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Effects of Nitrapyrin [2-Chloro-6-(Trichloromethyl)Pyridine] on the Obligate Methanotroph *Methylosinus trichosporium* OB3b

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Nitrapyrin inhibited growth, CH_4 oxidation, and NH_4^+ oxidation, but not the oxidation of CH_3OH, HCHO, or HCOONa, by *Methylosinus trichosporium* OB3b, suggesting that nitrapyrin acts against the methane monooxygenase enzyme system. The inhibition of CH_4 oxidation could be reversed by repeated washing of nitrapyrin-inhibited cells, indicating that its effect is bacteriostatic. The addition of Cu^{2+} did not release the inhibition. Methane oxidation was also inhibited by 6-chloro-2-picoline. These data suggest that the mode of action of nitrapyrin on *M. trichosporium* is different from that on chemoautotrophic NH_4^+ oxidizers or methanogens.

Nitrapyrin [2-chloro-6-(trichloromethyl)pyridine] inhibits chemoautotrophic ammonium oxidation (1, 3, 8), sulfate reduction in pure cultures of *Desulfovibrio* sp. and sediment (21), methanogenesis in marine sediments (19), and NO_3^- reduction by a denitrifying pseudomonad (10). We previously reported that it also inhibited CH_4 oxidation and CO_2 incorporation by two obligate methanotrophs, *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Texas) (25).

However, it was not known whether nitrapyrin specifically inhibited CH_4 oxidation or some other metabolic process. In this paper, we report that nitrapyrin inhibits NH_4^+ oxidation but not the oxidation of the intermediates of the CH_4 oxidation pathway, CH_3OH, HCHO, and HCOOH. Since NH_4^+ is a substrate for the methane monooxygenase (6, 17, 26), these data suggest that the target of inhibition is specifically associated with methane oxidation.

MATERIALS AND METHODS

Methylosinus trichosporium OB3b (obtained from T. Yoshinari) was grown at 30°C in 2-liter Erlenmeyer flasks containing 1.5 liters of nitrate mineral salts medium (26), agitated with a magnetic stirrer, and sparged continuously with 25% CH_4 in air at a flow rate of 225 to 250 ml min^{-1}. Mid-log phase cells were harvested by centrifugation at 16,000 × *g* for 12 min. Cells were washed twice with and resuspended in nitrate mineral salts medium, except for experiments in which HCHO was to be determined because the Sequestrene-Fe in nitrate mineral salts medium interfered with the HCHO assay. In such experiments, cells were washed and resuspended in 5 mM borate–10 mM phosphate buffer, pH 7.5 (11). Cells for NH_4^+ oxidation experiments were washed with and resuspended in 1 mM NH_4Cl–4 mM phosphate, and all other salts and trace elements were as described previously by Whittenbury et al. (26).

Ammonium oxidation experiments employed 25-ml samples of cell suspension in 160-ml serum bottles sealed with grey butyl rubber stoppers (Wheaton Scientific, Millville, N.J.). All other experiments used up to 10 ml of cell suspension in 60-ml serum bottles sealed as above, except for CH_3OH and HCHO oxidation experiments in which unsealed 60-ml serum bottles were used. All incubations were at 30°C and 200 or 250 rpm on a gyratory water bath shaker.

Pure nitrapyrin powder was dissolved in dimethyl sulfoxide (DMSO) for addition to cell suspensions. Pure 6-chloro-2-picoline was added directly to cell suspensions. Cell suspensions were incubated with these compounds for 2 to 4 h before the addition of substrate.

Gas-phase samples were removed by syringe, and CH_4 was determined by gas chromatography as previously described (4).

Methanol oxidation was determined as the initial velocity of HCHO production. Formaldehyde accumulation was linear during a 4-min incubation period after the addition of filter-sterilized CH_3OH. Formaldehyde was determined by a modification of the colorimetric method of Chrastil and Wilson (5). Samples (1 ml) of cell suspensions were removed at 1-min intervals and added directly to test tubes each of which contained 1 ml of tryptophan-ethanol reagent. Concentrated H_2SO_4 (1 ml) was then immediately added, and the tube was agitated on a vortex mixer. After all samples had been collected and processed as above, 200 μl of 0.2% (wt/vol) $FeCl_3 \cdot 6H_2O$ was added to each tube, and all tubes were incubated for 1 h at 70°C. After the tubes cooled, color development was measured at 575 nm on a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Formaldehyde stock solution was prepared by autoclaving 0.3 g of paraformaldehyde powder in 10 ml of deionized water for 1 h. Formaldehyde standards (20 to 150 μM) were prepared with heat-killed cell suspension and treated as above. Tryptophan-ethanol reagent, $FeCl_3 \cdot 6H_2O$ reagent, and HCHO stock solution were prepared daily.

Formaldehyde oxidation by cell suspensions supplemented with HCHO was determined by the method of Chrastil and Wilson (5) as described above.

Formate oxidation was measured as the decrease in radioactivity of cell suspensions supplemented with $H^{14}COONa$ (specific activity, 4.4 mCi $mmol^{-1}$, diluted to 44 μCi $mmol^{-1}$ with cold HCOONa; NEN Canada, Ltd., Lachine, Quebec) as $^{14}CO_2$ was evolved. Preliminary experiments with heat-killed cell suspensions indicated that loss of radioactivity due to substrate volatilization was not significant. Cell suspensions were incubated with 0.5 mM $H^{14}COONa$ for 15 min. Samples (50 μl) were transferred by Hamilton syringe directly into scintillation vials, and each was acidified with 100 μl of 4 N HCl to volatilize $^{14}CO_2$. Aquasol scintillation cocktail (NEN Canada, Ltd.) was added, and the radioactiv-

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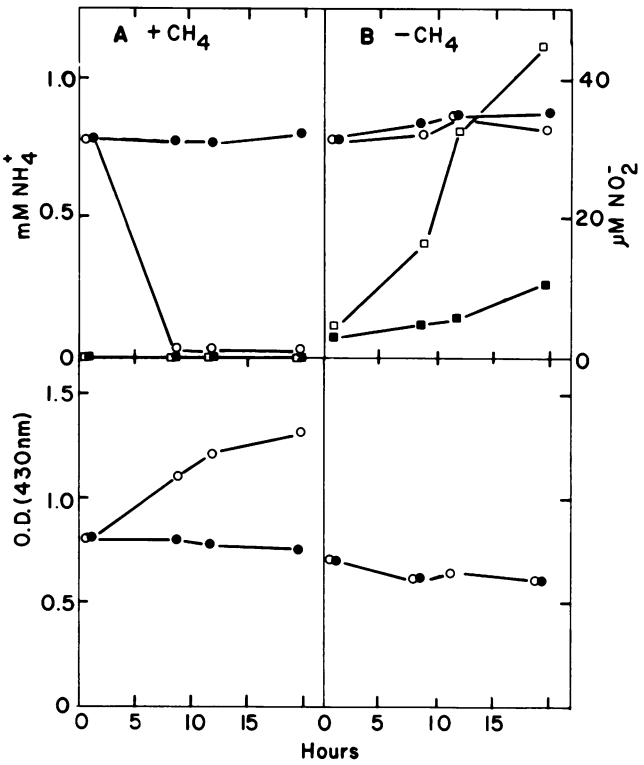

FIG. 1. Growth (optical density, O.D.), NH_4^+ consumption (●, ○), and NO_2^- production (■, □) by *Methylosinus trichosporium* OB3b with (closed symbols) or without (open symbols) the addition of 10 μg of nitrapyrin ml^{-1} in the presence (A) or absence (B) of 20% CH_4 in the gas phase. All flasks received 70 μl of DMSO $liter^{-1}$. Final protein concentration was 130 μg ml^{-1}.

TABLE 1. Production of HCHO from CH_3OH and the oxidation of HCOONa by *Methylosinus trichosporium* OB3b in the presence or absence of nitrapyrin[a]

Treatment	HCHO production[b] (μmol mg of protein^{-1} h^{-1})	HCOONa oxidation[c] (μmol mg of protein^{-1} h^{-1})
None	10.5	9.3
DMSO	11.3	9.3
DMSO + 1 μg of nitrapyrin ml^{-1}	ND[d]	9.0
DMSO + 5 μg of nitrapyrin ml^{-1}	10.8	9.9
DMSO + 10 μg of nitrapyrin ml^{-1}	12.1	9.5
Heat-killed cells + DMSO + 10 μg of nitrapyrin ml^{-1}	0.06	ND

[a] Formaldehyde production and HCOONa oxidation were determined in two separate experiments.

[b] The concentration of DMSO was 70 μl $liter^{-1}$. Initial concentration of CH_3OH was 2.0 mM. Final protein concentration was 170 μg ml^{-1}.

[c] The concentration of DMSO was 250 μl $liter^{-1}$. Final protein concentration was 135 μg ml^{-1}.

[d] ND, Not done.

ity was measured with a Nuclear Chicago Isocap 300 liquid scintillation spectrometer (Searle Instrumentation, Des Plaines, Ill.).

Ammonium and NO_2^- concentrations were determined by the indophenol and azo-dye colorimetric methods, respectively, with a Chemlab Instruments (Hornchurch, England) automated analysis system as previously described (14). Samples (3 ml) of cell suspension were removed by syringe, optical densities at 430 nm (Gilford 240 spectrophotometer) were determined, and the samples were frozen. Thawed samples were filtered (0.22-μm type GS Millipore filter) before NH_4^+ and NO_2^- analysis.

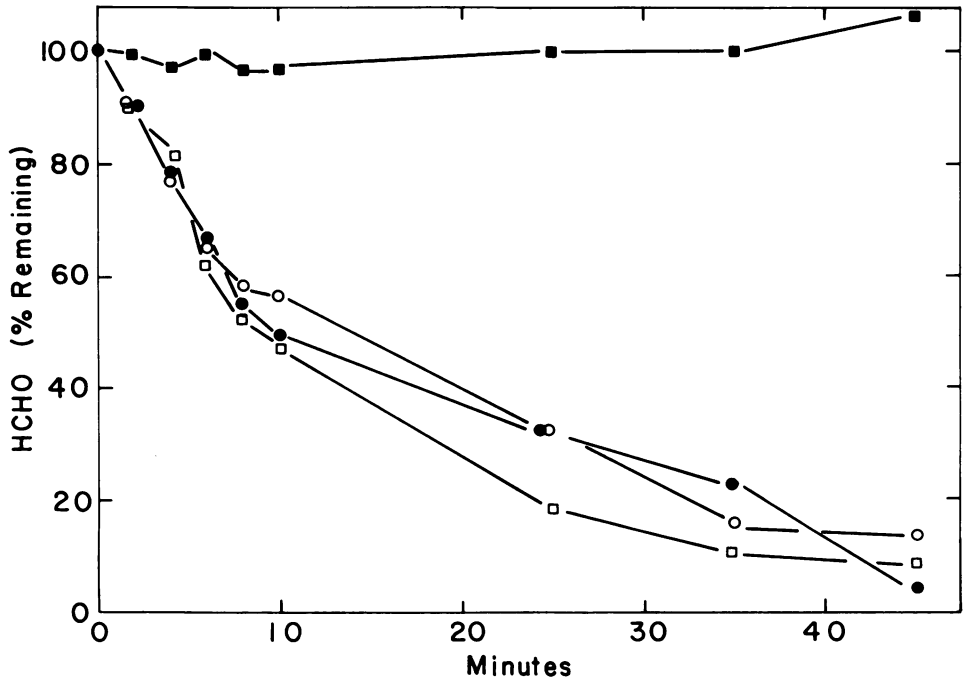

FIG. 2. Formaldehyde consumption by cell suspensions of *Methylosinus trichosporium* OB3b alone (□) or supplemented with 100 μl of DMSO $liter^{-1}$ (○) or DMSO + 5 μg of nitrapyrin ml^{-1} (●). Heat-killed cells also received DMSO and nitrapyrin (■). The initial HCHO concentration was 150 μM. Final protein concentration was 160 μg ml^{-1}.

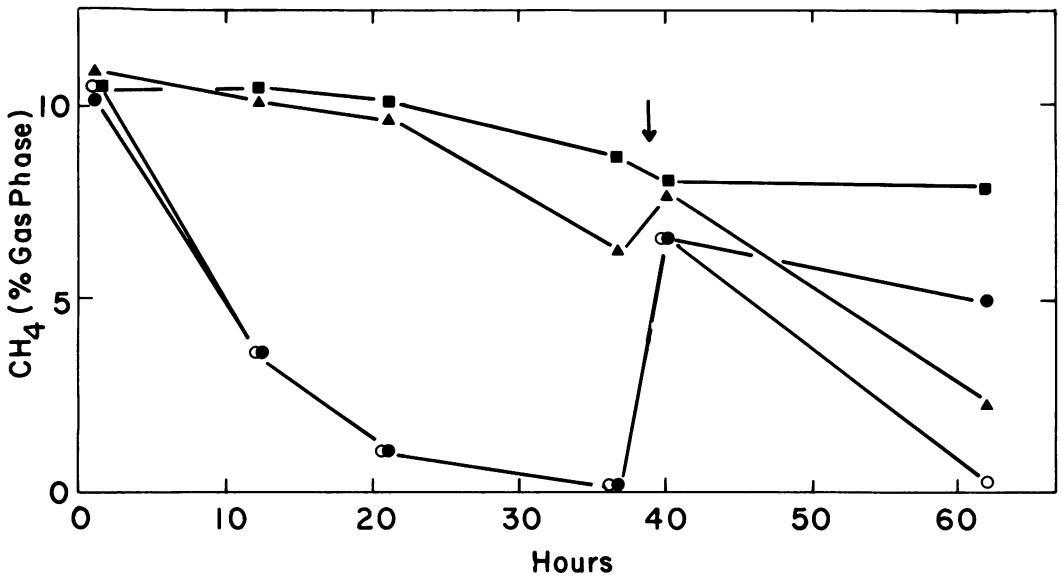

FIG. 3. Recovery from nitrapyrin inhibition of CH_4 oxidation by cell suspensions of *Methylosinus trichosporium* OB3b. Cells were washed three times after the addition of 10 μg of nitrapyrin ml^{-1} (●) or 100 μg of nitrapyrin ml^{-1} (▲). Controls received 10 μg of nitrapyrin ml^{-1} but not the washing treatment (■) or no addition of nitrapyrin and no washing treatment (○). At 39.5 h (arrow), all bottles were opened for 10 min. After the bottles were resealed, CH_4 concentrations were reestablished, and 10 μg of nitrapyrin ml^{-1} was added to treatment (●). Final protein concentration was 320 μg ml^{-1}.

Protein was estimated by the method of Lowry et al. (16) except that sodium citrate was used instead of sodium tartrate (7) with bovine serum albumin as the standard.

All treatments were in duplicate or triplicate. The standard error was always less than 10% of the mean.

Research grade nitrapyrin was a gift from the Dow Chemical Co. of Canada (Sarnia, Ontario). Paraformaldehyde and 6-chloro-2-picoline were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.); chromatographic grade CH_3OH was from Matheson, Coleman, and Bell (Cincinnati, Ohio); pure CH_4 was from Liquid Carbonic Canada (Montreal, Quebec); and Sequestrene NaFe (13% Fe [wt/wt]) was from CIBA-Geigy (Greensboro, N.C.). All other chemicals were reagent grade.

RESULTS

We previously reported (25) that nitrapyrin at concentrations as low as 2 μg ml^{-1} (8.7 μM) markedly inhibited CH_4 oxidation. We now show that CH_4-dependent growth and NH_4^+ consumption were inhibited by 10 μg of nitrapyrin ml^{-1} (43.3 μM) (Fig. 1A), as was NH_4^+-dependent NO_2^- production in the absence of CH_4 (Fig. 1B).

Nitrapyrin at concentrations that clearly inhibited CH_4 oxidation had no significant effect on the oxidation of CH_3OH (Table 1), HCHO (Fig. 2), and HCOONa (Table 1).

The inhibition of methane oxidation could be reversed by washing nitrapyrin-inhibited cells repeatedly with nitrate mineral salts medium (Fig. 3). Cell suspensions washed three times after the addition of 10 μg of nitrapyrin ml^{-1} metabolized CH_4 at a rate equivalent to that of controls receiving no nitrapyrin. Cell suspensions washed three times after the addition of 100 μg of nitrapyrin ml^{-1} recovered much more slowly, and cell suspensions receiving 10 μg of nitrapyrin ml^{-1} without the washing treatment did not recover during the course of the experiment. Inhibition of recovered cells by a second addition of 10 μg of nitrapyrin ml^{-1} without the washing treatment indicated that resistance to the inhibitor was not acquired.

Campbell and Aleem (3) demonstrated that the addition of 60 μM Cu^{2+} to cell suspensions of *Nitrosomonas* sp. inhibited by 1.5 μg of nitrapyrin ml^{-1} partially reversed the inhibition. The addition of 1 to 500 μM $CuSO_4$ to cell suspensions of *Methylosinus trichosporium* OB3b immediately before the addition of 5 μg of nitrapyrin ml^{-1} did not protect methane oxidation activity against inhibition (Table 2).

A derivative of pyridine, 6-chloropicolinic acid, which unlike 2-chloro-6-(trichloromethyl)pyridine (nitrapyrin) does not have a trichloro group, is not toxic for chemoautotrophic

TABLE 2. Effect of Cu^{2+} on the inhibition of CH_4 oxidation by nitrapyrin in cell suspensions of *Methylosinus trichosporium* OB3b[a]

Treatment	CH_4 oxidation (nmol of CH_4 mg of protein^{-1} h^{-1})	% Inhibition
DMSO	489.0	
DMSO + 5 μg of nitrapyrin ml^{-1}	71.0	85.5
DMSO + 5 μg of nitrapyrin ml^{-1} + 1 μM Cu^{2+}	29.8	93.9
DMSO + 5 μg of nitrapyrin ml^{-1} + 5 μM Cu^{2+}	14.8	97.0
DMSO + 5 μg of nitrapyrin ml^{-1} + 10 μM Cu^{2+}	0	100
DMSO + 5 μg of nitrapyrin ml^{-1} + 50 μM Cu^{2+}	0	100
DMSO + 5 μg of nitrapyrin ml^{-1} + 100 μM Cu^{2+}	0	100
DMSO + 5 μg of nitrapyrin ml^{-1} + 500 μM Cu^{2+}	25.5	94.8

[a] The concentration of DMSO was 85 μl $liter^{-1}$. Initial gas phase concentration of CH_4 was 4%. Final protein concentration was 51 μg ml^{-1}.

NH_4^+ oxidation (2) or for methanogenesis (19). Chloroform and DDT, compounds that contain a trichloro group, are toxic for methanogens (19). These findings suggest that the trichloro moiety is required for the toxicity of nitrapyrin (19). We found, however, that CH_4 oxidation was completely inhibited by 6-chloro-2-picoline (Table 3), a compound identical to nitrapyrin except for the replacement of the trichloromethyl group with a methyl group.

DISCUSSION

We previously reported that nitrapyrin inhibited CH_4 oxidation and CO_2 incorporation by *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Texas) (25). Results of CH_4-NH_4^+ competition studies (6, 17) as well as oxidation of NH_4^+ by pure methane monooxygenase from *Methylococcus capsulatus* (Bath) (H. Dalton, personal communication) suggest that the methane monooxygenase mediates the oxidation of both these substrates. Inhibition of this enzyme by nitrapyrin would therefore be expected to inhibit both these processes resulting in an impairment of growth, NH_4^+ assimilation, and NO_2^--production. Evidence is presented that low concentrations of nitrapyrin inhibit NH_4^+ oxidation in the absence of CH_4, as well as growth and consumption of NH_4^+ in the presence of CH_4. Although circumstantial, the insensitivity of the oxidation of CH_3OH, HCHO, and HCOONa to nitrapyrin suggests that the chemical exerts its effect directly on the methane monooxygenase and (or) on electron transfer component(s) specifically associated with this enzyme.

Campbell and Aleem (3) concluded that nitrapyrin was toxic for *Nitrosomonas* sp. because it chelated metal, probably copper, reaction centers in the ammonia oxidase complex. Conflicting reports have been published on the structure and apparent copper content of the methane monooxygenase of *Methylosinus trichosporium* OB3b (12, 22–24). The fact that the addition of Cu^{2+} or the removal of the trichloro group (i.e., 6-chloro-2-picoline) did not prevent the inhibition of methane oxidation indicates that the mode of action of nitrapyrin on methanotrophs is probably different from that on chemoautotrophic ammonium oxidizers or methanogens. This is perhaps not surprising as it is unlikely that there would be a common target of inhibition found in bacteria as diverse as methanogens, methanotrophs, chemoautotrophic ammonium oxidizers, sulfate reducers, and denitrifiers, but not in a large number of other bacteria, actinomycetes, and fungi surveyed and found to be insensitive to nitrapyrin (8, 15, 20).

Since removal of the trichloro group (6-chloro-2-picoline) did not affect the inhibition of methane oxidation, it would be interesting to test its effect on *Nitrosomonas* sp.

The reversal of the inhibition upon removal of 10 μg of nitrapyrin ml^{-1} indicates that it is bacteriostatic rather than bactericidal. Nitrapyrin was also bacteriostatic for a soil nitrifying population (18) and for pure cultures of *Nitrosomonas europaea* (20).

TABLE 3. Oxidation of CH_4 by *Methylosinus trichosporium* OB3b in the presence or absence of nitrapyrin and 6-chloro-2-picoline[a]

Treatment	CH_4 oxidation (μmol mg of protein^{-1} h^{-1})	% Inhibition
None	8.95	
Nitrapyrin	0.22	97.5
6-chloro-2-picoline	0.00	100

[a] Final concentrations of nitrapyrin and 6-chloro-2-picoline were 43 μM. Final concentration of DMSO was 400 μl liter^{-1}. Final protein concentration was 320 μg ml^{-1}.

Since nitrapyrin inhibits oxidation of NH_4^+ by *Methylosinus trichosporium* OB3b in pure culture, it would presumably inhibit methanotrophic NH_4^+ oxidation in situ. The association of CH_4 oxidation activity with an NO_2^- maximum in a stratified lake (9) and the oxidation of NH_4^+ by methanotrophs in vitro (13, 26) suggest that methanotrophs could contribute to in situ nitrification activities. Large methanotrophic populations in stratified metalimnia or aerobic sediments could possibly oxidize NH_4^+ after the complete consumption of CH_4 or simultaneously with CH_4 oxidation if the NH_4^+ concentrations are high and the CH_4 concentrations are low. Our present data indicate that nitrapyrin unfortunately cannot be used as a differential inhibitor to distinguish between chemoautotrophic and methanotrophic nitrification.

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