 $\pm$ 2.3	128.0 $\pm$ 7.5	82.2 $\pm$ 1.5
5	32.0 $\pm$ 9.9	86.9 $\pm$ 2.9	56.7 $\pm$ 1.8
10	7.8 $\pm$ 1.1	68.9 $\pm$ 1.0	46.1 $\pm$ 3.7

^a Production of nitrite from nitrate was determined during the first 5 min under anaerobic conditions.

^b The data are the means $\pm$ standard errors of the means for triplicate determinations.

FIG. 2. NO accumulation during reduction of nitrate (A) or nitrite (B) in the presence (●) or absence (○) of 5.0 mM chloramphenicol by *F. canadensis* cells (OD_{660} , 0.5) under anaerobic conditions. The data are the means of triplicate determinations, and the bars indicate ± 1 standard error of the mean. Where not shown, the error bars are within the symbols.

maximal level of production of nitric oxide in the presence of chloramphenicol was 89 nmol per bottle (i.e., 9% of the nitrite added). In addition, accumulation of NO from nitrite by whole cells of *F. canadensis* was linearly correlated with chloramphenicol concentration (Fig. 3). However, NO did not accumulate from nitrate or nitrite in "*P. denitrificans*" in the presence of chloramphenicol (data not shown). These results are consistent with the hypotheses that NO is an obligate intermediate of denitrification in *F. canadensis* and that the nitrite reductase and/or nitric oxide reductase of *F. canadensis* is quite different from the "*P. denitrificans*" enzyme(s).

Effect of chloramphenicol on NO or N_2O consumption. Chloramphenicol at a concentration of 5.0 mM inhibited NO consumption by intact cells of *F. canadensis* (Fig. 4) but not NO consumption by intact cells of "*P. denitrificans*" (data not shown). Inhibition of nitric oxide reductase by chloramphenicol in *F. canadensis* may explain the accumulation of NO after chloramphenicol was added to *F. canadensis* cells (Fig. 2 and 3). Chloramphenicol had no effect on N_2O consumption by the two organisms during the first 10 min (Fig. 5). We concluded that chloramphenicol did not inhibit nitrous oxide reductase.

DISCUSSION

In denitrification studies, chloramphenicol is sometimes used as a tool to measure the activity of denitrifying reductases

FIG. 3. Dependence of accumulation of NO from nitrite on the chloramphenicol (Cm) concentration in *F. canadensis* cells (OD_{660} , 0.5) during the first 3 min under anaerobic conditions. The data are the means of triplicate determinations, and the bars indicate ± 1 standard error of the mean. Where not shown, the error bars are within the symbols.

FIG. 4. NO consumption by *F. canadensis* cells (OD_{660} , 0.5) in the presence (●) or absence (○) of 5.0 mM chloramphenicol under anaerobic conditions. Autoclaved cells (■) were used as the control to measure NO consumption by chemical reaction with 5.0 mM chloramphenicol. The data are the means of triplicate determinations, and the bars indicate ± 1 standard error of the mean. Where not shown, the error bars are within the symbols.

since chloramphenicol inhibits de novo synthesis of enzymes and it is usually assumed that addition of this compound should not affect preexisting enzyme activity. In DEA assays performed with pure cultures or environmental samples, production of N_2O from nitrate should be measured during the short term in order to avoid the formation of new enzymes. Smith and Tiedje (19) found that in soil, two distinct phases of denitrification occur after anaerobiosis is imposed. Chloramphenicol does not decrease the denitrification rate during the first phase (1 to 3 h) since there is no formation of new enzymes and chloramphenicol has no effect on preexisting enzyme activity. However, chloramphenicol reduces denitrification during the second phase (4 to 8 h), when active derepression of new enzyme synthesis otherwise occurs. Rudaz et al. (16) found that de novo synthesis of enzymes involved in N_2O production occurred after 4 to 8 h of wetting of dry soils. Brooks et al. (2) measured production of N_2O from nitrate and nitrate disappearance in sediments and carbon-starved cultures of "*P. denitrificans*" ATCC 13867 in periods of less than 2.5 h. In our study, production of N_2O from nitrate was determined during the first 10 min since the rates of denitrification by the two organisms were relatively constant for the first 30 min of incubation.

The concentrations of chloramphenicol used in this study (0 to 10 mM) were chosen on the basis of the results of other

FIG. 5. N_2O consumption by intact cells (OD_{660} , 0.5) of *F. canadensis* (Fc) and "*P. denitrificans*" (Pd) in the presence or absence of 5.0 mM chloramphenicol (Cm) during the first 10 min under anaerobic conditions. The data are the means of triplicate determinations, and the bars indicate ± 1 standard error of the mean.

workers (6, 16, 18) and on the basis of our observations of chloramphenicol inhibition of denitrification in *F. canadensis* and "*P. denitrificans*." Our results show that the inhibition of production of nitrite and N_2O from nitrate by the two organisms and the stimulation of accumulation of NO from nitrite by *F. canadensis* were dependent on the chloramphenicol concentration. In pure cultures of some gram-negative and gram-positive bacteria, the minimal concentrations of chloramphenicol that resulted in inhibition of protein synthesis and cell growth ranged from 0.31 to 5.0 μM (13). In DEA assays performed with pure cultures or environmental samples, the chloramphenicol concentrations usually range from 0.31 mM (16, 18) to 15.5 mM (6). The chloramphenicol concentrations used in DEA assays are much higher than the minimal concentrations that inhibit protein synthesis and cell growth because some of the chloramphenicol added may be adsorbed to surfaces of soil or sediment, resulting in a decrease in antibiotic activity. In this study, concentrations of chloramphenicol ranging from 1 to 10 mM inhibited production of N_2O from nitrate by *F. canadensis* and "*P. denitrificans*," and similar results were obtained with *Paracoccus denitrificans* ATCC 19367 (data not shown). Similarly, Brooks et al. (2) observed that 0.92 mM chloramphenicol significantly decreased the rate of production of N_2O from nitrate in carbon-starved cultures of "*P. denitrificans*."

F. canadensis nitrate reductase is a membrane-bound enzyme, and methyl viologen or benzyl viologen, but not NADH or ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), is a good electron donor for nitrate reductase in membrane fractions (24). However, both methyl viologen (data not shown) and NADH (this study) are good electron donors for nitrate reductase in cell extracts of "*P. denitrificans*." Unfortunately, the chemical reaction of chloramphenicol with dithionite resulted in failure to maintain methyl viologen in the reduced state when the concentration of chloramphenicol was greater than 1.0 mM (data not shown). Thus, we determined nitrate reductase activity in cell extracts of "*P. denitrificans*" by using NADH as the electron donor. In a search for a suitable electron donor for *F. canadensis* nitrate reductase, we also examined hydrogen, formate, succinate, ascorbate-TMPD, and NADH, but no nitrate reductase activity was observed in cell extracts, membrane fractions, or cells treated with detergent (octyl β -glucoside, Triton X-100, toluene, or Tween 80) (data not shown). However, we observed significant rates of oxygen uptake when we used NADH or ascorbate-TMPD with cell extracts of *F. canadensis* (data not shown). The reason for this is not clear.

Chloramphenicol inhibited NO consumption in *F. canadensis*, indicating that chloramphenicol may be another inhibitor of nitric oxide reductase. Several nitric oxide reductase inhibitors have been recognized previously, including the inorganic nitrogen compounds azide, cyanide, NH_2OH , nitrite, and NO (22, 26), the ionophores carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and nigericin (11, 12, 25), the thiol reagent *N*-ethylmaleimide (12), and the detergent Triton X-100 (17). However, the mechanism(s) of inhibition of nitric oxide reductase has not been determined. The fact that chloramphenicol inhibited NO consumption in *F. canadensis* and the fact that this antibiotic may be an inhibitor of nitric oxide reductase explain our observation that there was net NO accumulation during reduction of nitrite in the presence of chloramphenicol. It is noteworthy that the presence of CCCP, FCCP, or nigericin also resulted in net accumulation of NO in *F. canadensis* (25). We concluded that the presence of high concentrations of chloramphenicol during DEA assays of environmental samples

may result in partial or complete inhibition of denitrification by at least some bacteria at the levels of nitrate reduction and NO reduction.

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