Nitrosomonas europaea ATCC25978は硝化抑制剤の検定に適切なアンモニア酸化細菌である

誌名	Journal of pesticide science
ISSN	1348589X
著者名	岡野,留衣子 高崎,宏寿 松葉,道知 徳山,龍明 佐藤,幸治 若林,攻
発行元	日本農薬学会
巻/号	29巻1号
掲載ページ	p. 50-52
発行年月	2004年2月

J. Pestic. Sci. **29**, 50–52 (2004)

Note

Nitrosomonas europaea ATCC25978 Is the Right Ammonia-Oxidizing Bacterium for Screening Nitrification Inhibitors

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(Received September 29, 2003; Accepted November 27, 2003)

To determine whether *Nitrosomonas europaea* is adequate to assay nitrification inhibitors as representative of ammonia-oxidizing bacteria or not, the inhibitory activity exhibited by known nitrification inhibitors in soil and the susceptibility of recently isolated ammonia-oxidizing bacteria to the inhibitors in cell suspension were compared. Nitrapyrin, MAST and Br-MAST completely inhibited nitrification in soil at 15 ppm for 15 days, whereas dicyanodiamide and thiourea were weak inhibitors. In order of effectiveness, the inhibitiors ranked as follows; Br-MAST > MAST > nitrapyrin ≫ dicyanodiamide ≥ thiourea. Ammonia-oxidation by ammonia-oxidizing bacteria in cell suspension was strongly inhibited by nitrapyrin, MAST and Br-MAST, whereas dicyanodiamide and thiourea were weak inhibitors. *N. europaea* is adequate to assay nitrification inhibitors as representative of ammonia-oxidizing bacteria.

Keywords: ammonia-oxidizing bacteria, susceptibility to nitrification inhibitors, nitrapyrin.

INTRODUCTION

Nitrification inhibitors such as 2-chloro-6-(trichloromethyl)-pyridine (nitrapyrin) and 2-amino-4-methyl-6-trichloromethyl-1,3,5-triazine (MAST), inhibit ammonia nitrification to nitrate, being expected maintenance of soil fertility, prevention of NO_x^- (x = 2 or 3) pollution in ground- and surface-water, and suppression of the stratospheric ozone depletion gas (N_2O) in the atmosphere.[1] In the nitrification process, NH_4^+–N → NH_2OH → NO_2^-–N → NO_3^-–N, in upland soil, inhibitors have been confirmed to interfere with the formation of NH_2OH from ammonia, possibly inhibiting the ammonia monooxygenase activity of ammonia-oxidizing bacteria.[2-5] These results have been obtained from studies using cells and cell-free extracts of a readily available ammonia-oxidizing bacterium, *Nitrosomonas europaea* ATCC25978, which has been used conveniently for the screening of new nitrification inhibitors at the cellular level.[3,5,6] However, several ammonia-oxidizing bacteria have recently been isolated from wastewater as well as soils and sludge, and their susceptibility to inhibitors is being assayed.[7] This study examined whether *N. europaea* is adequate to assay nitrification inhibitors, as representative of ammonia-oxidizing bacteria or not.

MATERIALS AND METHODS

1. *Chemicals*

2-Chloro-6-(trichloromethyl)pyridine (nitrapyrin) was kindly provided by Dow Elanco Japan Ltd. New 3-cyano-2-methyl-6-trifluoromethylpyridine (CMTP) was prepared by cyclization of 6-amino-5-cyano-1,1,1-trifluoro-3,5-heptadien-2-one in the presence of ammonium acetate, according to the method used to synthesize 2-trihalomethyl-5-cyanopyridines.[8] Physical properties are given in the footnote of Table 2. 2-Amino-4-methyl-6-trichloromethyl-1,3,5-triazine (MAST) and 2-amino-4-methyl-6-tribromomethyl-1,3,5-triazine (Br-MAST) were prepared by amination reaction of 2-methyl-4,6-bis(trichloromethyl)-1,3,5-triazine and bromination of MAST in glacial acetic acid, respectively, according to Murakami *et al.*[2] and Ohki *et al.*[6] Dicyanodiamide and thiourea were purchased from Wako Pure Chemical Industries Ltd., Tokyo. Analytical grade chemicals for the culture of ammonia-oxidizing bacteria and other chemicals were purchased from Kanto Chemical Co. Inc., Tokyo, and Dojindo Laboratories, Kumamoto, Japan.

2. *Ammonia-Oxidizing Bacteria Used in This Paper*

Three strains of *Nitrosomonas* (*N. europaea* ATCC25978, *N.* sp. TK794 and *N.* sp. B2), *Nitrosovibrio* sp. TYM9 and *Nitrosospira* sp. GS833 were used as representatives of ammonia-oxidizing bacteria isolated mostly from soils for susceptibility assays of nitrification inhibitors. *N. europaea* ATCC25978 was purchased from American Type Culture Collection, but the other ammonia-oxidizing bacteria were isolated by our group (Depository of strains: T. Tokuyama, Department of Agriculture and Biological Chemistry, Nihon University, Fujisawa-shi,

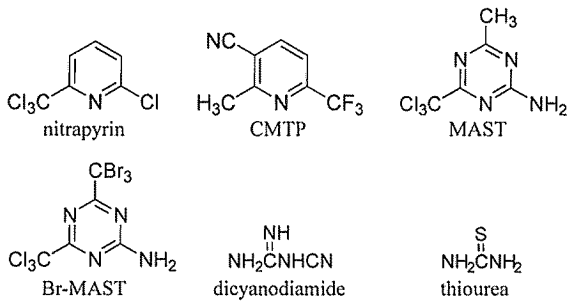

Fig. 1. Structures of nitrification inhibitors used in this study.

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Japan. 16S rRNA gene sequences: see NCBI, 2003[9]). All strains were incubated in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) medium before the inhibitory assay, according to Tomiyama *et al.*[10] The cells were centrifuged and washed twice with 0.02 M phosphate buffer at pH 8.0. A cell suspension was prepared with phosphate buffer containing ammonium sulfate (100 μg/ml) and adjusted to produce nitrite at a rate of 1 μg/ml/30 min.

3. Evaluation of Nitrification-Inhibitory Activity in Soil

Celite *545* and each inhibitor were mixed to prepare a 120 ppm stock soil treatment formulation. The soil used was upland soil from a field of Tamagawa University.[2,4] In each wide mouthed culture flask, the soil (25 g), urea (16.5 mg) and an appropriate amount of the inhibitor's formulation were mixed well. After adjusting the soil moisture content to 50% of the field capacity and pH to 6.8 using calcium carbonate, the flasks were covered with iron caps having 5 holes (ϕ: 2 mm) and incubated in the dark at 28℃ for 7 and 15 days. Then, 100 ml of distilled water was added to the flasks, which were subsequently shaken for 1 hr. The amount of nitrate (NO_3^--N) in the supernatant was determined with a nitrate ion-meter (Horiba Compact Ion-Meter C-141). Nitrification rates in the presence of inhibitors were calculated relative to the nitrification of urea applied in the inhibitor-free experiment, taking into account NO_3^--N formation in the blank. The nitrification rate of urea was 47.5% and 100% after 7 and 15 days, respectively.

4. Evaluation of Nitrification-Inhibitory Activity in Cell Suspension

The susceptibility of 5 strains of ammonia-oxidizing bacteria to nitrification inhibitors was examined using five nitrification inhibitors (Fig. 1). The inhibitors were dissolved in acetone and the final concentration of the solvent in the medium was kept below 0.1% (v/v). Ammonium sulfate in 0.02 M phosphate buffer (pH 8.0) was used as substrate, according to Takagi *et al.*[3] and Murakami *et al.*[4] After 30 min incubation at 37°C with the inhibitors, the amount of nitrite formed in the culture medium was determined by measuring optical density at 532 nm according to the Griess-Ilosvay method.[11] The molar concentration of the inhibitor, which shows 50% inhibition (molar I_{50}) against the nitrite formation by ammonia-oxidizing bacteria relative to the control, was calculated by probit analysis. The nitrification inhibition indices are expressed by pI_{50}, the negative logarithm of the molar I_{50}-concentration. The susceptibility of ammonia-oxidizing bacteria to the inhibitors is discussed using pI_{50}-values in this paper. For reference, the effect of the nitrification inhibitors on nitrite oxidation by *Nitrobacter agilis* ATCC14123 was also assayed according to Takagi *et al.*[3]

RESULTS AND DISCUSSION

1. Nitrification Inhibition by the Selected Inhibitors

The effect of five selected inhibitors, nitrapyrin, MAST, Br-MAST, dicyanodiamide and thiourea, on ammonia nitrification in soil was assayed using upland soil from the fields of Tamagawa University, the properties of which have already been described.[2] As shown in Table 1, dicyanodiamide and thiourea inhibited about 50% of nitrification at 30 ppm (3.6×10^{-4} and 4.0×10^{-4} mol/kg, respectively) after 15 days incubation. Nitrapyrin, MAST and Br-MAST completely controlled nitrification at 7.5 ppm (3.2×10^{-5}, 3.3×10^{-5} and 2.1×10^{-5} mol/kg, respectively) after 7 days incubation. Since no or little accumulation of NH_2OH and NO_2^--N was detected during incubation in the presence of inhibitors (data not cited), the inhibitors are considered to affect the oxidation of NH_4^+-N to NH_2OH by ammonia-oxidizing bacteria in soil. However, we have little information on the ammonia-oxidizing bacteria in the soil used.

To confirm the work of ammonia-oxidizing bacteria and reliability of using *N. europaea* ATCC25978 to screen nitrification inhibitors, the susceptibility of ammonia-oxidizing bacteria isolated already from soils to inhibitors was examined in cell suspensions.

2. Susceptibility of Ammonia-Oxidizing Bacteria to Nitrification Inhibitors

The susceptibility of five ammonia-oxidizing bacteria isolated from soils and wastewater, *N. europaea* ATCC25978 (1), *Nitrosomonas* sp. TK794 (2), *Nitrosomonas* sp. B2 (3), *Nitrosovibrio* sp. TYM 9 (4) and *Nitrosospira* sp. GS833 (5), to five nitrification inhibitors, nitrapyrin, MAST, Br-MAST,

Table 1. Effect of typical nitrification inhibitors on nitrate formation in soil

Inhibitor	Nitrification rate (%)[a,b]					
	7.5 ppm		15 ppm		30 ppm	
	7 days	15 days	7 days	15 days	7 days	15 days
Nitrapyrin	0	39.4 (±1.6)	0	0	0	0
MAST	0	14.2 (±2.2)	0	0	0	0
Br-MAST	0	3.8 (±2.3)	0	0	0	0
Dicyanodiamide	—[c]	—[c]	—[c]	—[c]	0	46.4 (±2.7)
Thiourea	—[c]	—[c]	—[c]	—[c]	10.3 (±1.9)	53.8 (±2.3)

[a] Percent nitrification was calculated relative to the nitrification of urea applied in the absence of inhibitors, based upon NO_3^--N (0.945 mg/100 g soil) in the blank soil. [b] Urea as the source of ammonia was mineralized extremely fast, and was completely converted to NH_4^+-N within 2 days irrespective of the presence or the absence of inhibitors. [c] Not tested.

Table 2. Susceptibility of inhibitors to nitrifying bacteria

	Nitrifying bacteria[a]	Habitat	pI_{50}-values for inhibitors					
			Nitrapyrin	CMTP[b]	MAST	Br-MAST	Dicyanodiamide	Thiourea
1	*Nitrosomonas europaea* ATCC25978 (AF353160)	upland soil	5.72 (±0.16)	4.93 (±0.24)	5.31 (±0.28)	7.06 (±0.16)	4.32 (±0.23)	4.03 (±0.23)
2	*Nitrosomonas* sp. TK794 (AF080185)	upland soil	5.54 (±0.25)	—[c]	4.96 (±0.24)	—[c]	—[c]	—[c]
3	*Nitrosomonas* sp. B2 (AB093545)	wastewater	6.14 (±0.18)	—[c]	5.47 (±0.12)	7.01 (±0.18)	<4.00	<4.00
4	*Nitrosovibrio* sp. TYM9 (AF080256)	woodland soil	5.49 (±0.04)	4.07 (±0.24)	5.39 (±0.13)	6.25 (±0.14)	4.20 (±0.29)	<4.00
5	*Nitrosospira* sp. GS833 (AF353162)	upland soil	5.86 (±0.15)	4.27 (±0.25)	5.77 (±0.12)	6.38 (±0.11)	4.07 (±0.23)	<4.00
6	*Nitrobacter agilis* ATCC14123 (AY055796)	upland soil	<3.00	—[c]	<3.00	—[c]	—[c]	—[c]

[a] 1–5 are ammonia-oxidizing bacteria, 6 is nitrite-oxidizing bacterium. [b] Mp: 61–62℃ ; ^{1}H NMR δ($CDCl_3$): 2.87 (3H, s, CH_3), 7.64 (1H, d, J=8.1 Hz, H-4 or H-5 of pyridine ring), 8.10 (1H, d, J=8.1 Hz, H-4 or H-5 of pyridine ring); MS m/z: 186 (M^+). [c] Not tested.

dicyanodiamide and thiourea, was assayed in cell cultures. Results are shown in Table 2. The oxidation of NH_4^+–N by the five bacteria was strongly inhibited by nitrapyrin, MAST and Br-MAST, indicating pI_{50}-values of 5.49–6.14, 4.96–5.77 and 6.25–7.06 (cf. *N. europaea*: 5.72, 5.31 and 7.06), respectively; whereas dicyanodiamide and thiourea were weak inhibitors ($pI_{50} \leq 4.32$). A new experimental pyridine (CMTP) exhibited an intermediate level of inhibition, with a pI_{50} of 4.07–4.93. For reference, the effect of nitrapyrin and MAST on a nitrite-oxidizing bacterium, *Nitrobacter agilis* ATCC14123, was also checked.[3,7] The two inhibitors were too insensitive to the *Nitrobacter* to prevent oxidation of NO_2^-–N in the cells. Thus, all ammonia-oxidizing bacteria isolated from soils and wastewater to date are confirmed to be susceptible to nitrification inhibitors.

Candidates for nitrification inhibitors are now usually screened through ammonia nitrification in cells of *N. europaea* ATCC25978 as well as in soil. From the results presented in this paper, it is now found that *N. europaea* is adequate to assay nitrification inhibitors, as representative of ammonia-oxidizing bacteria.

ACKNOWLEDGMENTS

The authors express their thanks to Dr. H. Ogawa and Dr. S. Ohki, Tamagawa University, and Dr. Vonk, EPP Consultancy, for helpful suggestion and discussion. They are grateful to Dr. T. Kawasaki, AGRO-KANESHO Co. Ltd., for synthesis of compounds assayed.

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