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Degradation of Pyridine by *Micrococcus luteus* Isolated from Soil†

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An organism capable of growth on pyridine was isolated from soil by enrichment culture techniques and identified as *Micrococcus luteus*. The organism oxidized pyridine for energy and released N contained in the pyridine ring as ammonium. The organism could not grow on mono- or disubstituted pyridinecarboxylic acids or hydroxy-, chloro-, amino-, or methylpyridines. Cell extracts of *M. luteus* could not degrade pyridine, 2-, 3-, or 4-hydroxypyridines or 2,3-dihydroxypyridine, regardless of added cofactors or cell particulate fraction. The organism had a NAD-linked succinate-semialdehyde dehydrogenase which was induced by pyridine. Cell extracts of *M. luteus* had constitutive amidase activity, and washed cells degraded formate and formamide without a lag. These data are consistent with a previously reported pathway for pyridine metabolism by species of *Bacillus*, *Brevibacterium*, and *Corynebacterium*. Cells of *M. luteus* were permeable to pyridinecarboxylic acids, monohydroxypyridines, 2,3-dihydroxypyridine, and monoamino- and methylpyridines. The results provide new evidence that the metabolism of pyridine by microorganisms does not require initial hydroxylation of the ring and that permeability barriers do not account for the extremely limited range of substrate isomers used by pyridine degraders.

Pyridine occurs in the environment as a by-product of coal gasification (21) and retorting of oil shale (13). The compound is mobile in soil (14) and persists in groundwater near underground coal gasification sites (21). Pyridine has moderately acute toxicity and is apparently teratogenic (11). Because pyridine occurs in the environment and is potentially dangerous to health, an understanding of its environmental fate is important.

Pyridine is readily degraded in soil (4), and a number of soil bacteria are capable of growth on pyridine as the sole source of carbon (7, 10, 12, 19, 20, 22). Several research groups have investigated bacterial metabolism of the unsubstituted pyridine ring (12, 19, 20, 22). Only aliphatic intermediates of pyridine metabolism were identified, because no researchers have produced cell extracts capable of degrading the intact pyridine ring. Therefore, the initial steps of ring fission remain uncertain. Generally, aerobic biodegradation of aromatic compounds such as benzene is thought to be initiated by hydroxylation of the ring. Unlike benzene, its homocyclic analog, pyridine resists electrophilic substitution. Hydroxylation was therefore considered an unlikely step in ring fission (19, 20, 22). The above hypothesis was supported by the nonreactivity of pyridine in a model system designed to imitate the enzymatic hydroxylation of aromatic compounds (10). Species of *Nocardia*, *Bacillus*, *Corynebacterium*, and *Brevibacterium* utilized pyridine as the sole source of carbon and energy but did not grow at the expense of monohydroxypyridines, and suspensions of cells or cell extracts did not oxidize mono- or dihydroxypyridines (10, 19, 20, 22). It should be remembered that cell extracts did not degrade pyridine, and thus the failure of cell extracts to oxidize hydroxypyridines does not exclude these compounds as potential intermediates. Similarly, the inability of pyridine-grown cells to oxidize hydroxypyridines could be due to permeability problems. The permeability of pyridine-grown cells to hydroxypyridines remained unknown.

Some experimental evidence suggests a role of hydroxypyridines in pyridine metabolism. Recently, Korosteleva et al. (12) described a *Nocardia* species which degraded pyridine by a pathway similar to that used by *Bacillus*, *Brevibacterium*, and *Corynebacterium* species. Growth of the organism on pyridine resulted in accumulation of 3-hydroxypyridine in the culture medium. Cells could not utilize 3-hydroxypyridine for growth, nor could washed suspensions of cells oxidize the compound. The inability of cells to transform 3-hydroxypyridine was attributed to impermeability of cells to the compound, although cell permeability was not tested.

Lack of information on the permeability of cells to substituted pyridines presents a serious limitation to the understanding of pyridine metabolism. Impermeability of cells to various pyridine derivatives could explain the extremely narrow range of substrate isomers utilized by pyridine degraders (10, 12, 18). If pyridine metabolism involves hydroxylated intermediates, cell permeability barriers could explain the inability of pyridine degraders to oxidize or utilize hydroxypyridines for growth. Conversely, if permeability barriers are not present, the inability of pyridine-grown cells to utilize hydroxypyridines gives strong evidence that hydroxylated intermediates are not involved.

The objectives of the study were (i) to isolate soil organisms capable of growth on pyridine, (ii) to determine the pathway for pyridine metabolism, (iii) to evaluate the specificity range for substrate isomers used, and (iv) to characterize the permeability of cells to substituted pyridines.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin, vitamins, DL-amino acids, isocitrate, succinate semialdehyde, coenzymes, dextran, and monoaminopyridines were obtained from Sigma Chemical Co. Other chemicals were obtained from Aldrich Chemical Co., Inc.

Isolation of organism. A Chalmers silt loam soil, not previously exposed to pyridine, was used as an inoculum for enrichment cultures. The medium contained basal salts (10),

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TABLE 1. Substituted pyridines tested as substrates for *M. luteus*

Functional group	Position(s) of substituent(s)								
—OH	2	3	4	2,3	2,4	2,6			
—COOH	2	3	4	2,3	2,4	2,5	2,6	3,4	3,5
—Cl	2	3	4	2,3	2,5	2,6	3,5		
—NH_2	2	3	4	2,3	2,5	2,6	3,4		
—CH_3	2	3	4	2,4	2,6	3,4	3,5		

yeast extract (150 mg liter^{-1}), and pyridine (0.01 M). The soil was perfused with pyridine enrichment medium in a rotary perfusion device. Perfusion of the soil resulted in enrichment of a gram-positive, aerobic, furazolidone-resistant coccus which was identified as a *Micrococcus* species. Results of tests for hydrolysis of lipid, casein, gelatin, and arginine, as well as reactions in tests for phosphatase, motility, and colony morphology (2) were consistent with the classification of the organism as *Micrococcus luteus* (G. K. Sims, Ph.D. thesis, Purdue University, West Lafayette, Ind., 1985). Cells were maintained on pyridine agar slants at 4°C. In the experiments described below, cells were cultured in the above salts medium with yeast extract and various carbon sources at 24°C unless otherwise indicated.

Measurement of growth. Growth was measured as optical density at 540 nm and expressed as cell density (dry weight) based on standard curves. Measurement at 540 nm avoided interference from a soluble yellow pigment produced by *M. luteus* when grown on pyridine.

Growth factor requirements of *M. luteus*. Cells grown on complex media without pyridine may express activity of enzymes associated with pyridine degradation pathways (20), which could mask induction of the enzymes by pyridine. Thus, a defined medium was used in enzyme induction studies. *M. luteus* required thiamine for growth on pyridine or succinate, and growth was enhanced by the addition of arginine, valine, leucine, and methionine. Defined media used in enzyme induction experiments thus contained thiamine (2 μM), arginine (0.3 mM), valine (0.4 mM), leucine (0.4 mM), and methionine (0.2 mM) to satisfy growth factor requirements and produce a high yield of cells. Succinate medium contained 0.5 mM N as $(NH_4)_2SO_4$ to provide an N source.

Pyridine degradation in cultures. Degradation of pyridine and substituted pyridines (Table 1) was measured as the decrease in absorbance at an appropriate wavelength in the UV, and samples were periodically scanned from 300 to 200 nm to detect the formation of UV-absorbing intermediates. Scans were performed with a Cary 17-D spectrophotometer, whereas measurements at fixed wavelengths were made with a Gilford 250. Cultures were incubated up to 3 weeks to allow for induction. An N source (0.5 mM N as ammonium sulfate) was added in case the organisms could not use N from substituted pyridines. Media containing substituted pyridines were filter sterilized. In addition to supplying needed growth factors, yeast extract produced a small amount of growth which served as a base line; this allowed the detection of toxic effects.

Degradation of formate, formamide, and pyridine derivatives by washed cells. Degradation of formate, formamide, and pyridine derivatives by washed cells was measured in cell extracts and suspensions of succinate- and pyridine-grown cells. Substrate solutions contained 4 mM formate, formamide, or pyridine derivatives in 33 mM phosphate buffer (pH 7). Buffered substrate solution (40 ml) was used for suspension of 12 mg (dry weight) of pyridine- or succinate-grown cells or was mixed with cell extract (2.5 mg protein) from pyridine- or succinate-grown cells. Samples were incubated at 24°C, agitated vigorously every 5 min with a Pasteur pipette, and sampled for formate, formamide, and pyridine over a 120-min period. Degradation of pyridine derivatives was also measured in reaction mixtures containing cell extract (0.25 mg of protein), phosphate buffer (100 μmol; pH 7), pyridine, 2-, 3-, 4-, or 2,3-hydroxypyridines (1 μmol) with or without NAD(P) (1 μmol), NAD(P)H (1 μmol), flavin adenine dinucleotide (1 μmol), or flavin mononucleotide (1 μmol) in a total volume of 3 ml. Formate was measured colorimetrically by reaction with thiobarbiturate (3), pyridine derivatives were measured by UV spectrophotometry, and formamide was measured by conversion to its hydroxamate (22).

Nitrogen. Ammonium was measured by Nesslerization of a steam distillate (1). Titration of NH_4^+ in the distillate was not possible owing to the presence of pyridine. Total N in cells was determined in Kjeldahl digests of washed cells harvested on polycarbonate filters.

Preparation of cell extracts. Large batch cultures (16 liters) grown on pyridine or succinate in defined medium were harvested in the growth phase (36 h) by continuous flow centrifugation at 15,000 × *g*. The cell paste was washed once in 33 mM phosphate buffer (pH 7), centrifuged at 15,000 × *g*, and stored at −20°C. Extracts were prepared by five cycles of sonication at 20 kHz (30 s), followed by cooling in ice (30 s). Whole cells were removed by centrifugation at 15,000 × *g* (4°C for 30 min). Cell debris removed by centrifugation at 100,000 × *g* (4°C for 1 h) was saved for later use. Protein was measured by the method of Lowry et al. (15), with bovine serum albumin as the standard. All manipulations of disrupted cellular materials were performed in an ice bath. Cell extracts stored at −20°C retained high levels of enzyme activity for 4 weeks or longer.

Enzyme assays. Isocitratase (EC 4.1.3.1) was determined as the semicarbazone of the product glyoxylate (17). Glutarate-dialdehyde dehydrogenase (EC 1.2.1.x), succinate-semialdehyde dehydrogenase (EC 1.2.1.24), and isocitrate dehydrogenase (EC 1.1.1.42) activities were determined by increase in A_{340} as a result of formation of reduced coenzyme [NAD(P)H]. Reaction mixtures contained cell extract (0 to 0.25 mg of protein), phosphate buffer (100 μmol; pH 7), NAD(P) (1 μmol), and substrate (0 to 2.5 μmol) in a total volume of 3 ml. Reactions were started by the addition of carbon substrate. Amidase was measured by the formation of NH_3 from formamide in reaction mixtures containing cell extract (2.5 mg of protein), phosphate buffer (1 mmol; pH 7), and formamide (160 μmol) in a total volume of 40 ml. Enzyme activities were expressed as micromoles of product formed · milligram of protein^{-1} · minute.$^{-1}$

Permeability to substituted pyridines. Permeability to substituted pyridines was measured by the thick suspension or space method of Conway and Downey (5), as described by Marquis (16). Resting cells were preincubated in 33 mM phosphate buffer (pH 7) at 4°C for 12 h to deplete cells of nascent pyridine. In the assay, 3 g of cells was mixed with 3 ml of 33 mM phosphate buffer (pH 7) containing a pyridine derivative (0.25 mM). Preliminary data indicated that 1 h of incubation was necessary to ensure equilibrium. Dilution effects of interstitial space were accounted for by determining space values for 20 mg of a dextran (molecular weight, >200,000) liter^{-1} and using this to correct data gathered for

test substrates as described by Marquis (16). Pyridine derivatives were measured by UV spectrophotometry, and dextran was measured by a colorimetric procedure (6). Permeability (space) values were calculated by the following equation: $S = (V_s/V_p)(C_0/C_f - 1)$, where V_s is the volume of solution added to the pellet, V_p is the volume of the pellet, C_0 is the initial concentration of the solute, and C_f is the final concentration. The space values were corrected for interstitial volume by the equation $R = (S_{sol} - S_{dex})/(1 - S_{dex})$, where S_{sol} is the space value for the solute and S_{dex} is the space value for the dextran. These equations are taken from Marquis (16). Values greater than zero indicate permeability of cells to the added substrate.

RESULTS

Utilization of pyridine by *M. luteus*. Detailed studies were conducted with *M. luteus*, since minimal information is available in the literature concerning pyridine metabolism by micrococci. *M. luteus* utilized pyridine as a carbon and energy source. Similar yields of cells were produced from either pyridine (8.5 mg of cells · mmol of substrate carbon^{-1}) or succinate (8.8 mg of cells · mmol of substrate carbon^{-1}). Growth kinetics were determined from the rate of cell dry weight production in the exponential phase at 1.6, 3.2, 6.4, and 12.8 mM pyridine. The result suggests that all the pyridine carbon was used. For growth on pyridine, K_s (substrate concentration at a growth rate of $0.5u_{max}$) and u_{max} (growth rate at a saturating concentration of substrate) from the Monod equation were 1.1 mmol · liter^{-1} and 0.09 h^{-1}. Maximum biomass of *M. luteus* was produced at pH 7.0 and 0.01 M pyridine. Increasing the pyridine concentration to 0.02 M extended the lag period before growth. No UV-absorbing compounds accumulated during growth on pyridine.

Pigment production. *M. luteus* produced an unidentified water-soluble pigment when grown on pyridine but not when grown on succinate, glucose, yeast extract, or nutrient broth. Incubation of *M. luteus* in media containing substituted pyridines did not result in pigment production.

Use of substituted pyridines. *M. luteus* was unable to grow at the expense of any of the substituted pyridines tested, including isomeric hydroxypyridines supplied at 0.01 M. Some compounds (3-chloropyridine, 3,4-dimethylpyridine, 2,6-pyridinedicarboxylic acid, and 2-, 3-, and 4-aminopyridines) appeared to be toxic to *M. luteus* and abolished the small amount of growth produced by yeast extract (150 mg · liter^{-1}) present in all treatments. Analysis of pyridine derivatives in cultures showed no difference between inoculated and uninoculated treatments for all substituted pyridines.

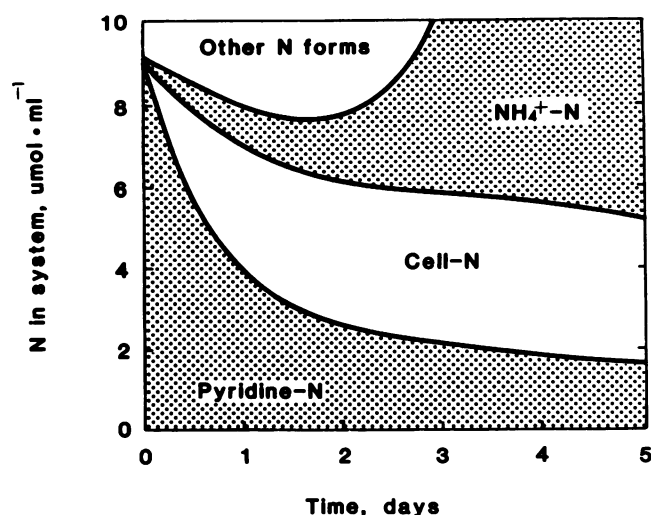

FIG. 1. Release of nitrogen from the pyridine ring by *M. luteus*. Defined medium containing 0.01 M pyridine was inoculated with pyridine-grown cells (2 mg [dry wt] liter^{-1}) and assayed for N forms after 0, 1, 2, 3, 4, and 5 days.

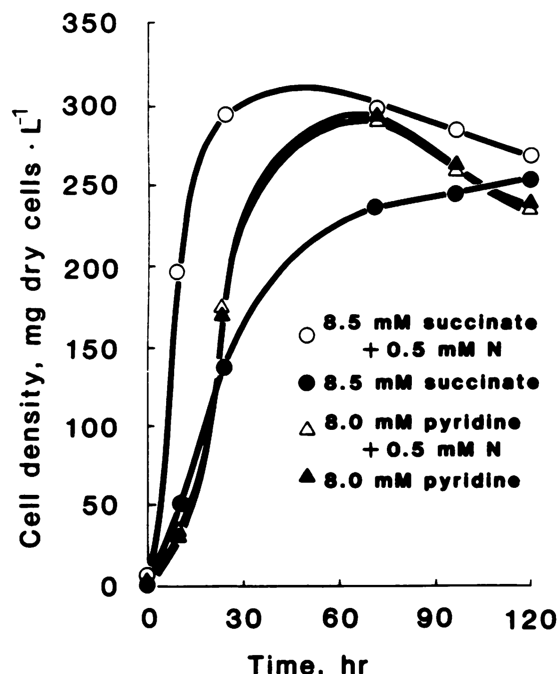

FIG. 2. Growth of *M. luteus* in media containing pyridine or succinate in the presence and absence of 0.5 mM N as $(NH_4)_2SO_4$.

Release of pyridine nitrogen. During the first 2 days of incubation, 70% of the added pyridine was degraded in cultures of *M. luteus*. Over 60% of the nitrogen contained in pyridine was retained by cells until the culture entered the stationary phase at 3 days of incubation. During the stationary phase, 79% of the pyridine N was released into the medium as NH_3. Other N forms reported in Fig. 1 were obtained by subtraction of pyridine N, cell N, and NH_4^+N from a total of 10.1 mmol of N · liter^{-1} consisting of 9.1 mmol of N · liter^{-1} as pyridine and 1.0 mmol of N · liter^{-1} as yeast extract. Growth of *M. luteus* on succinate (in the presence of 150 mg of yeast extract · liter^{-1}) was enhanced by the addition of 0.5 mM N as ammonium sulfate (Fig. 2). Cell yield on succinate was increased from 7.2 to 8.8 mg of cells · mmol of substrate carbon^{-1}, and N content was increased from 4.0 to 8.5 mol of N · kg of cells^{-1} by addition of ammonium sulfate. Growth of *M. luteus* on pyridine was not stimulated by the addition of ammonium sulfate (Fig. 2). Cell yield (8.5 mg of cells · mmol of substrate carbon^{-1}) and N content (7.2 mol of N · kg of cells^{-1}) of *M. luteus* were unaffected by the addition of ammonium sulfate to medium containing pyridine. The results suggest that *M. luteus* assimilated some N from pyridine.

Metabolism of pyridine carbon. Pyridine was degraded by pyridine-grown cells resuspended in buffer containing 4 mM pyridine. No degradation occurred when succinate-grown

TABLE 2. Induction of enzymes by pyridine in *M. luteus*

Enzyme	Product measured	Sp act (μmol of product per min/mg of protein) of:	
		Pyridine-grown cells	Succinate-grown cells
Isocitrate dehydrogenase	NADPH	0.04	0.05
Isocitratase	Glyoxylate semicarbazone	<0.01	<0.01
Succinate-semialdehyde dehydrogenase	NADPH, NADH	0.39 0.52	<0.01 <0.01
Glutarate-dialdehyde dehydrogenase	NADPH, NADH	0.01 0.01	<0.01 <0.01
Formamidase	NH_3	0.21	0.23

cells were used. Neither pyridine- nor succinate-grown cell extracts could degrade pyridine, regardless of added cofactors [NAD(P), NAD(P)H, flavin adenine dinucleotide, or flavin mononucleotide]. Because cell extracts did not degrade pyridine and extracts from succinate-grown cells had lower protein yield, a constitutive enzyme was assayed to determine whether pyridine- and succinate-grown cell extracts were comparable. Isocitrate dehydrogenase was not expected to be induced by pyridine and was assayed as a control for the above reasons. The activity of isocitrate dehydrogenase was comparable in the two preparations (Table 2).

Resuspended cells or cell extracts from pyridine- and succinate-grown cells did not degrade 2-, 3-, or 4-hydroxypyridine, nor did they degrade 2,3-dihydroxypyridine. Addition of the 100,000 × *g* cell fraction to cell extracts did not confer the ability to degrade pyridine or hydroxypyridines.

Pyridine induced succinate-semialdehyde dehydrogenase activity in *M. luteus* cells. Activity was not detected in succinate-grown cells (Table 2). The activity of the enzyme was dependent on the presence of substrate and followed saturation kinetics. Double-reciprocal plots indicated a K_m of 0.015 mM and V_{max} values ranging from 0.24 to 0.52 μmol of NADH · mg of protein^{-1} · min^{-1} for different extract preparations. At saturation (0.5 mM), NADH produced by the reaction was proportional to protein added (R^2 = 0.999), supporting an enzyme catalysis mechanism. Succinate-semialdehyde dehydrogenase of *M. luteus* was classified as EC 1.2.1.24, because NAD was a better coenzyme for the reaction than was NADP (Table 2). Neither isocitratase nor glutarate-dialdehyde dehydrogenase was induced by pyridine (Table 2). Succinate-semialdehyde dehydrogenase is involved in pyridine metabolism by species of *Bacillus*, *Brevibacterium*, and *Corynebacterium* (19, 20, 22), whereas isocitratase and glutarate-dialdehyde dehydrogenase are involved in pyridine metabolism by *Nocardia* species (22). The data indicate that the mechanism of pyridine degradation in *M. luteus* is similar to the pathway used by species of *Bacillus*, *Brevibacterium*, and *Corynebacterium*.

Formate and formamide were degraded rapidly by suspensions of pyridine- or succinate-grown cells (Fig. 3). During assays of formamide degradation, NH_3 was released only from pyridine-grown cells (Fig. 3), although pyridine- and succinate-grown cells were shown previously to contain similar amounts of total N. Amidase activity was observed in both pyridine- and succinate-grown cell extracts (Table 2), indicating that the enzyme is constitutive. In contrast to previous reports for *Bacillus* spp., which have a cytochrome-linked formate dehydrogenase activity (22), cells of *M. luteus* poisoned with 0.2 mM KCN did not release formate or degrade pyridine (data not shown).

Uptake of substituted pyridines. Uptake values (*R*) corrected for interstitial volume were greater than zero for all substituted pyridines tested (Table 3). The data show that *M. luteus* is permeable to substituted pyridines.

DISCUSSION

The results suggest that the system for the metabolism of pyridine by *M. luteus* is inducible and specific for pyridine. The above findings reflect properties reported for other pyridine degraders (12, 20, 22). *M. luteus* could not use substituted pyridines without a lag.

The failure of *M. luteus* to degrade hydroxypyridines suggests the compounds are not intermediates in pyridine metabolism. However, permeability barriers cannot be excluded. For example, 2,5-dihydroxypyridine, which has been identified as an intermediate in nicotinic acid metabo-

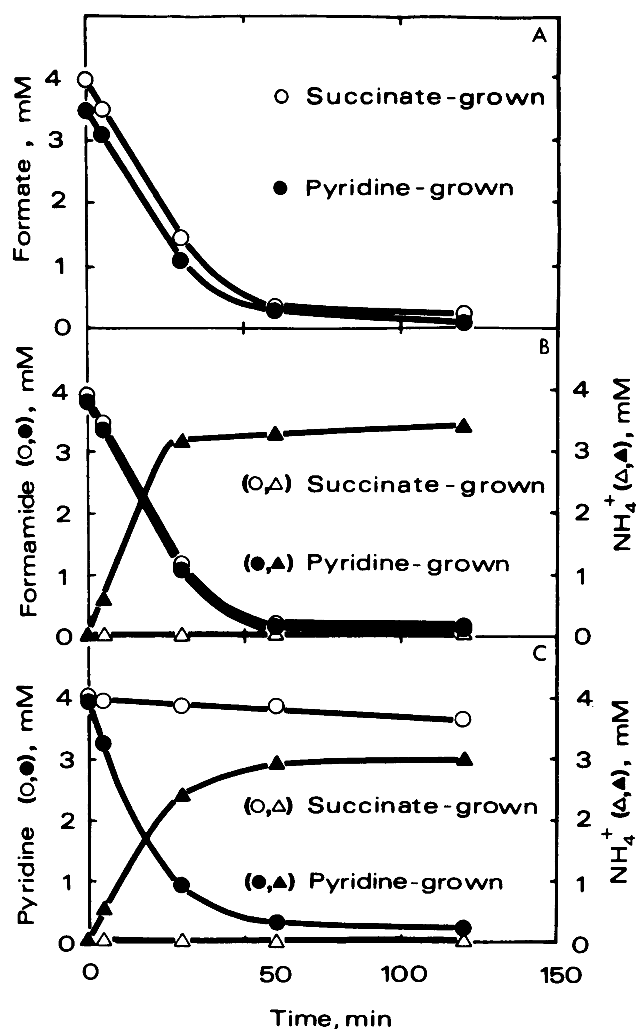

FIG. 3. Degradation of formate, formamide, and pyridine by resuspended cells (0.3 g [dry wt] liter^{-1}) of *M. luteus*. (A) Formate degradation. (B) Formamide degradation and release of ammonium. (C) Pyridine degradation and release of ammonium.

TABLE 3. Permeability of *M. luteus* to substituted pyridines

Substituent	Position(s) of substitutent(s)	R^a
None (Pyridine)		9.5
—OH	2	1.7
	3	6.1
	4	6.3
	2,3	2.1
—COOH	2	2.2
	3	2.7
	4	12.1
$—CH_3$	2	10.2
	3	4.4
	4	6.3
$—NH_2$	2	4.1
	2,6	2.4

[a] R values corrected for interstitial volume by determining uptake for high-molecular-weight dextran.

lism, cannot penetrate nicotinate-grown cells of *Bacillus* spp. or *Pseudomonas fluorescens*, although 2,5-dihydroxypyridine is oxidized by cell extracts of *P. fluorescens* (8). The failure of cell extracts of *M. luteus* to degrade hydroxypyridines does not prove that hydroxy intermediates are not involved in pyridine metabolism, since the extracts could not degrade pyridine. It was therefore that *M. luteus* was found to be permeable to hydroxypyridines in order to exclude the compounds as intermediates.

Hydroxypyridines apparently are not involved in pyridine metabolism by species of *Bacillus*, *Brevibacterium*, *Corynebacterium*, or *Nocardia* (19, 20, 22). For the results of studies with *M. luteus* to be applicable to other pyridine degraders, it was necessary to investigate the pathway for pyridine metabolism. Two distinct pathways for the metabolism of pyridine are recognized. The more common pathway involves cleavage of the ring between C-2 and C-3 to yield a 6-member semialdehyde, which is hydrolyzed to formamide and succinate semialdehyde (Fig. 4). Formamide is converted to formate and ammonia by a specific amidase. Succinate semialdehyde is oxidized to succinate by a NAD-linked dehydrogenase, which is usually induced by pyridine (20, 22). Pyridine induction of NAD-linked succinate-semialdehyde dehydrogenase, as well as the presence of a constitutive amidase and the ability to degrade both formate and formamide, suggest that *M. luteus* uses the above pathway to degrade pyridine. Another pathway for pyridine metabolism involves C-2–N ring cleavage and is accompanied by pyridine-induced glutarate-dialdehyde dehydrogenase, glutarate-semialdehyde dehydrogenase, acyl coenzyme A synthetase, and isocitratase activities (22). Since glutarate-dialdehyde dehydrogenase and isocitratase were not induced by growth of *M. luteus* on pyridine, the pathway is probably not present in the organism.

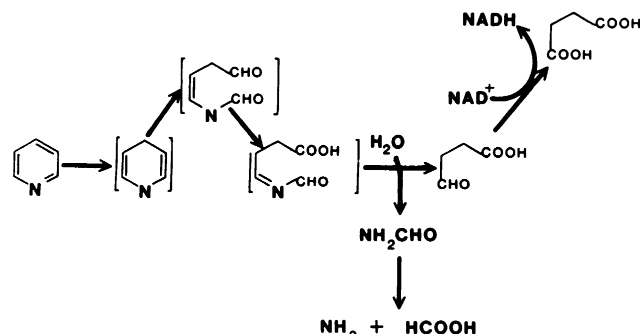

FIG. 4. Proposed pathway for pyridine metabolism (adopted from reference 22).

The results suggest a common pathway for pyridine metabolism in *M. luteus* and species of *Bacillus*, *Brevibacterium*, and *Corynebacterium*. Permeability of *M. luteus* to hydroxypyridines is important, since *M. luteus*, *Bacillus* spp., *Brevibacterium* spp., and *Corynebacterium* spp. do not degrade hydroxypyridines (19, 20, 22). The results do not explain a recent finding that 3-hydroxypyridine accumulates in medium during the growth of *Nocardia* sp. strain KM-2 on pyridine (12). The pathway for pyridine degradation by *Nocardia* sp. strain KM-2 is essentially the same as that reported for *M. luteus* and species of *Bacillus*, *Brevibacterium*, and *Corynebacterium*. The *Nocardia* sp. did not degrade the proposed 3-hydroxy intermediate. Hydroxylation of pyridine by *Nocardia* sp. strain KM-2 could be nonspecific, since pyridine is nonspecifically hydroxylated by several enzymes. Peroxidase and catalase enzymes of plant and animal origin catalyze nonspecific hydroxylation of pyridine to 3-hydroxypyridine (9). *Nocardia* sp. strain Z1 carries out nonspecific hydroxylation of 3-hydroxypyridine to 2,3- and 3,4-diols. The specific role of 3-hydroxypyridine in pyridine metabolism by *Nocardia* sp. strain KM-2 remains unclear.

The results presented in this paper provide new evidence that metabolism of pyridine by microorganisms does not require initial hydroxylation of the ring. In addition, permeability barriers do not account for the extremely limited range of substrate isomers used by pyridine degraders.

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