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Screening for Microorganisms Producing D-Malate from Maleate

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More than 300 microorganisms were screened for their ability to convert maleate into D-malate as a result of the action of maleate hydratase. Accumulation of fumarate during incubation of permeabilized cells with maleate was shown to be indicative of one of the two enzymes known to transform maleate. The ratio in which fumarate and malate accumulated could be used to estimate the enantiomeric composition of the malate formed. Many strains (n = 128) were found to be capable of converting maleate to D-malate with an enantiomeric purity of more than 97%. *Pseudomonas pseudoalcaligenes* NCIMB 9867 was selected for more detailed studies. Although this strain was not able to grow on maleate, permeabilized cells were able to degrade maleate to undetectable levels, with a concomitant formation of D-malate. The D-malate was formed with an enantiomeric purity of more than 99.97%.

Biological formation of optically active synthons (starting compounds for chemical synthesis) for the fine-chemicals industry is one of the major application areas in biotechnology (12). Lyases (e.g., hydratases, ammonia lyases) are very interesting enzymes in this respect. They are generally cofactor independent, quite stable, and have a theoretical yield of 100% compared to only 50% for enantiomeric resolutions.

Our goal was to screen microorganisms for novel lyase activities to ultimately produce optically active amino acids or hydroxy acids on a commercial scale.

Lyases have been used on a commercial scale for some decades. L-Malate has been produced since 1974 by the Japanese firm Tanabe Seiyaku in a continuous process that uses immobilized *Brevibacterium* sp. cells containing high fumarase activity (36). An example of an ammonia lyase which is used on a commercial scale is aspartase. This enzyme stereospecifically adds ammonia to fumarate, forming L-aspartate. Since 1958, aspartate has been produced on an industrial scale by the same Japanese firm by using *Escherichia coli* containing high aspartase activity. Since 1973, this process has been operated continuously by using immobilized cells (6).

A chiral α-hydroxy acid with potential commercial applications is D(*R*)-malate. D-Malate can be used as a chiral synthon (37) or as a resolving agent in racemic resolutions (3, 29). D-Malate can be formed from maleate (*cis* isomer of fumarate) with maleate hydratase (malease) (EC 4.2.1.31). This enzyme has been purified from rabbit kidneys (11) and has also been detected in several *Pseudomonas* spp. (17, 27, 34).

Maleate can be used as a carbon and energy source by microorganisms (24) and is an intermediate in one of the degradation pathways of gentisate (7, 17) and in the degradation of the pyridine ring (1, 5). Two degradation pathways for maleate have been described (see Fig. 1). In the first pathway, maleate is transformed into fumarate by maleate *cis-trans*-isomerase (24). Fumarate is then further degraded in the citric acid cycle. In the second degradation pathway, maleate is directly hydrated to D-malate (17, 27). D-Malate is degraded by several microorganisms via an inducible, NAD^+-dependent D-malic enzyme (14, 16, 18–20, 33). This enzyme catalyzes the oxidative decarboxylation of D-malate to pyruvate and CO_2. D-Malate can also be cleaved by a coenzyme A- and ATP-dependent enzyme to acetyl-coenzyme A and glyoxylate (32). D-α-Hydroxy acid dehydrogenase from yeasts (8) and animal mitochondria (38) and succinate dehydrogenase from pig hearts (10) were reported to transform D-malate into oxaloacetate, but it is not certain if these enzymes also play a role in the in vivo degradation of D-malate. Besides these three pathways, an $NADP^+$-dependent degradation pathway for D-malate has also been suggested (18).

In this report, we describe a screening procedure to select strains exhibiting malease activity but devoid of maleate *cis-trans*-isomerase activity. These strains should theoretically be capable of stoichiometric formation of D-malate from maleate. One strain, *Pseudomonas* sp. strain NCIMB 9867, was studied in more detail especially with respect to the enantiomeric composition of the D-malate formed.

MATERIALS AND METHODS

Isolation of microorganisms. Soil samples (5 g) collected from different polluted sites were incubated in 30 ml of mineral salts medium (15) containing 2 g of maleate per liter in serum bottles (130 ml) under different conditions. For standard conditions, the pH of the medium was adjusted to 7.0 and the enrichment culture was incubated statically at 30°C. For denitrifying conditions, 5 g of sodium nitrate per liter was added to the medium, the serum bottle was completely filled with the medium, and a syringe was placed in the rubber septum to allow simple monitoring of N_2 evolution. For low pH, the medium was adjusted to a pH of 4 with phosphoric acid. For the selective isolation of enterobacteria, 1.5 g of bile salts per liter and 5 g of NaCl per liter were added to the medium. For the selective isolation of yeasts, 100 mg of streptomycin per liter and 200 mg of chloramphenicol per liter were added to the medium. For conditions of

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high temperature, the enrichment cultures were incubated at 45 or 58°C.

The enrichment cultures were incubated for 2 weeks after which 5 ml was transferred to fresh medium and the new enrichment culture was incubated for another 2 weeks. After a second transfer and 2-week incubation period, a loopful was streaked onto mineral salts medium agar plates containing 2 g of maleate per liter. Colonies were streaked to purity and maintained on yeast extract-glucose agar slants.

Culture collection strains were obtained from the culture collections of both the Department of Microbiology and the Department of Food Science, Wageningen Agricultural University, Wageningen, The Netherlands. *Pseudomonas* sp. strain NCIMB 9867 was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Medium and cultivation. Strains were cultivated in 300-ml Erlenmeyer flasks containing 75 ml of rich medium. Rich medium contained the following (per liter of demineralized water): 10 g of casein peptone, 7.5 g of yeast extract, 7.5 g of malt extract, 5 g of glucose, and 2 g of maleate. The pH of the medium was adjusted to 7.0 with sodium hydroxide. Cells were cultivated for 30 h at 30°C on a reciprocal shaker oscillating at 1 Hz with an amplitude of 10 cm.

Cells of *Pseudomonas pseudoalcaligenes* used to determine if maleate was oxidized and degraded by intact cells were grown on the mineral salts medium containing 3 g of dicarboxylic acid per liter (pH 7.0) as the sole carbon source. Final cell densities and growth rates on dicarboxylic acid were determined by monitoring the optical density at 660 nm. In these growth experiments, the substrate concentrations were 1 g of dicarboxylic acid per liter or 1 g of dicarboxylic acid plus 1 g of maleate (each per liter) (pH 7.0).

The growth substrate range of *P. pseudoalcaligenes* NCIMB 9867 was determined by cultivating this strain on mineral salts medium containing 1 g of substrate per liter.

Permeabilization of cell suspensions. The cells were permeabilized by the method of Miozzari et al. (23). The strains used in the screening were harvested (10 min at 16,000 × *g*) and washed once with 50 mM potassium phosphate buffer (pH 7.0). The cells were then resuspended in 2.5 ml of 50 mM potassium phosphate buffer (pH 7.0) with 0.1% (wt/vol) Triton X-100 and stored overnight at −20°C.

Enzyme assays with permeabilized cells. To determine maleate hydratase and maleate *cis-trans*-isomerase activities, 2.5 ml of permeabilized cells (100 to 350 mg [dry weight]) was diluted in a 35-ml serum bottle with 7.5 ml of double-strength mineral salts medium containing 50 mM Tris hydrochloride (pH 7.0) and 20 mM maleate. The serum bottles were closed with rubber septa and incubated statically in a water bath at 30°C. Because of the high cell densities, these cell suspensions quickly became anaerobic. Samples were taken from the incubation mixtures after 0.5, 1.5, and 4 h, and the supernatants were analyzed for maleate, malate, and fumarate by high-performance liquid chromatography (HPLC).

HPLC analysis. Malate, fumarate and maleate were analyzed by HPLC by using the method described by Skelly (30). The dicarboxylic acids were separated on a C18 column (200 by 3 mm; Chrompack, Middelburg, The Netherlands) at room temperature. The mobile phase was 2 mM octylamine–25 mM potassium phosphate buffer (pH 7.0) in distilled water. The flow rate was 0.4 ml/min, and the organic acids were detected at 210 nm by using a variable-wavelength detector. The column had to be equilibrated for at least 3 h with the mobile phase before a reproducible separation of the organic acids was achieved.

Dry weight. The cell suspension (1 ml) was put in a glass tube with a predetermined weight. The tube was placed overnight in a stove at 130°C. The glass tube was allowed to cool in a desiccator containing dried kiesel gel. The weight of the tube was again determined. Dry weights were corrected for the dry weight of the screening buffer which was determined in the same way.

Determination of L-malate. L-Malate concentrations were determined enzymatically. To 1.6 ml of 50 mM glycylglycine–10 mM glutamate buffer (pH 10.0) were added 100 μl of supernatant sample diluted with distilled water resulting in L-malate concentrations lower than 2 mM, 200 μl of 20 mM NAD^+, 50 μl of glutamate-oxaloacetate transaminase (~10 U), and 50 μl of L-malate dehydrogenase (~15 U). The absolute absorption increase after the addition of L-Malate dehydrogenase was determined at 340 nm, and the L-malate concentration was calculated by using L-malate standards.

Respiration experiments. Dicarboxylic-acid-dependent oxygen uptake experiments were performed as described previously (15) by determining the difference in oxygen uptake rates of whole cells before (endogenous oxygen uptake rate) and after the addition of substrate (final concentration, 0.33 mM).

Production of malate. Malate used for the determination of optical rotation and enantiomeric purity was produced from either maleate, maleic anhydride, or fumarate by permeabilized cells of *P. pseudoalcaligenes* grown on the rich medium. Ten grams (dry weight) of permeabilized cells was suspended in 30 ml of substrate solution (1.0 M) which had been adjusted to pH 7.0 with sodium hydroxide. This suspension was incubated for 3 days in a static water bath at 30°C. The suspension was then spun down (15 min at 27,000 × *g*), and the supernatant was used to determine the optical purity and the optical rotation of the malate formed.

Optical rotation measurements. The optical rotation of enzymatically produced malate was assayed by the method described by Krebs and Eggleston (21). Supernatant was made up to a total volume of 10 ml with 5 ml of 20% (wt/vol) $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 ml of 50% (vol/vol) acetic acid, 1 ml of 1.25 M sodium citrate, and deionized water. The mixture was centrifuged when precipitation was observed. The optical rotation (final malate concentration in polarimeter, approximately 50 mM) was measured at room temperature at 589 nm in a 10.0-cm cuvette with a Perkin-Elmer 241 polarimeter.

Determination of the enantiomeric purity. The D-malate concentration of a 500-times-diluted supernatant sample was determined by using the enzymatic D-malic acid test kit of Boehringer with D-malate standards. A correction was made for the 1.3% L-malate determined enzymatically to be present in the commercially available D-malate. The L-malate concentration was determined in the undiluted sample (containing ~500 mM malate). The total absorption increase in the enzymatic L-malate assay was not influenced by the presence of these high concentrations of D-malate.

Chemicals. L-Malate dehydrogenase (for analytical purposes, solution in glycerol), glutamate-oxaloacetate transaminase, and NAD^+ were from Boehringer. Octylamine, maleate, and glycylglycine were from Aldrich. Fumarate, maleic anhydride, ammonium molybdate, and D-malate were from Janssen Chimica. Triton X-100, L-malate, and sodium citrate were from Merck. Câsein peptone and yeast extract were from GIBCO, and malt extract and agar (no. 3) were from Oxoid.

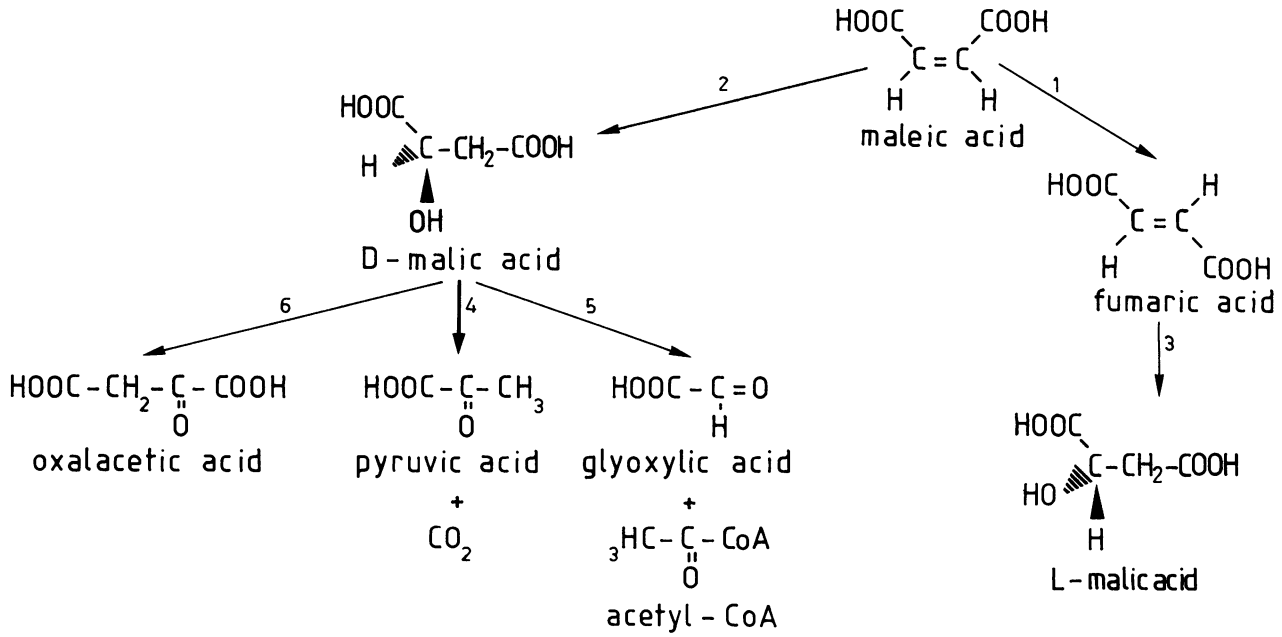

FIG. 1. Microbial degradation pathways of maleate. Enzymes involved in pathways: 1, maleate *cis-trans*-isomerase; 2, maleate hydratase; 3, fumarase; 4, D-malic enzyme; 5, D-malate synthase; 6, D-malate dehydrogenase.

RESULTS

Screening. Two hundred microorganisms isolated on maleate under different environmental conditions (see Materials and Methods) and 116 microorganisms from culture collections were screened for their ability to convert maleate into D-malate. No effort was made to identify the isolated strains.

Cells used in the screening were permeabilized with Triton X-100. A concentration of 0.1% was found to be optimal for most types of microorganisms. Fumarate accumulation during incubation of permeabilized cells with maleate under anaerobic conditions was used as the selection criterion to discriminate between the two known maleate degradation pathways (Fig. 1). In Table 1, the distribution of the capacity of permeabilized cells of microorganisms from culture collections to degrade maleate and the apparent distribution of the maleate degradation pathways are shown. The data in Table 1 indicate that the maleate-hydrating activity (no fumarate accumulation) is more widely distributed than maleate *cis-trans*-isomerase activity. The strains which did not accumulate fumarate showed a stoichiometric formation of malate from maleate.

Of the 315 strains screened, 55 strains which were isolated on maleate and 74 strains from culture collections consumed maleate without any significant (less than 0.1 mM) accumulation of fumarate.

Determination of the enantiomeric composition of the malate formed by the screened microorganisms. After this initial screening, the enantiomeric composition of the malate formed by the microorganisms which did not accumulate fumarate was determined. This was done by determining the total malate concentration by HPLC and by determining the L-malate concentration by using L-malate dehydrogenase. The stereochemical composition of the malate formed by 88 strains (55 strains isolated on maleate and 33 strains from culture collections with the highest maleate-degrading activity) was determined. These strains all produced D-malate with an enantiomeric purity of at least 97%.

Strains which accumulated fumarate during incubation with maleate formed a much higher percentage of L-malate than strains which did not. The 15 fumarate-accumulating strains tested produced malate which contained between 20 and 90% L-malate. When the L-malate concentration, as determined by the enzymatic assay, is plotted on a graph against the concentration of fumarate, as determined by HPLC, a straight line could be drawn through these points (Fig. 2). The slope of the line obtained by linear regression analysis was 4.6 ($r(x,y) = 0.96$).

Description of *Pseudomonas* sp. strain NCIMB 9867. *Pseudomonas* sp. strain NCIMB 9867 was selected for further studies because it had one of the highest specific maleate hydratase activities and did not show as many contaminants on the HPLC chromatogram as most of the other screened microorganisms did.

This cream-colored *Pseudomonas* sp. was further characterized. It was a gram-negative motile rod (0.3 to 0.5 by 1.3 to 2.9 μm) with one polar flagellum. It was oxidase positive and did not produce a fluorescent pigment. The strain did not contain arginine dihydrolase, urease, or β-galactosidase activity. It was able to reduce nitrate to nitrite but was not able to denitrify. The strain did not hydrolyze esculin, gelatin, or Tween 80. It could grow at 41°C and did not ferment glucose. It could utilize fructose, L-arginine, L-serine, L-phenylalanine, β-alanine, itaconate, mesaconate, fumarate, citraconate, succinate, glutarate, glycerate, L-malate, D-malate, β-hydroxybutyrate, glycerol, ethanolamine, or betaine as the sole source of carbon and energy for growth. Glucose, sucrose, maleate, malonate, acrylate, crotonate, 2,5-dimethylphenol, and *m*-cresol were not utilized. On the basis of these results, strain NCIMB 9867 was identified as a *P. pseudoalcaligenes* strain according to *Bergey's Manual of Systematic Bacteriology* (25).

Although permeabilized cells of *P. pseudoalcaligenes* could convert maleate into D-malate and Hopper et al. (17) showed that gentisate is degraded via maleate in this micro-

TABLE 1. Distribution of the different maleate-transforming activities among various microorganisms from culture collections

Genera	Total no. of strains tested	No. of strains exhibiting:		
		No maleate consumption	Maleate consumption	
			Fumarate accumulation	No fumarate accumulation
Bacteria				
Acinetobacter	1			1
Actinoplanes	1			1
Arthrobacter	3		1	2
Bacillus	4	1	1	2
Brevibacterium	4			4
Citrobacter	1	1		
Corynebacterium	4		1	3
Enterobacter	2		2	
Escherichia	2	2		
Hyphomicrobium	1	1		
Klebsiella	1			1
Lactobacillus	2	2		
Lactococcus	1	1		
Leuconostoc	2	2		
Micrococcus	1			1
Mycobacterium	5			5
Nocardia	11			11
Proteus	1			1
Pseudomonas	11		3	8
Rhodococcus	3			3
Serratia	1		1	
Streptococcus	1	1		
Streptomyces	4			4
Xanthobacter	2			2
Yeasts				
Arxula	1	1		
Candida	4	1		3
Hansenula	1			1
Kluyveromyces	1	1		
Octosporus	1	1		
Pichia	2	1		1
Rhodotorula	10	3	2	5
Saccharomyces	3	1	1	1
Schizosaccharomyces	1			1
Sporobolomyces	2	1		1
Stephanoascus	1	1		
Trichosporiella	1		1	
Trichosporon	3		1	2
Yarrowia	1			1
Fungi				
Aspergillus	3	1		2
Botrytis	1	1		
Dipodascus	1		1	
Exophilia	1			1
Mortriella	1		1	
Penicillium	3			3
Phanerochaete	1			1
Pleurotus	1			1
Rhizopus	1			1
Trichoderma	1	1		

organism, this strain was not able to grow on maleate as the sole source of carbon and energy, presumably because of its inability to transport maleate into growing cells. Of the other screened strains which did not accumulate fumarate during maleate degradation, not one of the 33 strains with the highest maleate-degrading activity from the culture collections and only 11 of the 55 strains isolated on maleate were actually able to grow on maleate.

Intact cells of *P. pseudoalcaligenes* grown on several dicarboxylic acids (fumarate, succinate, L-malate, D-malate, itaconate, mesaconate, or citraconate) did not oxidize or degrade maleate, although maleate was converted into D-malate by permeabilized cells grown on these substrates.

In growth experiments with dicarboxylic acids (fumarate, succinate, L-malate, D-malate, itaconate, mesaconate, and citraconate), no increase in the optical density was observed

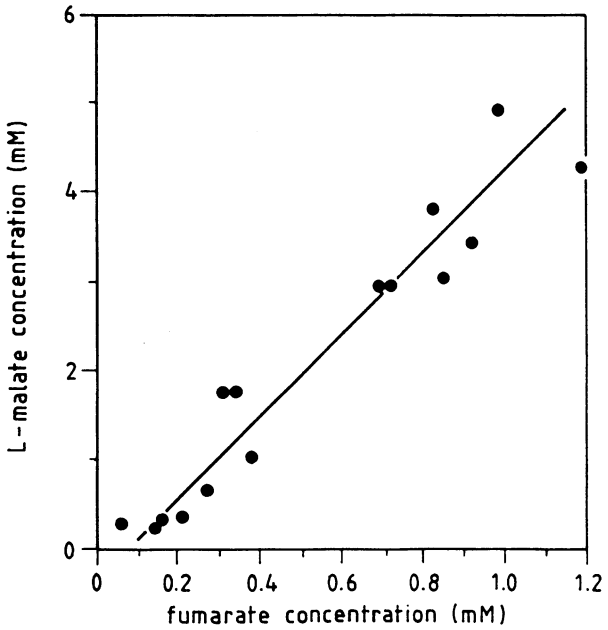

FIG. 2. Relationship between L-malate and fumarate accumulated from maleate by permeabilized cells.

when maleate was added in comparison with a control without maleate. The presence of maleate did not influence the growth rates of *P. pseudoalcaligenes* on these dicarboxylic acids.

Characterization of D-malate production by *P. pseudoalcaligenes*. When permeabilized cells of *P. pseudoalcaligenes* were incubated with 15 mM maleate under anaerobic conditions, maleate was degraded within 1 h to a level below the detection limit (<0.01 mM), with a stoichiometric formation of D-malate. No fumarate (detection limit of fumarate, ~0.002 mM) or L-malate could be detected. D-Malate consumption was not observed during the time of the experiment (5 h). Under aerobic conditions, a similar yield of D-malate from maleate was observed.

Maleic anhydride, which chemically hydrates to maleic acid and which is more than two times cheaper than maleic acid, was also completely transformed into D-malate by permeabilized cells of *P. pseudoalcaligenes*.

The optical rotation of the malate formed from maleate, maleic anhydride, and fumarate by *P. pseudoalcaligenes* was determined after complexation with molybdate and citrate (Table 2). The specific optical rotation of the products of the hydratase reactions was in agreement with the specific rotation of the expected products. Also, an increase in optical rotation due to the presence of citrate, specific for malate (21), was observed. The observed specific optical rotations indicate an enantiomeric purity which is close to 100% for the malate formed.

TABLE 2. Specific optical rotations ($[\alpha]_D^T$) of the products formed from the unsaturated acids by *P. pseudoalcaligenes* after complexation with molybdate and citrate

Product	$[\alpha]_D^T$
From maleate	−111.8
From maleic anhydride	−111.9
From fumarate	+111.9
D-Malate (Janssen Chimica)	−111.6
L-Malate (Merck)	+111.0

The enantiomeric excess of the D-malate formed from maleate and maleic anhydride was determined more precisely by using commercially available L-malate dehydrogenase and D-malic enzyme. An enantiomeric purity of 99.97% was determined for the D-malate formed from maleate, and an enantiomeric purity of 99.996% was determined for the D-malate formed from maleic anhydride.

DISCUSSION

In this report, the production of D-malate from maleate was demonstrated for a number of culture collection strains and environmental isolates.

Two enzymes are known that transform maleate: (i) maleate hydratase, yielding D-malate, and (ii) maleate *cis-trans*-isomerase, yielding fumarate, and as a consequence, the undesired L-malate is formed by the action of the citric acid cycle enzyme fumarase (Fig. 1). As we wanted to select for strains containing only the first enzyme, a simple screening method was used in which incubations of permeabilized cells with maleate were analyzed by HPLC for malate and fumarate accumulation. The microorganisms used in the screening were permeabilized with Triton X-100 since the uptake of dicarboxylic acids (e.g., fumarate, L-malate, D-malate, and succinate) into the microbial cell is known to be dependent on specific transport mechanisms (28). Because we wanted to select microorganisms which do not produce maleate *cis-trans*-isomerase under any circumstances, maleate, which has been reported to be an inducer of maleate *cis-trans*-isomerase (35), was included in the growth medium.

Accumulation of fumarate during incubation of permeabilized cells with maleate was shown to be indicative for the presence of the unwanted maleate *cis-trans*-isomerase activity. The fumarase reaction has an equilibrium constant (K_{eq} = [L-malate]/[fumarate]) of 4.5 under the assay conditions used (2), and, as a consequence, both fumarate and L-malate will accumulate under anaerobic conditions. As fumarate can be detected with a 70-times-higher sensitivity than malate, low levels of maleate *cis-trans*-isomerase activity could already be detected.

Fumarate was shown to accumulate at about 22% of the L-malate concentration (Fig. 2) in experiments were the L-malate concentration was determined separately. This ratio is in good agreement with the equilibrium constant for fumarase. The enantiomeric composition of the malate formed can therefore be estimated fairly accurately from one HPLC run in which the total malate and fumarate concentrations are determined.

The enantiomeric composition of the malate formed by some of the fumarate-accumulating microorganisms showed a remarkable variation (between 20 and 90% L-malate was detected). Apparently all of these strains containing maleate *cis-trans*-isomerase also contained maleate hydratase activity. Rahatekar et al. (27) already reported a *Pseudomonas* strain that contained both maleate hydratase and maleate *cis-trans*-isomerase activity.

All strains degrading maleate without a significant accumulation of fumarate produced D-malate with an enantiomeric purity of at least 97%. These strains probably only contain maleate hydratase activity for the degradation of maleate. When maleate is degraded via maleate hydratase, it is transformed into D-malate, which is not oxidized any further under anaerobic conditions. Theoretically, D-malate could be converted into L-malate via oxidation to oxaloace-

tate and a subsequent reduction to L-malate. D-Malate dehydrogenase has to our knowledge, however, not yet been described in bacteria. Furthermore, no interconversion of D- and L-malate has been reported in the literature even when the enantiomers are degraded by a common pathway (18, 31–33). Fumarate formation from D-malate under anaerobic conditions is, therefore, not very likely to occur. This was also confirmed by the fact that we were able to select maleate hydratase-containing microorganisms which exclusively accumulated D-malate from maleate.

Maleate hydratase activity appeared to be widespread among bacteria, yeasts, and fungi (Table 1). Of the culture collection strains tested, at least 63% contained maleate hydratase activity and no *cis-trans*-isomerase activity. This percentage was much lower (28%) in the strains isolated on maleate. All strains reported in the literature and most of our own isolates capable of growth on maleate as the sole source of carbon and energy contained the undesired maleate *cis-trans*-isomerase activity. Therefore, the selection of maleate-utilizing strains for the production of D-malate from maleate was not very worthwhile.

The percentage of microorganisms containing maleate hydratase activity was much higher than we expected, indicating that the enzyme may play a role in central metabolism, especially since most of these microorganisms were unable to grow on maleate. Maleate is known to be an intermediate in the aerobic degradation pathway of nicotinic acid, and, therefore, maleate hydratase might play a role in the salvaging of pyridine nucleotides under conditions of nutrient depletion (22). The aerobic degradation pathway for nicotinic acid described by Behrman and Stanier (1) involves maleate conversion into fumarate by maleate *cis-trans*-isomerase; however, on the basis of the results in Table 1, the hydratation of maleate to D-malate is much more likely to occur in most microorganisms.

Only 20% of the maleate-hydratase-positive, *cis-trans*-isomerase-negative strains were able to grow on maleate as the sole source of carbon and energy. In addition, most of the maleate-hydratase-positive, *cis-trans*-isomerase-negative strains which were isolated from enrichment cultures with maleate also did not grow on maleate. Possibly, the enrichment cultures still contained a large number of microorganisms which were unable to grow on maleate but which could grow on impurities in the agar plates. Evans et al. (13) found similar results. Of 32 strains positive for phenylalanine ammonia lyase that were isolated on *trans*-cinnamic acid, not one strain was able to grow on *trans*-cinnamic acid as the sole source of carbon and energy.

Pseudomonas sp. strain NCIMB 9867, which was selected for further studies, was previously reported to be a *Pseudomonas alcaligenes* by Poh and Bayly (26). Strain NCIMB 9867 does not, however, contain arginine dihydrolase activity and was able to grow on fructose, β-hydroxybutyrate, mesaconate, itaconate, glycerate, glutarate, L-serine, L-phenylalanine, glycerol, ethanolamine, and betaine and is therefore reclassified as *P. pseudoalcaligenes* according to *Bergey's Manual of Systematic Bacteriology* (25). Growth on 2,5-dimethylphenol and *m*-cresol, as reported by Hopper et al. (18), was not observed. Poh and Bayly (26) already found that spontaneous mutants which had lost the ability to grow on these two substrates were isolated readily, and they suggested that a plasmid is involved in the degradation of these two compounds.

P. pseudoalcaligenes did not grow on maleate, although it was able to grow on 3-hydroxybenzoate and gentisate, compounds which are known to be degraded through maleate and D-malate by this organism (17). Even when *P. pseudoalcaligenes* was grown on other dicarboxylic acids, which might induce a transport mechanism also capable of maleate transportation (28), intact cells were not able to oxidize or degrade maleate. The most likely explanation for the inability of *P. pseudoalcaligenes* to grow on maleate, therefore, is that it is not capable of synthesizing a transport mechanism for maleate.

Maleate is degraded by permeabilized *P. pseudoalcaligenes* to undetectable levels under anaerobic conditions with a concomitant formation of D-malate. The molar yield is close to 100%. From the equilibrium constant of the fumarase reaction (2) and the free energy for the maleate *cis-trans*-isomerase reaction (9), a K_{eq} ([D-malate]/[maleate]) of 275 can be calculated (30°C). This equilibrium constant corresponds to a maximum theoretical yield of 99.6%.

This conversion is significantly higher than that observed for the fumarase reaction (82%) (2) and that observed for the mesaconate (2-methylfumarate) hydratase reaction (85%) (4). Citraconate (2-methylmaleate) can, however, be converted to D-citramalate, with a yield which is also close to 100% (34).

The stoichiometric formation of D-malate from maleate, even under aerobic conditions, indicates that there is no D-malate-degrading activity present in permeabilized cells of this strain. Hopper et al. (18) already reported that there was no D-malate-degrading activity detectable in cell extracts of this strain.

The stereospecificity of the maleate hydratase reaction is very high, and the small amount of L-malate which is formed (0.03%) can be accounted for almost completely by the contamination with fumarate (0.02%) of the maleate used. The contaminating fumarate is converted into L-malate by fumarase, resulting in a reduced enantiomeric purity. By using maleic anhydride, which contains less fumarate, D-malate with an enantiomeric purity of 99.996% was produced. The enantiomeric purity of the D-malate produced with the maleate hydratase reaction is very good compared with that of the commercially obtained D-malate, which was 98.7%.

Subsequent work will focus on the optimization of D-malate production from maleate with the selected *P. pseudoalcaligenes* strain.

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