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Distribution of Aldoxime Dehydratase in Microorganisms

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The distribution of phenylacetaldoxime-degrading and pyridine-3-aldoxime-degrading ability was examined with intact cells of 975 microorganisms, including 45 genera of bacteria, 11 genera of actinomyces, 22 genera of yeasts, and 37 genera of fungi, by monitoring the decrease of the aldoximes by high-pressure liquid chromatography. The abilities were found to be widely distributed in bacteria, actinomyces, fungi, and some yeasts: 98 and 107 strains degraded phenylacetaldoxime and pyridine-3-aldoxime, respectively. All of the active strains exhibited not only the aldoxime-dehydration activity to form nitrile but also nitrile-hydrolyzing activity. On the other hand, all of 19 nitrile-degrading microorganisms (13 species, 7 genera) were found to exhibit aldoxime dehydration activity. It is shown that aldoxime dehydratase and nitrile-hydrolyzing activities are widely distributed among 188 aldoxime and 19 nitrile degraders and that the enzymes were induced by aldoximes or nitriles.

Nitrile compounds are discharged into the environment as industrial waste water, agricultural chemicals, etc. (23). Many microorganisms can use nitriles as a source of carbon and/or nitrogen for growth. Asano et al. have isolated various nitrile-degrading microorganisms from soil (2, 5, 24) and clarified that nitriles are converted to carboxylic acids either by a combination of nitrile hydratase (1, 3, 6) and amidase (4) or by nitrilase (8). These enzymes have been extensively evaluated from the viewpoint of chemical industry: the industrial production of acrylamide, nicotinamide, and 5-cyanovalelamide are typical examples (5, 6, 16, 26). Despite the importance of the nitrile-hydrolyzing enzymes in industry, information about their distribution in microorganisms is quite limited, and their physiological role has never been well understood since they have been screened only from nitrile-degrading microorganisms.

We have been studying aldoxime-degrading enzymes and isolated various aldoxime-degrading microorganisms, e.g., *Bacillus* sp. strain OxB-1 (7) and *Rhodococcus* sp. strain YH3-3 (12), from soil. The isolated strains metabolized aldoximes through nitriles into the corresponding carboxylic acid by a combination of a novel aldoxime dehydratase and nitrile-hydrolyzing enzymes (7, 12) (Fig. 1). The novel aldoxime dehydratase was purified and characterized from *Bacillus* sp. strain OxB-1 (7, 15). The enzyme from *Rhodococcus* sp. strain YH3-3 was applied to the enzymatic synthesis of nitriles from aldoximes under a mild condition (12, 13). A nitrile hydratase responsible for aldoxime metabolism was purified and characterized from *Rhodococcus* sp. strain YH3-3, and its properties were compared with the known nitrile hydratases (14).

To elucidate the generality of the relationship of aldoxime dehydratase and nitrile-hydrolyzing enzymes in microorganisms, we examined the distribution of both enzyme activities with the intact cells of a variety of aldoxime- or nitrile-degrading microorganisms, monitoring the decrease of the aldoximes, and we studied the relationship among the enzymes.

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MATERIALS AND METHODS

Chemicals. Meat and malt extracts were obtained from Kyokuto (Tokyo, Japan). Polypepton and yeast extract were purchased from Nippon Seiyaku (Tokyo, Japan). High-pressure liquid chromatography (HPLC) columns ODS-80Ts (4.6 by 150 mm) and Hibar LiChrosorb-NH_2 (4.0 by 250 mm) were from Tosoh Corp. (Tokyo, Japan) and Kanto Chemicals (Tokyo, Japan), respectively. Aldoximes were synthesized as described previously (7, 12). All other chemicals were from commercial sources and used without further purification.

Microorganisms and culture media. A total of 975 strains from the following type culture collections were used: the Institute of Molecular and Cellular Biosciences (IAM), University of Tokyo, Tokyo, Japan; the Institute of Fermentation (IFO), Osaka, Japan; the Japan Collection of Microorganisms (JCM), Tokyo, Japan; the National Collections of Industrial Food and Marine Bacteria (NCIMB), Aberdeen, Scotland; the National Collections of Type Cultures and Pathogenic Fungi (NCTC) London, United Kingdom; the American Type Culture Collection (ATCC); and our own laboratory (TPU). These included 407 strains of 45 genera of bacteria, 133 strains of 11 genera of actinomyces, 333 strains of 22 genera of yeasts, and 102 strains of 37 genera of fungi.

The culture medium for the bacteria was composed of 1.0% of meat extract, 1.0% of Polypepton, and 0.5% NaCl (pH 7.2). The medium for actinomyces contained 1.0% malt extract, 0.4% yeast extract, and 0.4% D-glucose (pH 7.2). The medium for yeasts consisted of 1.0% D-glucose, 0.5% Polypepton, 0.3% yeast extract, and 0.3% malt extract (pH 5.6). The medium for fungi contained 20 g of sucrose and boiled extract from 200 g of potato per liter (pH 6.0). Tap water was used for the media described above, and the pH was adjusted by using HCl and NaOH. Each microorganism was inoculated into a test tube containing 10 ml of the medium with 0.05% concentrations of the inducers, i.e., phenylacetaldoxime (PAOx), pyridine-3-aldoxime (PyOx), phenylacetonitrile (PAN), and 3-cyanopyridine (CyPy), and then incubated with shaking for 2 to 14 days at 30°C until their growth reached maximum levels. For facultative anaerobic bacteria, the strains were also grown under static conditions.

Enzyme assay and definition of units. Microbial degradation of aldoxime was qualitatively analyzed by thin-layer chromatography (TLC). After the strain was cultured in the medium containing PAOx or PyOx, 1 μl of the culture was spotted onto silica gel TLC plates (Kieselgel 60; Merck) and then developed with 20% (vol/vol) ethyl acetate in hexane. The remaining aldoxime in the medium was visualized with iodine vapor.

Aldoxime dehydration and nitrile-hydrolyzing activities were quantitatively assayed by measuring the rate of consumption of aldoxime and nitrile, respectively. A standard assay solution contained 50 μmol of potassium phosphate buffer (pH 7.0), 25 μmol of substrate, and washed cells from 5 ml of culture in a total volume of 500 μl. After an incubation at 30°C with shaking, the reaction mixture was centrifuged (18,000 × *g*, 2 min) at 4°C to remove the cells. The supernatant was analyzed with a Waters 600E HPLC apparatus equipped with a Waters 486 absorbance detector (254 nm) at a flow rate of 1.0 ml/min. PAOx, PAN, phenylacetamide (PAAm), and phenylacetic acid (PAA) were detected with an ODS-80Ts column using an elution solvent consisting of 10 mM H_3PO_4 in 40% (vol/vol) CH_3CN, and PyOx and 3-CyPy were measured with the same column using 10 mM H_3PO_4 in 10% (vol/vol) CH_3CN. Nicotinamide (NAm) and nicotinic acid (NA) were detected with Hibar LiChrosorb-NH_2 column with 2.5 mM potassium phosphate buffer (pH 2.8) in 75% (vol/vol) CH_3CN. Single units of aldoxime dehydration and nitrile-hydrolyzing activities were defined as the amount of enzyme that catalyzed the dehydration of aldoxime and hydrolysis of

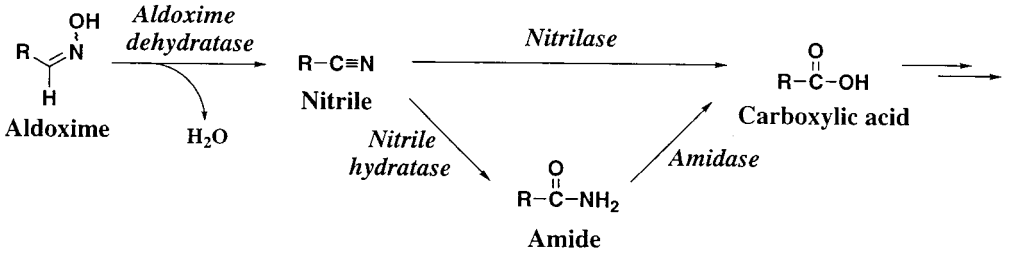

FIG. 1. Microbial metabolism of aldoximes. *Bacillus* sp. strain OxB-1 metabolizes PAOx (R = $PhCH_2$) to form PAN, which is successively hydrolyzed to PAA by the action of nitrilase (7). On the other hand, *Rhodococcus* sp. strain YH3-3 metabolizes PyOx (R = 3-pyridyl) as follows: the aldoxime is dehydrated to form CyPy, which is converted to NAm by nitrile hydratase, and the NAm is successively hydrolyzed to NA by amidase (12).

nitrile, respectively, at a rate of 1 μmol/min. Reaction mixtures with the cells but without substrates served as blanks for both the assays. Under the assay conditions, the measurable detection limit of the enzyme activity was about 0.01 (in units/liter of culture).

RESULTS

Distribution of PAOx and PyOx dehydration activity in bacteria and actinomyces. We selected PAOx and PyOx as a model compound of alkylaldoxime and arylaldoxime, respectively. Bacterial and actinomycetal strains in our stock cultures were aerobically grown in the medium containing PAOx or PyOx, and the ability to degrade the aldoximes was screened by qualitative measurement of aldoxime disappearance in the culture medium by using TLC assay. Among the 540 strains tested, 37 strains were able to degrade PAOx, and this activity was distributed across 7 genera, while 96 strains, distributed across 30 genera, could degrade PyOx. Although some aerobically grown facultative anaerobes could degrade PyOx, none of the statically grown cells could degrade PAOx nor PyOx. We measured not only the aldoxime dehydration activities but also the PAN- and CyPy-hydrolyzing activities in the cells of PAOx- or PyOx-degrading strains. Since all of the aldoxime-degrading strains contained both aldoxime dehydratase and nitrile-hydrolyzing enzymes, nitriles formed by the dehydration of aldoximes were subsequently converted to amides and/or acids by the action of the latter enzymes. We therefore analyzed the dehydration activity by measuring the consumption of aldoxime in the assay. Nitrile-hydrolyzing activity was estimated by measuring the rate of consumption of nitrile, which was equal to the sum of nitrile hydratase and nitrilase activities. As shown in Tables 1 and 2, all of the strains grown with PAOx or PyOx exhibited the aldoxime dehydration activities, together with nitrile-hydrolyzing enzyme activities. The 21 strains marked with a number symbol (#) exhibited both PAOx and PyOx dehydration activities.

By measuring the formation of PAAm and NAm in the reaction mixture, we determined the mode of nitrile degradation by the active strains. Totals of 10 and 21 strains, marked with an asterisk (*) in Tables 1 and 2, respectively, converted nitrile to acid-accumulating amide, indicating that the strains hydrolyzed the nitriles by a sequential action of nitrile hydratase and amidase. These results do not exclude the possibility of a coexistence of nitrilase in the strains. The other strains degraded PAN and CyPy without forming the corresponding amides. In these strains, the nitriles are hydrolyzed by nitrilase and/or the combination of nitrile hydratase and amidase in which amidase activity was stronger than that of nitrile hydratase.

Distribution of aldoxime dehydration activity in fungi. We next screened for fungal strains which degrade PAOx or PyOx. PAOx-degrading activity was widely distributed in various gen-

TABLE 1. PAOx dehydration and PAN-hydrolyzing activities in bacteria and actinomyces grown with PAOx[a]

Strain[b]	Enzyme activity (U/liter of culture)	
	PAOx dehydration	PAN hydrolysis
Rhodococcus sp. strain TPU 3458*#	5.13	0.52
Aureobacterium testaceum IAM 1561#	4.55	15
Rhodococcus erythropolis IAM 1463#	3.68	12.3
Nocardia asteroides IFO 3423*#	3.55	0.2
Rhodococcus erythropolis TPU 3201*	3.43	7.41
Nocardia asteroides TPU 3036*#	3.35	0.31
Rhodococcus sp. strain TPU 3455*#	2.24	0.37
Rhodococcus erythropolis IAM 1400*#	1.61	4.35
Rhodococcus erythropolis IAM 1428*#	1.49	9.2
Kocuria varinus IAM 12146	1.45	2.32
Rhodococcus erythropolis IAM 1440*#	1.39	1.03
Bacillus subtilis ATCC 21697	1.22	4.56
Pseudomonas sp. strain TPU 7162*	0.52	2.03
Rhodococcus sp. YH3-3 TPU 3453[c]*#	22.4	288
Bacillus sp. OxB-1 TPU 5563[d]	12.2	2.59

[a] The strains marked with an asterisk (*) hydrolyzed PAN accumulating PAAm. The strains marked with a number sign (#) degraded not only PAOx but also PyOx. All the active strains did not exhibit PAOx dehydration activity when they were grown without PAOx. The group of inactive bacteria and actinomyces for PAOx degradation in the TLC assay included 4 species of *Achromobacter*, 4 species of *Acinetobacter*, 1 species of *Actinomyces*, 1 species of *Aerobacter*, 2 species of *Aeromonas*, 2 species of *Agrobacterium*, 7 species of *Alcaligenes*, 1 species of *Amycolatpsis*, 18 species of *Arthrobacter*, 2 species of *Azotobacter*, 12 species of *Bacillus*, 11 species of *Brevibacterium*, 1 species of *Comamonas*, 3 species of *Cedecea*, 2 species of *Cellulomonas*, 3 species of *Chromobacterium*, 4 species of *Citrobacter*, 9 species of *Corynebacterium*, 1 species of *Curtobacterium*, 2 species of *Edwardsiella*, 5 species of *Enterbacter*, 1 species of *Erwinia*, 7 species of *Escherichia*, 3 species of *Flavobacterium*, 1 species of *Gordona*, 1 species of *Hafinia*, 5 species of *Klebsiella*, 2 species of *Kluyvera*, 4 species of *Methylobacterium*, 2 species of *Microbacterium*, 7 species of *Micrococcus*, 1 species of *Moraxella*, 1 species of *Morganella*, 6 species of *Mycobacterium*, 18 species of *Nocardia*, 1 species of *Nocardioides*, 1 species of *Ochrobactrum*, 2 species of *Pimelobacter*, 4 species of *Proteus*, 1 species of *Pantoea*, 3 species of *Providencia*, 22 species of *Pseudomonas*, 1 species of *Pseudonocardia*, 5 species of *Rhodococcus*, 1 species of *Rothia*, 4 species of *Salmonella*, 7 species of *Serratia*, 1 species of *Sprosarcina*, 1 species of *Staphylococcus*, 16 species of *Streptomyces*, 1 species of *Streptoverticillium*, 1 species of *Variovorax*, 3 species of *Xanthomonas*, 3 species of *Yersinia*, and unidentified coryneform rods.

[b] Low PAOx dehydration activity (~0.03 U/liter of culture) was seen in the 4 strains of *Nocardia asteroides* (IFO 3384*, TPU 3035*#, TPU 3037*#, and TPU 3038*), the 16 strains of *Rhodococcus erythropolis* (IAM 1399*#, IAM 1414*#, IAM 1452*#, IAM 1474*, IAM 1484*#, IAM 1494*, IAM 12122*, IFO 12320*#, IFO 12538, IFO 12540*, IFO 12541#, JCM 3132*, JCM 3201*, TPU 3206*, TPU 3218*, and TPU 3219*), and 4 strains of *Rhodococcus* sp. (TPU 3454*, TPU 3456*, TPU 3457*#, and TPU 3463*#) with PAN-hydrolyzing activities.

[c] Isolated as PyOx degrader from soil (12).

[d] Isolated as PAOx degrader from soil (7).

TABLE 2. PyOx dehydration and CyPy-hydrolyzing activities in bacteria and actinomyces grown with PyOx[a]

Strain[b]	Enzyme activity (U/liter of culture)	
	PyOx dehydration	CyPy hydrolysis
Rhodococcus erythropolis IAM 1484	1.00	37.0
Cellulomonas fimi IAM 12107*	0.72	3.50
Aureobacterium testaceum IAM 1561#	0.65	1.23
Xanthomonas flavus NCIMB 10071*	0.62	0.92
Flavobacterium aquatile IFO 3772*	0.51	0.03
Micrococcus ureae TPU 6656	0.50	0.33
Rhodococcus rhodochrous NCIMB 11273*	0.47	0.54
Brevibacterium butanicum TPU 5710*	0.45	1.3
Stenotrophomonas maltophilia IFO 12020*	0.45	1.00
Bacillus coagulans IFO 3887	0.44	0.14
Nocardia asteroides IFO 3423#	0.42	0.33
Serratia marcescens IAM 12143*	0.38	0.13
Rhodococcus rhodochrous IAM 12121	0.38	0.67
Bacillus megaterium IAM 13418*	0.33	0.24
Rhodococcus erythropolis IAM 1399*#	0.31	0.61
Arthrobacter crystallopoietes IFO 14235	0.30	0.04
Pseudomonas fluorescens TPU 7128	0.29	0.23
Corynebacterium rathayi IFO 12161*	0.22	0.56
Corynebacterium paurometabolum IFO 12160*	0.20	0.04
Proteus vulgaris IAM 12542	0.18	0.04
Flavobacterium suaveolens IFO 3752*	0.16	0.05
Flavobacterium rigense IAM 1238	0.12	0.14
Alcaligenes faecalis IAM 12563*	0.09	0.35
Micrococcus luteus IAM 1097	0.08	0.07
Flavobacterium lutescens IFO 3751	0.07	0.16
Bacillus subtilis ATCC 21697	0.06	0.06
Arthrobacter ramosus IFO 12958	0.05	0.19
Klebsiella pneumoniae IFO 3318	0.04	0.35
Agrobacterium radiobacter IAM 13256	0.04	0.19
Rhodococcus sp. strain YH3-3 TPU 3453[c]*#	2.83	144

[a] The strains marked with an asterisk (*) hydrolyzed CyPy accumulating NAm. The strains marked with a number sign (#) degraded not only PyOx but also PAOx. All the active strains did not exhibit PyOx dehydration activity when they were grown without PyOx. The group of inactive bacteria and actinomyces for PyOx degradation in the TLC assay included 3 species of *Achromobacter*, 3 species of *Acinetobacter*, 1 species of *Actinomyces*, 2 species of *Aeromonas*, 2 species of *Agrobacterium*, 6 species of *Alcaligenes*, 1 species of *Amycolatpsis*, 9 species of *Arthrobacter*, 2 species of *Azotobacter*, 10 species of *Bacillus*, 10 species of *Brevibacterium*, 1 species of *Comamonas*, 3 species of *Cedecea*, 1 species of *Cellulomonas*, 3 species of *Chromobacterium*, 4 species of *Citrobacter*, 5 species of *Corynebacterium*, 1 species of *Curtobacterium*, 1 species of *Edwardsiella*, 4 species of *Enterbacter*, 6 species of *Escherichia*, 1 species of *Gordona*, 3 species of *Klebsiella*, 1 species of *Kocuria*, 4 species of *Methylobacterium*, 2 species of *Microbacterium*, 4 species of *Micrococcus*, 1 species of *Moraxella*, 1 species of *Morganella*, 5 species of *Mycobacterium*, 18 species of *Nocardia*, 1 species of *Nocardioides*, 1 species of *Ochrobactrum*, 1 species of *Pimelobacter*, 2 species of *Proteus*, 1 species of *Pantoea*, 3 species of *Providencia*, 17 species of *Pseudomonas*, 1 species of *Pseudonocardia*, 4 species of *Rhodococcus*, 1 species of *Rothia*, 3 species of *Salmonella*, 5 species of *Serratia*, 1 species of *Sprosarcina*, 16 species of *Streptomyces*, 1 species of *Streptoverticillium*, 1 species of *Variovorax*, 2 species of *Xanthomonas*, 3 species of *Yersinia*, and unidentified coryneform rods.

[b] Low PyOx dehydration activity (~0.03 U/liter of culture) was seen in *Achromobacter cycloclastes* IAM 1013, *Agrobacterium radiobacter* IAM 1527*, 3 strains of *Alcaligenes faecalis* (IFO 13111*, IAM 12369*, and IAM 12562*), *Arthrobacter aurescens* IFO 12340, *Arthrobacter citreus* IFO 12957*, *Arthrobacter pascens* IAM 12346, *Arthrobacter polychromogenes* JCM 2523, 2 strains of *Arthrobacter sulfureus* (IFO 12678* and JCM 1338), *Arthrobacter ramosus* JCM 1334, *Arthrobacter* sp. strain TPU 5407, *Arthrobacter variabillis* JCM 2154, 2 strains of *Bacillus cereus* (IFO 3001* and IAM 1226*), *Corynebacterium aquaticum* IFO 12154*, *Corynebacterium rathayi* IFO 12161*, *Edwardsiella tarda* JCM 1656*, *Enterobacter aerogenes* IFO 13534*, 2 strains of *Erwinia carotovora* (IFO 3380* and IFO 12380*), *Escherichia blattae* ATCC 33430*, *Hafinia alvei* IFO 3731*, *Klebsiella planticola* IFO 14939*, *Kluyvera ascorbata* JCM 1681*, *Kluyvera cryocrescens* JCM 1682*, *Methylobacillus glycogenes* JCM 2850*, *Micrococcus roseus* IFO 3768*, *Mycobacterium phlei* IFO 13160, 4 strains of *Nocardia asteroides* (TPU 3001, TPU 3035*#, TPU 3036*#, and TPU 3037*#), *Pimelobacter simplex* IFO 12679*, *Proteus mirabilis* IFO 13300*, 3 strains of *Proteus vulgaris* (IFO 3851*, IFO 3988*, and IAM 1025), *Pseudomonas diminuta* IAM 1513*, *Pseudomonas fluorescens* TPU 7171*, *Pseudomonas putida* IFO 12996, 2 strains of *Pseudomonas syncyanea* (IFO 3757* and IFO 3906*), 2 strains of *Pseudomonas taetrolens* (IFO 3460* and IFO 12691*), 11 strains of *Rhodococcus erythropolis* (IAM 1400#, IAM 1414#, IAM 1428#, IAM 1440#, IAM 1452#, IAM 1463*#, IAM 1484*#, IFO 12320*#, IFO 12541*#, JCM 3201#, and TPU 3209), 6 strains of *Rhodococcus* sp. (TPU 3451, TPU 3452, TPU 3455*#, TPU 3457, TPU 3458*#, and TPU 3463#), *Salmonella choleraesuis* JCM 8721*, *Serratia plymuthica* IFO 3055, *Serratia marcescens* IFO 3054*, and *Staphylococcus aureus* IFO 3060* with CyPy-hydrolyzing activities.

[c] Isolated as PyOx degrader from soil (12).

era. Of the 102 strains, 52 among 28 genera were able to degrade PAOx, whereas PyOx-degrading activity was seen in only a limited number of fungal strains; it was seen in 11 strains among 3 genera. Tables 3 and 4 show both of the enzyme activities in all of the active strains. Some active fungal strains degraded PAN accumulating PAAm, suggesting that the strains hydrolyzed PAN by the combinatorial action of nitrile hydratase and amidase, whereas PyOx-degrading fungi converted CyPy into NA without forming NAm.

Distribution of aldoxime dehydration activity in yeasts. Aldoxime degradation activity in yeasts is rare. We screened 333 strains, including 2 species of *Brettanomyces*, 29 species of *Candida*, 3 species of *Cryptococcus*, 3 species of *Debaryomyces*, 1 species of *Endomycopsis*, 1 species of *Hanseniaspora*, 24 species of *Hanenula*, 3 species of *Kloeckera*, 1 species of *Lipomyces*, 19 species of *Pichia*, 3 species of *Rhodosporidium*, 10 species of *Rhodotorula*, 32 species of *Saccharomyces*, 1 species of *Saccharomycodes*, 1 species of *Saccharomycopsis*, 3 species of

TABLE 3. PAOx dehydration and PAN-hydrolyzing activities in fungi grown with PAOx[a]

Strain[b]	Enzyme activity (U/liter of culture)	
	PAOx dehydration	PAN hydrolysis
Fusarium oxysporum f. sp. *nicotianae* IFO 6386#	7.88	7.74
Schizophyllum sp. strain TPU 4435*	3.41	6.00
Schizophyllum commune IFO 4929*	3.32	1.16
Neosartorya fisheri IAM 13864*	1.82	1.60
Rhizopus oryzae IFO 4705*	1.62	1.49
Gibberella fujikuroi IFO 6605	1.47	0.42
Talaromyces flavus IAM 13770*	1.16	0.81
Mortierella sp. TPU 4801*	0.95	1.59
Mortierella ramanniana var. *angulispora* IFO 8187*	0.94	2.28
Flammulina sp. strain TPU 4675*	0.82	0.04
Fusarium solani var. *martii* IFO 5900	0.81	0.17
Aspergillus celluosae TPU 4040	0.78	0.69
Absidia corymbifera IFO 4009	0.63	0.53
Rhizopus oryzae IFO 5438	0.61	0.09
Gibberella fujikuroi IFO 6356#	0.56	0.48
Fusarium solani IFO 5232#	0.54	1.64
Mortierella isabellina IFO 7875*	0.51	2.48
Pycnoporos coccineus TPU 4405	0.45	0.94
Keratinomyces ajelloi IFO 7865	0.44	0.73
Mucor fragilis IFO 6449*	0.42	1.32
Flammulina velutipes IFO 8329	0.33	0.97
Coprinus phlyctidosporus TPU 4614	0.29	2.25
Flammulina sp. strain TPU 4674*	0.27	0.11
Aureobasidium pullulans IFO 4464	0.25	0.83
Aspergillus candidus TPU 4037	0.23	1.18
Rhizopus nigricans TPU 4701*#	0.15	0.35
Cunninghamella echinulata var. *elegans* IFO 6334	0.14	0.88
Aspergillus pulverulentus IAM 2084*	0.12	0.04
Phycomyces nitens IFO 9422	0.06	0.13
Aspergillus amstelodami IAM 2025*	0.05	0.40
Fusarium culmorum IFO 5902	0.04	2.00

[a] The strains marked with an asterisk (*) hydrolyzed PAN accumulating PAAm. The strains marked with a number sign (#) degraded not only PAOx but also PyOx. All the active strains did not exhibit PAOx dehydration activity when they were grown without PAOx. The group of inactive fungi for PAOx degradation in the TLC assay included 1 species of *Arthroderma*, 11 species of *Aspergillus*, 2 species of *Aureobasidium*, 2 species of *Beauveria*, 1 species of *Chaetomium*, 1 species of *Chrysosporium*, 1 species of *Eurotium*, 1 species of *Exophiala*, 1 species of *Gloeophyllum*, 1 species of *Lentinus*, 6 species of *Mucor*, 1 species of *Neurospora*, 3 species of *Penicillium*, 1 species of *Pholiota*, 1 species of *Pycnoporus*, 3 species of *Rhizopus*, and 1 species of *Trametes*.

[b] Low PAOx dehydration activity (~0.03 U/liter of culture) was seen in *Absidia cylindrospora* IAM 6187, *Aspergillus cellulosae* TPU 4044, 2 strains of *Chaetomium globosum* (IFO 4822 and IAM 8059), 2 strains of *Coriolus versicolor* (IFO 30388 and IFO 4938), *Daedaleopsis styracina* IFO 4910, *Flammulina velutipes* TPU 4670, 2 strains of *Flammulina* sp. (TPU 4671 and TPU 4672), *Ganoderma lucidum* TPU 4607, *Gloeophyllum sepiarium* IFO 4944, *Irpex lacteus* IFO 5367, *Laetiporus sulphureus* TPU 4310, *Mortierella humicola* IFO 8289, 2 strains of *Mortierella isabellina* (IFO 7884* and IFO 8572), *Mortierella ramanniana* var. *angulispora* IFO 8287, 3 strains of *Mortierella* sp. (TPU 4800, TPU 4801*, and TPU 4802), *Penicillium notatum* IFO 4640, *Pholiota nameko* TPU 4370, and *Phytophthora infestans* IFO 4872, 2 strains of *Schizophyllum commune* (IFO 4928* and IFO 6502*), *Schizophyllum* sp. strain TPU 4436, and *Zygorhynchus californiensis* IFO 6663 with PAN-hydrolyzing activities.

TABLE 4. PyOx dehydration and CyPy-hydrolyzing activities in fungi grown with PyOx[a]

Strain[b]	Enzyme activity (U/liter of culture)	
	PyOx dehydration	CyPy hydrolysis
Fusarium oxysporum f. sp. *narcissi* IFO 5265	1.2	0.35
Fusarium oxysporum f. sp. *gladioli* IFO 5894#	0.96	0.56
Gibberella fujikuroi IFO 6356#	0.84	3.35
Fusarium solani TPU 4505#	0.79	0.06
Rhizopus nigricans TPU 4701#	0.51	2.43
Fusarium merismoides TPU 4500#	0.39	0.11
Fusarium oxysporum f. sp. *nicotianae* IFO 6386#	0.18	0.42
Fusarium solani IFO 5232#	0.12	0.96

[a] The strains degraded PyOx without forming NAm. The strains marked with a number sign (#) degraded not only PyOx but also PAOx. All of the active strains did not exhibit PyOx dehydration activity when they were grown without PyOx. The group of inactive fungi for PyOx degradation in the TLC assay included 2 species of *Absidia*, 1 species of *Arthroderma*, 15 species of *Aspergillus*, 2 species of *Aureobasidium*, 2 species of *Beauveria*, 2 species of *Chaetomium*, 1 species of *Chrysosporium*, 1 species of *Coriolus*, 1 species of *Coprinus*, 1 species of *Cunninghamella*, 1 species of *Daedaleopsis*, 1 species of *Eurotium*, 1 species of *Exophiala*, 1 species of *Flammulina*, 1 species of *Ganoderma*, 2 species of *Gloeophyllum*, 1 species of *Irpex*, 1 species of *Keratinomyces*, 1 species of *Laetiporus*, 1 species of *Lentinus*, 4 species of *Mortierella*, 6 species of *Mucor*, 1 species of *Neosartorya*, 1 species of *Neurospora*, 4 species of *Penicillium*, 1 species of *Pholiota*, 1 species of *Phycomyces*, 1 species of *Phytophthora*, 1 species of *Pycnoporus*, 2 species of *Rhizopus*, 2 species of *Schizophyllum*, 1 species of *Talaromyces*, 1 species of *Trametes*, and 1 species of *Zygorhynchus*.

[b] Low PyOx dehydration activity (~0.03 U/liter of culture) was seen in *Fusarium solani* TPU 4505, *Fusarium culmorum* IFO 5902, and *Fusarium merismoides* TPU 4506 with CyPy-hydrolyzing activities.

Schizosaccharomyces, 1 species of *Sporidiobolus*, 4 species of *Sporobolomyces*, 12 species of *Torulopsis*, 3 species of *Trichosporon*, and 1 species of *Zygosaccharomyces*. Of these, two were active in degrading PAOx: *Candida methanolica* TPU 1217 and *Pichia miso* TPU 1306 showed PAOx dehydration activity at 1.31 and 0.9 (U/liter of culture), coexisting with a PAN-hydrolyzing activity at 0.5 and 0.2 (U/liter of culture), respectively, when they were grown with 0.05% of PAOx. It was also shown that both the strains degraded PAOx by a successive combination of aldoxime dehydration and nitrile-hydrolyzing enzymes. *P. miso* degraded PAN accumulating PAAm, while *C. methanolica* degraded it without the formation of PAAm. On the other hand, no yeast strain could degrade PyOx.

Based on these results, it is shown that all of the PAOx or PyOx degraders degraded the aldoximes by a combination of aldoxime dehydratase and nitrile-hydrolyzing enzymes.

Aldoxime dehydration enzyme activity in nitrile-degrading microorganisms. Nineteen microorganisms (13 species, 7 genera) which had been isolated as nitrile degraders were grown with aldoximes, such as PAOx and PyOx, or nitriles, such as PAN and CyPy, and the aldoxime dehydration and nitrile-hydrolyzing activities were measured. As shown in Table 5, all of the nitrile degraders thus examined showed either PAOx or PyOx dehydratase activities. A high PAOx dehydration activity was seen in alkylnitrile-degrader, e.g., *Rhodococcus* sp. strain N-774 (23), *Corynebacterium* sp. strain C-5 (22), *R. rhodochrous* J-1 (3, 19), and *R. erythropolis* BG-16 (Y. Asano, T. Yasuda, Y. Tani, and H. Yamada, Abstr. Ann. Meet. Japan Soc. Ferment. Technol., 1981). No aldoxime dehydratase activity was seen when the strains were grown without aldoximes or nitriles, although PAN- or CyPy-hydrolyzing activity was detected in the cells.

DISCUSSION

Aldoximes are considered to be intermediates in the biosynthesis of certain biologically active compounds such as indoleacetic acid, cyanogenic glucosides, and glucosinolates in plants (17, 20, 21); however, very little is known about aldoxime-degrading enzymes: indoleacetaldoxime (IAOx)

TABLE 5. Aldoxime dehydration and nitrile-hydrolyzing activities in nitrile-degrading microorganisms when they were grown with or without inducers[a]

Strain[a]	Enzyme activity (U/liter of culture)[b]												Source or reference
	PAOx dehydration			PAN hydrolysis			PyOx dehydration			CyPy hydrolysis			
	PAOx	PAN	None	PAOx	PAN	None	PyOx	CyPy	None	PyOx	CyPy	None	
Alcaligenes faecalis IFO 13111 (ATCC 8750)	–	–	–	–	–	–	0.76	0.39	–	0.41*	0.65*	0.29*	27
Brevibacterium butanicum ATCC 21196	–	–	–	–	–	–	0.45	0.20	–	1.30*	0.08*	0.09*	27
Corynebacterium alkanum ATCC 21194	0.23	0.08	–	4.21*	0.83*	0.67*	0.51	0.21	–	1.33*	0.66*	0.38*	27
Corynebacterium nitrilophilus NCIMB 11594	–	–	–	–	–	–	0.21	0.13	–	0.35	1.10	0.06	18
Corynebacterium sp. strain C5 TPU 6015	3.32	6.08	–	10.7*	27.2*	8.39*	–	–	–	–	–	–	22
Pseudomonas fluorescens IFO 3925	–	–	–	–	–	–	0.53	0.11	–	0.62*	0.14*	0.09*	27
Pseudomonas sp. strain K-9 TPU 7177	0.52	0.78	–	2.03*	1.31*	0.76*	0.29	0.12	–	0.23	0.39	0.41	25
Rhodococcus erythropolis BG-13 TPU 3220	0.19	0.14	–	0.40*	0.84*	0.55*	0.50	0.75	–	1.47*	0.28*	0.10*	Asano et al.
Rhodococcus erythropolis BG-16 TPU 3221	1.71	1.15	–	1.58*	1.53*	0.31*	–	–	–	–	–	–	Asano et al.
Rhodococcus rhodochrous J-1 TPU 3311	2.08	0.17	–	13.4*	10.8*	3.81*	0.43	0.11	–	7.52	1.38	1.73	3, 8, 19
Rhodococcus rhodochrous NCIMB 11273 (ATCC 21197)	–	–	–	–	–	–	0.47	0.16	–	0.54*	0.13*	0.04*	11
Rhodococcus sp. strain AK-32 TPU 3466	1.4	6.3	–	28.0*	13.8*	1.15*	1.15	0.12	–	189	9.5	–	27
Rhodococcus sp. strain I-9 TPU 3467	0.72	0.16	–	1.25	0.61	1.33	0.84	0.12	–	0.31	14.6	3.75	24
Rhodococcus sp. strain N-774 TPU 3465	7.97	27.2	–	208*	158*	70.2*	7.02	0.81	–	42.0*	17.6*	23.5*	23
Rhodococcus sp. strain NCIMB 11215	–	–	–	–	–	–	1.44	1.33	–	3.23	1.80	0.52	10
Rhodococcus sp. strain NCIMB 11216	–	–	–	–	–	–	0.25	0.31	–	1.62	0.97	0.32	9
Fusarium merismoides TG-1 TPU 4500	0.36	0.17	–	2.07	4.26	3.86	0.39	0.25	–	0.11	0.10	0.12	2
Fusarium solani TG-2 TPU 4501	1.05	0.18	–	4.53	2.65	2.35	0.79	0.10	–	0.10	0.37	0.18	2
Candida guilliermondii IFO 0454	–	–	–	–	–	–	0.11	0.10	–	0.85*	0.10*	0.14*	28
Rhodococcus sp. strain YH3-3[c] TPU 3453	22.4	4.21	–	288*	89.3*	1.17*	2.83	0.08	–	144*	56.3*	0.15*	7
Bacillus sp. strain OxB-1[d] TPU 5563	12.2	0.91	–	2.59	4.11	0.94	–	–	–	–	–	–	12

[a] The strains were grown with PAOx, PAN, PyOx, or CyPy or without inducers. The activity marked with an asterisk hydrolyzed PAN and CyPy accumulating PAAm and NAm, respectively.
[b] None, no inducer. A dash (–) indicates that no activity was detected, under the assay conditions.
[c] Isolated as PAOx degrader from soil.
[d] Isolated as PyOx degrader from soil.

hydrolyase (EC 4.2.1.29), catalyzing a specific dehydration reaction of IAOx to form indoleacetonitrile, has been detected in limited genera of fungi and higher plants (20, 21), and the enzyme has only been partially purified from *Gibberella* spp. (17). In our previous report, we isolated various aldoxime-degrading bacteria from soil by several-months' acclimation and clarified that the isolated strains degraded aldoxime by the combination of aldoxime dehydratase and nitrile-hydrolyzing enzymes (7, 12). *Rhodococcus* sp. strain YH3-3, isolated in a medium containing PyOx as a sole source of nitrogen, degrades PyOx to NA by a combination of aldoxime dehydratase, nitrile hydratase, and amidase (12) (Fig. 1), whereas *Bacillus* sp. strain OxB-1 degrades PAOx to PAA by the action of aldoxime dehydratase and nitrilase (7) (Fig. 1), although it could not utilize PAOx as a nitrogen or carbon source due to the toxicity of PAOx. In this report, we discovered that the novel aldoxime dehydratases acting on the aldoximes are distributed among a variety of microorganisms, together with nitrile-hydrolyzing enzymes. We also screened for the aldoxime dehydratase activity from the nitrile degraders hitherto known and clarified that all of the nitrile degraders contained not only nitrile-hydrolyzing enzymes but also aldoxime dehydratase by culturing with aldoxime. None of the active strains showed aldoxime dehydration activity when they were grown in the medium without aldoximes, suggesting that aldoxime dehydratase was inducibly formed in these strains. Although a number of studies had been done on the nitrile-hydrolyzing enzymes, their induction mechanisms remained unknown. Nitrile hydratases of *P. chlororaphis* B23 and *R. rhodochrous* J-1 are strongly induced by amides and their derivatives (26), although that of *Rhodococcus* sp. strain N-774 (23) is formed constitutively. Nitrilases are inducibly formed (2, 9, 10, 18, 24, 25, 27), except that the enzyme is constitutively produced in *Acinetobacter* sp. strain AK 226 (27). In the present study we found that aldoximes act as good inducers not only for aldoxime dehydratase but also for the nitrile-hydrolyzing enzymes. From the results presented here, we cannot clarify whether aldoxime dehydratase and nitrile-hydrolyzing enzymes are regulated separately or not in these strains. We have recently discovered that the genes for aldoxime dehydratase and nitrilase coexisted as a gene cluster in the genome of *Bacillus* sp. strain OxB-1 (15). It is important to genetically clarify the general relationship between aldoxime dehydratase and nitrile-hydrolyzing enzymes in the aldoxime- and nitrile-degrading strains.

A total of 31 bacterial and 9 fungal aldoxime and nitrile degraders showed both the PAOx and PyOx dehydration activities, together with PAN and CyPy hydrolyzing activities. It is not clear whether the differences between PAOx and PyOx dehydration activities observed in the strains were simply due to the substrate specificity of aldoxime dehydratase or to the possible existence of two or more aldoxime dehydratases having different substrate specificities. Purification and characterization of aldoxime dehydratase and nitrile-hydrolyzing enzymes from the strains will solve this problem.

It has been reported that about 70 strains of microorganisms were isolated to degrade nitrile compounds, and they were distributed across 37 species of 19 genera (6, 24). However, they appear frequently in limited genera, i.e., *Arthrobacter*, *Bacillus*, *Rhodococcus*, *Fusarium*, since the most common screening protocols involve enrichment isolation in the media containing nitriles as a carbon or a nitrogen source: this is inevitably skewing positive clones to groups of readily culturable microorganisms. By culturing the strains with aldoxime, we showed here that the occurrence of nitrile hydratase in various species of facultative anaerobes and fungi. Although it is reported that *Rhodococcus* sp. strain AK32 has only nitrilase (27), culturing the strain with PAOx showed that the strain also contains nitrile hydratase and amidase in addition to nitrilase. These findings encourage us to screen further cryptic nitrile-hydrolyzing enzymes in microorganisms by culturing with aldoxime.

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REFERENCES

1. **Asano, Y., Y. Tani, and H. Yamada.** 1980. A new enzyme "nitrile hydratase" which degrades acetonitrile in combination with amidase. Agric. Biol. Chem. **44:**2251–2252.
2. **Asano, Y., S. Ando, Y. Tani, H. Yamada, and T. Ueno.** 1981. Fungal degradation of triacrylonitrile. Agric. Biol. Chem. **45:**57–62.
3. **Asano, Y., K. Fujishiro, Y. Tani, and H. Yamada.** 1982. Aliphatic nitrile hydratase from *Arthrobacter* sp. J-1. Purification and characterization. Agric. Biol. Chem. **46:**1165–1174.
4. **Asano, Y., M. Tachibana, Y. Tani, and H. Yamada.** 1982. Purification and characterization of amidase which participates in nitrile degradation. Agric. Biol. Chem. **46:**1175–1181.
5. **Asano, Y., T. Yasuda, Y. Tani, and H. Yamada.** 1982. A new enzymatic method of acrylamide production. Agric. Biol. Chem. **46:**1183–1189.
6. **Asano, Y.** 1991. Studies on the synthesis of amides and amino acids by a novel microbial enzymes. Nippon Nogeikagaku Kaishi **65:**1617–1626.
7. **Asano, Y. and Y. Kato.** 1998. *Z*-Phenylacetaldoxime degradation by a novel aldoxime dehydratase from *Bacillus* sp. strain OxB-1. FEMS Microbiol. Lett. **158:**185–190.
8. **Bandyopadhyay, A. K., T. Nagasawa, Y. Asano, K. Fujishiro, Y. Tani, and H. Yamada.** 1986. Purification and characterization of benzonitrilases from *Arthrobacter* sp. strain J-1. Appl. Environ. Microbiol. **51:**302–306.
9. **Harper, D. B.** 1976. Purification and properties of an unusual nitrilase from Nocardia NCIMB 11216. Biochem. Soc. Trans. **4:**502–504.
10. **Harper, D. B.** 1985. Characterization of a nitrilase from *Nocardia* sp. (*rhodochrous* group) NCIMB 11215, using *p*-hydroxybenzonitrile as sole carbon source. Int. J. Biochem. **17:**677–683.
11. **Kakeya, H., N. Sakai, T. Sugai, and H. Ohta.** 1991. Microbial hydrolysis as a potent method for the preparation of optically active nitriles, amides, and carboxylic acids. Tetrahedron Lett. **32:**1343–1346.
12. **Kato, Y., R. Ooi, and Y. Asano.** 1998. Isolation and characterization of a bacterium possessing a novel aldoxime-dehydration activity and nitrile-degrading enzymes. Arch. Microbiol. **170:**85–90.
13. **Kato, Y., R. Ooi, and Y. Asano.** 1999. A new enzymatic method of nitrile synthesis by *Rhodococcus* sp. strain YH3-3. J. Mol. Catal. B Enzymatic **6:**249–256.
14. **Kato, Y., T. Tsuda, and Y. Asano.** 1999. Nitrile hydratase involved in aldoxime metabolism from *Rhodococcus* sp. strain YH3-3—purification and characterization. Eur. J. Biochem. **263:**662–670.
15. **Kato, Y., K. Nakamura, H. Sakiyama, S. G. Mayhew, and Y. Asano.** 2000. A novel heme-containing lyase, phenylacetaldoxime dehydratase from *Bacillus* sp. strain OxB-1: purification, characterization, and molecular cloning of the gene. Biochemistry **39:**800–809.
16. **Kobayashi, M., T. Nagasawa, and H. Yamada.** 1992. Enzymatic synthesis of acrylamide: a success story not yet over. Trends Biotechnol. **10:**402–408.
17. **Mahadevan, S.** 1973. Role of oximes in nitrogen metabolism in plants. Annu. Rev. Plant Physiol. **24:**69–88.
18. **Mimura, A., T. Kawano, and K. Yamaga.** 1969. Application of microorganisms to petrochemical industry. (I) Assimilation of nitrile compounds by microorganisms. J. Ferment. Technol. **47:**631–638.
19. **Nagasawa, T., C. D. Mathew, J. Mauger, and H. Yamada.** 1988. Nitrile hydratase-catalyzed production of nicotinamide from 3-cyanopyridine in *Rhodococcus rhodochrous* J1. Appl. Environ. Microbiol. **54:**766–1769.
20. **Rajagopal, R., and P. Larsen.** 1972. Metabolism of indole-3-acetaldoxime in plants. Planta **103:**45–54.
21. **Sibbesen, O., B. Koch, P. Rouzé, B. L. Møller, and B. A. Halkier.** 1995. Biosynthesis of cyanogenic glucosides. Elucidation of the pathway and characterization of the cytochrome P-450 involved, p. 227–241. *In* R. M. Wallsgrove (ed.), Amino acids and their derivatives in higher plants. Cambridge University Press, Cambridge, England.
22. **Tani, Y., M. Kurihara, H. Nishise, and K. Yamamoto.** 1989. Bioconversion of dinitrile to mononitrile, a tranexamic acid intermediate, by *Corynebacterium* sp. Agric. Biol. Chem. **53:**3143–3149.
23. **Watanabe, I., Y. Satoh, and K. Enomoto.** 1987. Screening, isolation and taxonomical properties of microorganisms having acrylonitrile-hydrating activity. Agric. Biol. Chem. **51:**3193–3199.
24. **Yamada, H., Y. Asano, T. Hino, and Y. Tani.** 1979. Microbial utilization of

acrylonitrile. J. Ferment. Technol. **57:**8–14.
25. **Yamada, H., Y. Asano, and Y. Tani.** 1980. Microbial utilization of glutaronitrile. J. Ferment. Technol. **58:**495–500.
26. **Yamada, H., and M. Kobayashi.** 1996. Nitrile hydratase and its application to industrial production of acrylamide. Biosci. Biotechnol. Biochem. **60:**1391–1400.
27. **Yamamoto, K., Y. Ueno, K. Otsubo, K. Kawakami, and K. Komatsu.** 1990. Production of *S*-(+)-ibuprofen from a nitrile compound by *Acinetobacter* sp. strain AK226. Appl. Environ. Microbiol. **56:**3125–3129.
28. **Yamamoto, K., K. Oishi, I. Fujimatsu, and K. Komatsu.** 1991. Production of *R*-(−)-mandelic acid from mandelonitrile by *Alcaligenes faecalis* ATCC 8750. Appl. Environ. Microbiol. **57:**3028–3032.