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Glutathione-Independent Isomerization of Maleylpyruvate by *Bacillus megaterium* and Other Gram-Positive Bacteria

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Maleylpyruvate, the ring fission product of gentisic acid, was found to be isomerized to fumarylpyruvate without a requirement for glutathione by an enzyme activity found in cell extracts of *m*-hydroxybenzoate-grown *Bacillus megaterium* 410. The isomerization reaction was detected as a shift in the absorbance maximum from 330 nm, the maximum for maleylpyruvate, to 345 nm, the maximum for fumarylpyruvate, when assayed at pH 8.0. Ammonium sulfate precipitation and dialysis of *B. megaterium* cell extracts resolved the isomerase activity from low-molecular-weight compounds such as glutathione but did not eliminate the isomerase activity. Iodoacetate and *p*-chloromercuribenzoate were potent inhibitors of the isomerase from *B. megaterium*. However, *N*-ethylmaleimide and iodoacetamide did not significantly inhibit this activity. In addition, fumaric acid was demonstrated as a product of gentisate oxidation by dialyzed cell extracts of *B. megaterium*. Glutathione-independent maleylpyruvate isomerases with properties similar to the isomerase found in *B. megaterium* were also found in other genera of gram-positive organisms. Eleven different organisms representing the genera *Bacillus*, *Arthrobacter*, *Corynebacterium*, *Nocardia*, and *Rhodococcus* were all found to possess this novel type of glutathione-independent maleylpyruvate isomerase.

The ring fission product of gentisate oxidation, maleylpyruvate [2,4-diketo-(*cis*)-5-hepta-enedioic acid], was first shown to be isomerized to fumarylpyruvate [2,4-diketo-(*trans*)-5-hepta-enedioic acid] with a requirement for a catalytic amount of glutathione by a *Pseudomonas* isolate (Fig. 1A) (17, 18). The product of this isomerization, fumarylpyruvate, was then hydrolyzed to fumaric acid and pyruvate. Later reports on the bacterial catabolism of gentisate have also described the requirement for glutathione as a cofactor of maleylpyruvate isomerases in other gram-negative organisms (5, 15, 27). However, in the case of the glutathione-dependent maleylpyruvate isomerase from *Moraxella* sp. strain OA3, no enzymatic activity was observed with substituted maleylpyruvates (6).

Organisms which oxidize aromatic substrates to substituted maleylpyruvates via substituted gentisates have been observed to utilize direct hydrolytic fission of the maleylpyruvate rather than isomerization (Fig. 1B). Studies by Hopper et al. of the metabolism of xylenols by *Pseudomonas alcaligenes* 25X first demonstrated that the hydrolysis of maleylpyruvate (12) and its substituted derivatives (13) could occur without prior isomerization. This hydrolytic activity had no requirement for glutathione. A maleylpyruvate hydrolase pathway has also been demonstrated in a strain of *Arthrobacter* which catabolizes 2,4,5-trimethoxybenzoate via 4-methoxygentisate (19). 4-Methoxygentisate was oxidized to a methoxylated maleylpyruvate which undergoes direct hydrolysis without isomerization.

Bacteria of the genus *Bacillus* have been frequently reported to possess a maleylpyruvate hydrolase when grown on aromatic substrates which are oxidized via gentisic acid (4, 5, 7). In only two of the strains of *Bacillus* examined, however, was maleic acid reported to be identified as a product of gentisate oxidation (4). A rapid spectrophotometric assay has been described (5) which has been used to show maleylpyruvate hydrolase activity in other strains of *Bacillus* (4, 5, 7). This assay makes use of the transient accumulation of a metabolic product of gentisate oxidation as monitored by UV absorbance at 330 nm. In the absence of an effect of either glutathione or *N*-ethylmaleimide (NEM), it is assumed that the decrease in absorbance at 330 nm is a measure of maleylpyruvate hydrolase.

In this report, we present evidence for an enzymatic activity in a strain of *Bacillus megaterium* which catalyzes the isomerization of maleylpyruvate to fumarylpyruvate and which is insensitive to the addition of either glutathione or NEM. This type of glutathione-independent isomerase has also been detected in 10 other strains of gram-positive organisms representing five different genera.

(This work was presented in part previously [S. R. Hagedorn, S. Keenan, and P. J. Chapman, Annu. Meet. Am. Soc. Microbiol. 1977, Q86, p. 275]).

MATERIALS AND METHODS

Growth of organisms. Organisms were grown in minimal medium (11) supplemented with 0.005% yeast extract and Casamino Acids (Difco Laboratories, Detroit, Mich.). The principal carbon source was added to a final concentration of 0.05%. *B. megaterium* 410, a laboratory isolate, was grown with *m*-hydroxybenzoate as the principal carbon source. All other organisms were grown on carbon sources as indicated in the text. One liter of this medium was inoculated with a 5-ml inoculum, and the culture was grown overnight at 30°C with shaking. Cells were harvested by centrifugation, washed with 50 mM Na-KH_2PO_4 buffer (pH 7), and stored frozen at −10°C.

Preparation of cell extracts. Cell extracts were prepared by crushing a frozen cell pellet, as described by Hughes (14). The ruptured cell paste was suspended in 1.0 ml of 50 mM Na-KH_2PO_4 buffer (pH 7.0) per g of cell paste. The cell extract was treated with 100 μg of RNase and DNase per ml for 10 min, followed by centrifugation at 27,000 × *g* for 15 min. The recovered supernatant was used for enzyme as-

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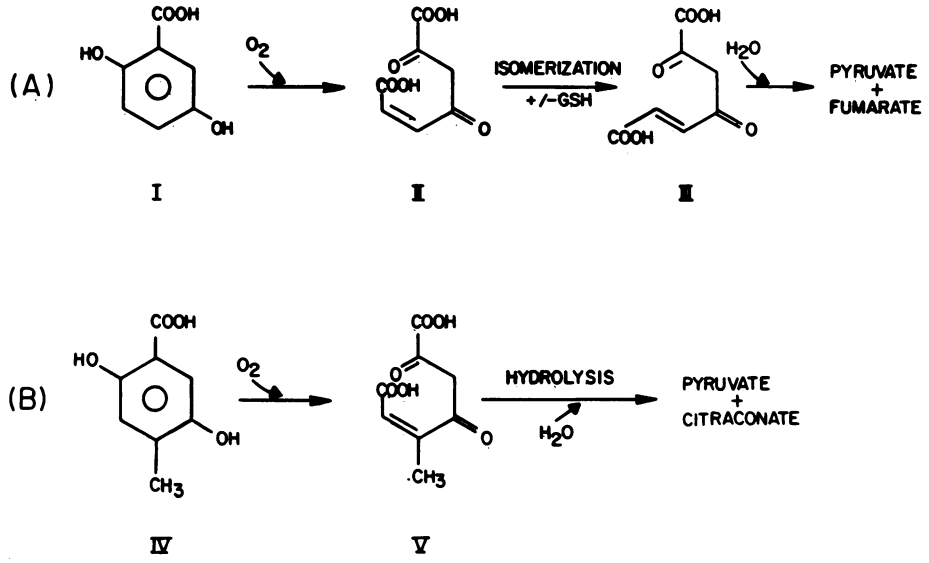

FIG. 1. Pathways of gentisate and substituted gentisate metabolism in bacteria. (A) Isomerization pathway (17, 18). (B) Hydrolytic pathway (13). Compound: gentisate (I), maleylpyruvate (II), fumarylpyruvate (III), 4-methylgentisate (IV), and 5-methylmaleylpyruvate (V).

says. Protein was determined by the method of Lowry et al. (20). Ammonium sulfate precipitation was accomplished by the slow addition of cold, neutral, saturated $(NH_4)_2SO_4$ solution to stirred cell extracts at 0°C to the desired percent saturation. Precipitated protein was centrifuged at 27,000 × *g* after 1 h of equilibration and dissolved in the original volume of buffer.

Preparation of substrates. Maleylpyruvate was prepared by the oxidation of gentisic acid with a dialyzed cell extract of *Moraxella* sp. strain OA3 grown on salicylate by the procedure of Lack (18). One milliliter (28 mg of protein) of a dialyzed preparation of *Moraxella* sp. strain OA3 grown on salicylate was incubated with 0.1 ml of 0.1 M $Fe(NH_4)_2SO_4$ for 30 min to reactivate the gentisate dioxygenase. This reactivated preparation of *Moraxella* sp. strain OA3 was added to 20 ml of Na-KH_2PO_4 buffer (0.05 M, pH 7.5) containing 900 μmol of gentisic acid and 900 μmol of ammonium chloride. The reaction mixture was monitored by diluting a sample 1:600 and scanning the spectrum from 360 to 280 nm for the presence of maleylpyruvate. The pH was monitored and periodically adjusted to pH 7.5 by the addition of 1.0 N NaOH. Gentle aeration with stirring was found to accelerate the rate of gentisate oxidation. As the reaction proceeded, the color of the incubation was observed to go from purple to a yellow-orange color. When no further increase in absorbance was seen, the reaction was stopped by the addition of 100 ml of cold 95% ethanol. The precipitate was centrifuged, and the denatured protein pellet was discarded. To the supernatant was added 360 ml of absolute diethyl ether, and the mixture was allowed to stand for 2.0 h. The yellow flocculent precipitate gradually settled, and the majority of the supernatant was decanted. The remaining precipitate was recovered and placed under vacuum overnight. This desiccated preparation was stored in the cold and was stable for several years. Maleylacetoacetate was similarly prepared with a dialyzed cell extract of *Moraxella* sp. strain OA3 grown on tyrosine. Fumarylpyruvate was prepared by continuous extraction with diethyl ether of a solution of maleylpyruvate acidifed with *meta*-phosphoric acid (26). The recovered fumarylpyruvate was crystallized from ethylacetate-petroleum ether. Dessicated maleylpyruvate, maleylacetoacetate, and fumarylpyruvate were stored at 4°C and were stable for several years.

Enzyme assays. Maleylpyruvate isomerase, maleylacetoacetate isomerase, and fumarylpyruvate hydrolase were assayed with solutions of substrates prepared each day. Assays were initiated by the addition of cell extract to 0.2 μmol of maleylpyruvate, maleylacetoacetate, or fumarylpyruvate in 3.0 ml of 0.1 M Tris buffer (pH 8.0). Absorbance changes were then measured at 330, 320, or 345 nm, respectively. When maleylpyruvate isomerase was at a greater specific activity than fumarylpyruvate, the assay was monitored by alternating between 330 and 345 nm. Rates of isomerization of maleylpyruvate to fumarylpyruvate were calculated with a value of 2,400 cm^{-1} M^{-1} for the extinction changes at 330 nm. This value was used whenever an extract was found to have a higher specific activity of maleylpyruvate isomerase than of fumarylpyruvate hydrolase. The extinction coefficient used for maleylpyruvate was 13,000 cm^{-1} M^{-1} at 330 nm. Maleylacetoacetate was 14,000 cm^{-1} M^{-1} at 320 nm, and fumarylpyruvate was 12,000 cm^{-1} M^{-1} at 345 nm. When assaying for a glutathione-dependent isomerase, glutathione was added to a final concentration of 17 μM in a cuvette.

Analysis for glutathione content. The amount of glutathione present in cell extracts was determined by the procedure of Fahey et al. (9) with glutathione reductase.

Recovery of products of gentisate oxidation. The recovery of products of gentisate oxidation for analysis by paper chromatography and gas chromatography-mass spectrometry (GC-MS) was performed by the following procedure. Gentisate (60 μmol) was oxidized by 2.0 to 5.0 mg of cell extract protein in 30.0 ml of 50 mM phosphate buffer (pH 7.0). The reaction was monitored spectrophotometrically at 330 and 345 nm. When no further decrease in absorbance was observed at these wavelengths, the reaction was acidified with 0.3 ml of 2 N sulfuric acid. The volume was reduced by rotary evaporation at 60°C to a final volume of 2.0 to 3.0 ml, and 4.0 g of anhydrous magnesium sulfate was added slowly to sequester the remaining water. Diethyl ether (30 ml) was added, and the magnesium sulfate was triturated therein. After standing in ether overnight, the diethyl ether

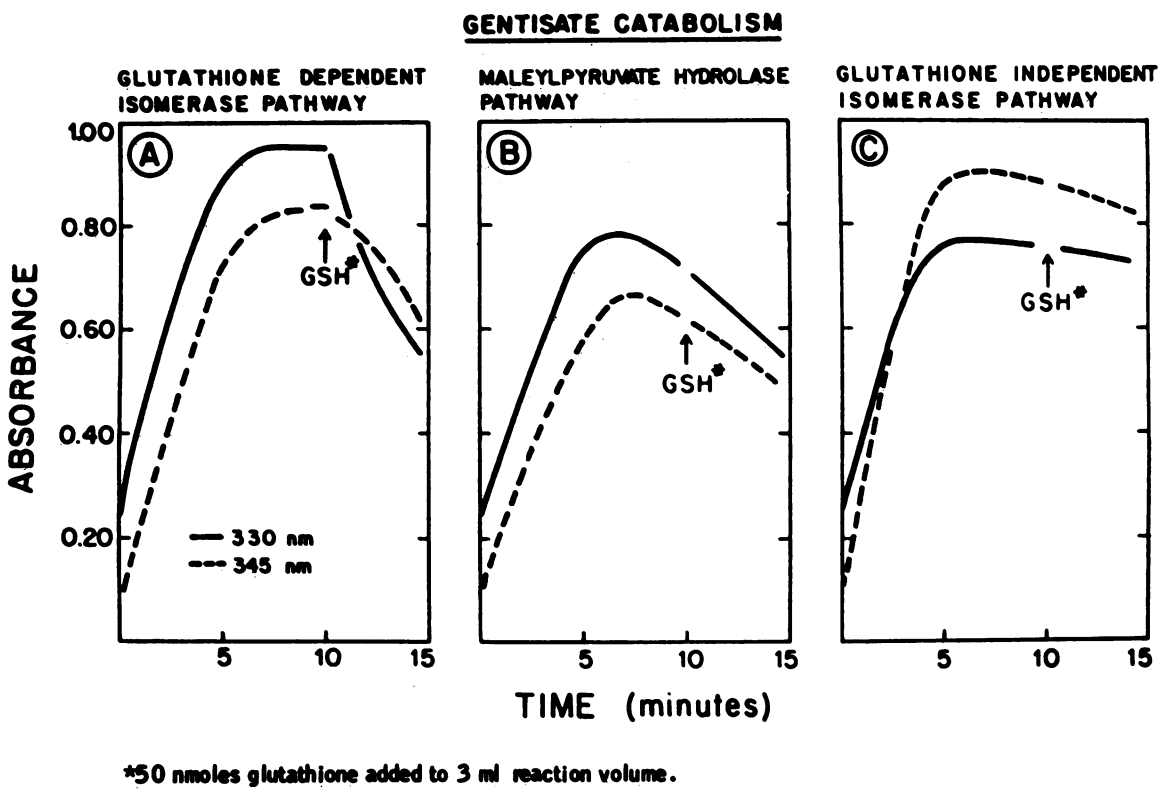

FIG. 2. Spectrophotometric tracings of gentisate oxidation by cell extracts of bacteria possessing various mechanisms of maleylpyruvate metabolism. Each assay contained 0.22 μmol of gentisate and 300 μmol of Tris buffer (pH 8) in a 3.0-ml final volume. Assays were initiated by the addition of 0.2 to 0.8 mg of protein of cell extracts. (A) Glutathione-dependent maleylpyruvate isomerase (*Moraxella* sp. strain OA3). (B) Maleylpyruvate hydrolase (*P. alcaligenes* 25X). (C) Glutathione-independent maleylpyruvate isomerase (*B. megaterium* 410). Arrow indicates time at which glutathione was added to the assay to a final concentration of 17 μM.

was decanted and evaporated, and the recovered residue was analyzed by paper chromatography (see below). Cell extract added to solutions of fumaric acid and maleic acid in buffer served as controls which were subjected to the same diagnostic procedure. Neither fumaric acid nor maleic acid was chemically altered by this method. Metabolites analyzed by GC-MS were purified by descending paper chromatography and extracted from the paper with diethyl ether.

Paper chromatography. Descending paper chromatography was performed with eucalyptol–*n*-propanol–formic acid–water (10:10:4:1) as the solvent. Fumaric acid and maleic acid could be detected initially as UV absorbing spots which gave yellow spots against a blue background when sprayed with a neutral ethanolic solution of 0.05% bromocresol green. Fumaric acid (R_f = 0.81) was readily resolved from maleic acid (R_f = 0.50) by this system.

GC-MS analysis. Samples of fumarate analyzed by GC-MS were first derivatized with a 1:1 mixture of hexamethyldisilazane-trimethylchlorosilane to give the bistrimethylsilyl ester of fumarate. Derivatized samples were then chromatographed by gas-liquid chromatography on 3% OV17 at 100°C, and mass spectra were obtained on the resolved peak of the bistrimethylsilyl ester of fumaric acid. GC-MS analysis was performed on an LKB 9000 instrument.

Deuterium oxide isotope experiment. The effect of deuterium oxide (D_2O) as a solvent on isomerization was determined by measuring the rate of isomerization and the products of isomerization in the presence of either water or D_2O. Enzyme assays and the recovery of fumaric acid were performed as described above, except that the buffer was prepared with anhydrous phosphate salts and 99% enriched deuterium oxide. The percent enrichment of deuterium in the fumarate was determined by analysis of the mass spectrum of the bistrimethylsilyl ester of fumarate after being resolved by gas chromatography. The isotope effect on the rate of isomerization was determined by taking the ratio of the rate of isomerization measured in water versus the rate measured in D_2O. (Isomerase isotope effect = rate of isomerization in water/rate of isomerization in D_2O.)

Materials. Glutathione reductase, RNase, DNase, enzymes, cofactors, inhibitors, gentisate, homogentisate, and Tris were obtained from the Sigma Chemical Co., St. Louis, Mo. All other materials were of the highest grade commercially available.

RESULTS

Glutathione-independent isomerization of maleylpyruvate. When gentisate was oxidized by cell extracts from *B. megaterium*, an absorbance change (Fig. 2C) was observed. Fumarylpyruvate was identified as the accumulating metabolite by the retention of its absorbance at 330 nm when the assay mixture was acidified. This was a characteristic of fumarylpyruvate which distinguished it from maleylpyruvate (17, 18). The UV absorbance of maleylpyruvate was abolished in the region of 330 nm under acidic conditions. When maleylpyruvate was used as a substrate, it was found to be rapidly isomerized to fumarylpyruvate without the addition of exogenous glutathione. The isomerization of maleylpyruvate to fumarylpyruvate was detected as an increase in

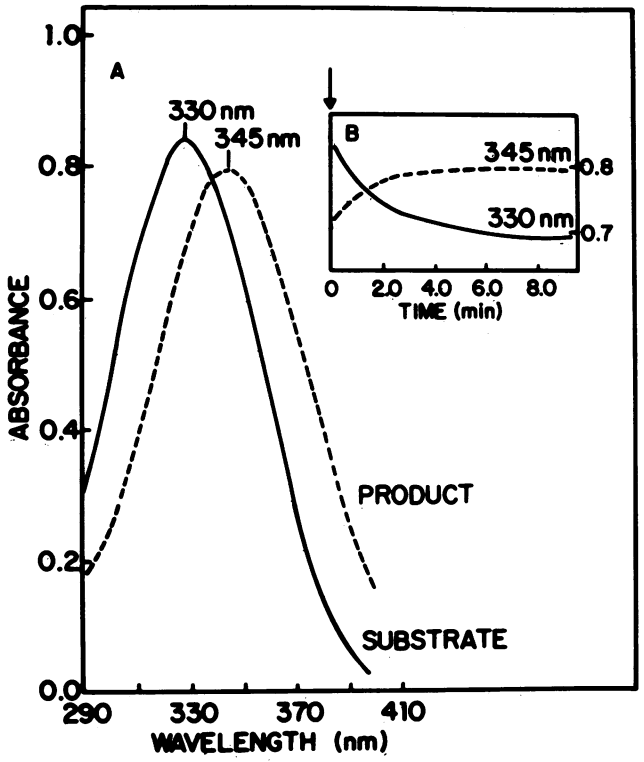

FIG. 3. Isomerization of maleylpyruvate by 40 to 60% ammonium sulphate protein precipitate of *B. megaterium* cell extract. The assay contained 0.2 μmol of maleylpyruvate and 300 μmol of Tris buffer (pH 8) in a 3.0-ml final volume. The assay was initiated at the arrow by the addition of 0.5 mg of a 40 to 60% ammonium sulfate fraction of *B. megaterium*. (A) Spectrum of the substrate, maleylpyruvate (—). Spectrum of the product, fumarylpyruvate (– –). (B) Spectrophotometric monitoring of the enzymatic isomerization of maleylpyruvate at 330 nm (—) and 345 (– –).

absorbance at 345 nm relative to the absorbance at 330 nm (Fig. 3B). Comparison of the spectra reveals a shift in the absorbance maximum from 330 nm for the substrate to 345 nm for the product (Fig. 3A). When gentisate was used as a substrate with a cell extract of *B. megaterium* 410, fumaric acid was extracted and identified as a reaction product. Both paper chromatography and GC-MS were used to establish its identity. Maleic acid was recovered unchanged in control experiments.

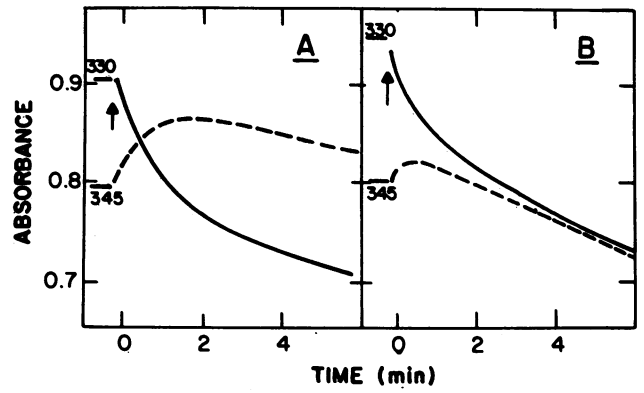

FIG. 4. Effect of pH on the spectrophotometric assay for maleylpyruvate isomerization. Each assay contained 0.2 μmol of maleylpyruvate in 1.0 ml of 100 mM Tris buffer at pH 8.0 (A) and pH 7.0 (B). Isomerization was monitored at 330 nm (—) and 345 nm (– – –). The arrow indicates initiation of the assay by the addition of a cell extract of *B. megaterium*.

TABLE 1. Effect of ammonium sulfate precipitation and dialysis on maleylpyruvate isomerase

Treatment	Glutathione concn (mM)	Sp act (μmol/min per mg of protein) of maleylpyruvate isomerase from:	
		Moraxella sp. strain OA3	*B. megaterium* 410
Cell extract	0	0.03	4.70
	0.02	5.02	5.00
Ammonium sulfate precipitation (80% saturated)	0	<0.004	0.70
	0.02	4.30	1.00
Dialysis (12 h against 500 volumes)	0	<0.004	1.65
	0.02	5.22	2.44

Substrate specificity. The glutathione-independent maleylpyruvate isomerase in cell extracts of *B. megaterium* 410 demonstrated the highest activity toward maleylpyruvate and greatly reduced activity toward maleylacetoacetate. The specific activity of the isomerase towards maleylpyruvate was 4.90 μmol/min per mg of protein, whereas the specific activity towards maleylacetoacetate was only 0.002 μmol/min per mg of protein. This demonstrated that the maleylpyruvate isomerase in *B. megaterium* 410 had an activity which differed from that of a maleylacetoacetate isomerase known to be involved in homogentisate catabolism.

Effect of pH on the assay of maleylpyruvate isomerase. The ability to detect the isomerization of maleylpyruvate was found to be strongly pH dependent due to the spectral properties of the product, fumarylpyruvate. The absorption maximum of fumarylpyruvate was found to vary from 335 nm at pH 7.0 to 345 nm at pH 8.0. However, the absorption maximum of maleylpyruvate remained relatively constant at 330 nm in this pH range. Isomerization was observed optimally by measuring an increase in absorbance at 345 nm with a decrease at 330 nm at pH 8.0 (Fig. 4A). A shift in absorption maximum was barely observed when maleylpyruvate isomerase was assayed at pH 7.0 (Fig. 4B). This was due to the similar absorption maxima of fumarylpyruvate and maleylpyruvate at pH 7.0.

Effect of ammonium sulfate precipitation and dialysis on maleylpyruvate isomerases. Ammonium sulfate precipitation and dialysis were used to compare the effects of these procedures on the glutathione-dependent isomerase from *Moraxella* sp. strain OA3 and the glutathione-independent isomerase from *B. megaterium* (Table 1). The low level of maleylpyruvate isomerase activity found in cell extracts of *Moraxella* sp. strain OA3 measured in the absence of added glutathione was completely eliminated by ammonium sulfate precipitation or dialysis. Maleylpyruvate isomerase activity of *Moraxella* sp. strain OA3 was increased by a factor of at least 10^3 when glutathione was added to the assay solution at a final concentration of 0.020 mM. This concentration permitted complete isomerization of 0.067 mM maleylpyruvate. In contrast, the isomerase from *B. megaterium* was only

partially inactivated by dialysis and ammonium sulfate precipitation. This partial inactivation appeared to be due to irreversible denaturation, since the addition of 0.02 mM glutathione only partially restored activity. A similar effect of cysteine was observed with cell extract preparations that had been inactivated by repeated freezing and thawing (unpublished data). The partial reactivation of maleylpyruvate isomerase from *B. megaterium* by glutathione may be due to a restoration of oxidized sulfhydryl groups in the partially denatured enzyme.

Effect of sulfhydryl inhibitors. NEM and iodoacetamide were found not to inhibit the maleylpyruvate isomerase activity from *B. megaterium*. This demonstrated that a freely dissociable sulfhydryl compound such as glutathione was not required by this isomerase (Table 2). However, *p*-chloromercuribenzoate and iodoacetate were both found to be potent irreversible inhibitors of this isomerase. The addition of excess glutathione to extracts inhibited by *p*-chloromercuribenzoate or iodoacetate did not restore isomerase activity in cell extracts of *B. megaterium*. The maleylpyruvate isomerase from *Moraxella* sp. strain OA3 was inhibited by all of these sulfhydryl inhibitors, but activity was restored by the addition of glutathione in excess of the amount of sulfhydryl inhibitor added.

Effect of deuterium oxide as a solvent for the isomerase. Fumaric acid recovered from the oxidation of gentisic acid by extracts in buffered solutions of deuterium oxide was found not to be enriched with deuterium when analyzed by GC-MS. This demonstrated that no solvent protons were incorporated when maleylpyruvate was isomerized to fumarylpyruvate. However, an isotope effect of 1.25 was observed on the rate of isomerization of maleylpyruvate when assayed in buffered solutions containing deuterium oxide.

Comparison of gentisate oxidation by UV spectrophotometry. Differences in the spectrophotometric changes for alternate pathways of gentisate oxidation at pH 8.0 are illustrated in Fig. 2. When gentisate is oxidized by a diluted cell extract of an organism known to possess a glutathione-dependent isomerase, maleylpyruvate accumulates and is not further metabolized until glutathione is added (Fig. 2A). Evidence of isomerization is shown by the crossover of 330- and 345-nm traces. Oxidation of gentisate by an extract of an organism possessing maleylpyruvate hydrolase results in a transient accumulation of maleylpyruvate which is degraded without the addition of glutathione (Fig. 2B). In this case, no crossover is observed. A very different pattern is observed with extracts of *B. megaterium* oxidizing gentisate (Fig. 2C). It can be seen that there is an early and rapid increase in absorbance at 345 nm, relative to that at 330 nm when assayed at pH 8.0 (Fig. 2C), which is indicative of isomerization and the accumulation of fumarylpyruvate.

Survey of bacteria. Sixteen different bacterial strains were examined for the pathway of gentisate oxidation to determine the mechanism of maleylpyruvate metabolism in other procaryotic organisms (Table 3). All of the gram-positive organisms examined appeared to possess a maleylpyruvate isomerase similar to that found in *B. megaterium* 410. Isomerase activity from all of the gram-positive organisms was not stimulated by glutathione (0.02 mM), nor was it inhibited by NEM (1.0 mM). In all of the gram-positive organisms, maleylpyruvate was shown to be isomerized to fumarylpyruvate as determined by a shift of the absorption maximum from 330 nm toward 345 nm and by the identification of fumaric acid as a product of gentisate metabolism. In all cases, control experiments demonstrated that fumaric acid did not arise from either the enzymatic or chemical isomerization of maleic acid.

TABLE 2. Effect of sulfhydryl inhibitors on maleylpyruvate isomerase activity from *B. megaterium*

Inhibitor	Concn in assay (mM)	% Inhibition
p-Chloromercuribenzoate	0.67	100
NEM	170.0	<4
Iodoacetate	33.0	100
Iodoacetamide	170.0	<4

With the exception of *P. alcaligenes* 25X, the gram-negative organisms examined all possessed glutathione-dependent maleylpyruvate isomerase. Glutathione (0.02 mM) increased the isomerase activity by a factor of 10^2 to 10^3, and 1.0 mM NEM reversibly inhibited isomerization. The inhibition by NEM was reversed by the addition of excess glutathione (1.1 mM). The situation in *P. alcaligenes* 25X is more complicated. Maleylpyruvate hydrolase activity is evident in extracts of cells grown with either *m*-hydroxybenzoate or 3,5-xylenol, but evidence of its isomerization by glutathione stimulation is also seen with extracts from *m*-hydroxybenzoate-grown cells. Crawford and Frick (5) noted the same effect. The presence of fumarylpyruvate hydrolase is also observed in *m*-hydroxybenzoate-grown cells as previously shown by Poh and Bayly (22); this enzyme has been purified by Bayly et al. (1). Despite this evidence for an isomerizing pathway under certain growth conditions, maleic acid formation is still possible in the absence of added glutathione.

Glutathione content. A number of the organisms examined in Table 3 were assayed for the presence of glutathione plus glutathione disulfide (GSH + 1/2 GSSG) by the use of glutathione reductase (Table 4) (9). Significant levels of glutathione were detected in the gram-negative organisms that were tested (4.98 to 0.33 μmol of GSH + 1/2 GSSG per g [dry weight]). However, glutathione was not detected in any of the three gram-positive organisms tested, which included *B. megaterium* 410, *Corynebacterium* sp., and *Bacillus* sp. strain B4 (<0.003 μmol of GSH + 1/2 GSSG per g [dry weight]). These results were in agreement with the levels of glutathione in gram-negative and gram-positive bacteria previously reported by Fahey et al. (10).

DISCUSSION

The maleylpyruvate isomerase in extracts of *B. megaterium* 410 has been demonstrated to be active, independent of glutathione or any other low-molecular-weight thiol. The isomerization of maleylpyruvate is in contrast to the previously reported hydrolysis of maleylpyruvate in other strains of *Bacillus* (3, 5). Glutathione-independent isomerases have also been demonstrated in 10 other strains of gram-positive bacteria representing five different genera. In contrast, the gram-negative bacteria examined in this report and in previous reports (6, 15, 17, 18, 27) all required glutathione for the isomerization of maleylpyruvate.

The substrate specificity of the glutathione-independent maleylpyruvate isomerase was similar to that described by Lack (18) for the glutathione-dependent maleylpyruvate isomerase from a gram-negative soil isolate. The maleylpyruvate isomerase in cell extracts of *B. megaterium* 410 had a greatly reduced activity toward maleylacetoacetate. This confirms the belief that the maleylpyruvate isomerase in this

TABLE 3. Survey of gentisate oxidation in bacteria

Organisms	Source or reference	Growth substrate[a]	Metabolic characteristics[g]						
			MPI[b]	MPH[b]	λ max[c] (nm)	FPH[b]	GSH[d]	NEM[e]	Product[f]
Gram positive									
Bacillus megaterium 410	Laboratory isolate	mHBA	1.5	NA	345	0.12	–	–	F
Bacillus sp. strain B4	Laboratory isolate	mHBA	2.2	NA	345	0.43	–	–	F
Bacillus stearothermophilus 6T-5	Laboratory isolate	mHBA	2.8	NA	345	0.11	–	–	F
Nocardia globerula CL1	P. W. Trudgill	mHBA	2.3	NA	345	0.15	–	–	F
Nocardia rhodnii	P. W. Trudgill	mHBA	3.2	NA	345	0.14	–	–	F
Corynebacterium sp.	P. W. Trudgill	mHBA	2.0	NA	345	0.17	–	–	F
Arthrobacter sp. strain 4CB/5-9	Laboratory isolate	mHBA	5.2	NA	345	0.38	–	–	F
Arthrobacter sp. strain 4CB/2-1	Laboratory isolate	mHBA	1.3	NA	345	0.15	–	–	F
Rhodococcus sp. strain A81	R. Crawford	mANS	7.2	NA	345	0.41	–	–	F
Bacterium PCI	Laboratory isolate	mHBA	5.0	NA	345	0.50	–	–	F
Nocardia sp. strain 3CS	Laboratory isolate	SAL	4.2	NA	345	0.30	–	–	F
Gram negative									
Pseudomonas acidovorans 16	ATCC 15666	mHBA	0.20	NA	330	0.41	+	NA	NA
Alcaligenes eutrophus 325	ATCC 17697 (15)	mHBA	1.60	NA	345	0.20	+	+	F
Moraxella sp. strain OA3	(6)	mHBA	1.90	NA	345	0.43	+	+	F
Moraxella sp. strain OA3	(6)	SAL	0.50	NA	330	0.79	+	NA	NA
Acinetobacter sp. strain CHC	NCIB 9870	mHBA	0.19	NA	330	0.58	+	NA	NA
Pseudomonas alcaligenes 25X	NCIB 9867	mHBA	NA	0.24	330	0.22	+	–	M
Pseudomonas alcaligenes 25X	NCIB 9867	3,5-Xyl	NA	0.13	330	0.06	–	–	M

[a] Growth substrate abbreviations: mHBA, *m*-hydroxybenzoate; pHBA, *p*-hydroxybenzoate; SAL, salicylate; 3,5-Xyl, 3,5-xylenol; and mANS, *m*-anisate.
[b] Specific activity (micromoles per minute per milligram of protein) for maleylpyruvate isomerase (MPI), maleylpyruvate hydrolase (MPH), and fumarylpyruvate hydrolase (FPH).
[c] λmax, Absorbance maximum during maleylpyruvate degradation. Values: 330 nm, constant absorbance maximum; 345 nm, shift in absorbance during assay.
[d] GSH, Effect of the addition of 20 μM glutathione on maleylpyruvate metabolism. Symbols: (+) stimulation; (−) no effect.
[e] NEM, Effect of the addition of 1.0 mM NEM on maleylpyruvate degradation. Symbols: (+) inhibition; (−) no effect.
[f] Product of gentisate oxidation by cell extracts of fumaric (F) or maleic (M) acid.
[g] NA, Not assayed.

gram-positive organism is unique from a maleylacetoacetate isomerase and is not being recruited from a homogentisate catabolic pathway.

The presence of glutathione-independent isomerases in the gram-positive organisms was correlated with the absence of glutathione in extracts of these organisms. Gram-positive organisms examined for the presence of glutathione were found to contain less than 0.003 μmol of GSH + 1/2 GSSG per g (dry weight). In contrast, the gram-negative bacteria examined in this report all possessed readily detectable levels of glutathione (0.33 to 4.98 μmol of GSH + 1/2 GSSG per g [dry weight]). These observations were in agreement with the report of Fahey et al. (10), who reported that most gram-positive bacteria had low or undetectable levels of glutathione, even though they possessed significant soluble thiols.

Previous reports on the metabolism of gentisate by *Bacillus* isolates (4, 5, 7) have used a spectrophotometric assay to distinguish maleylpyruvate isomerase from maleylpyruvate hydrolase. Since an organism may possess a maleylpyruvate isomerase which does not require glutathione, caution must be taken in interpreting these spectrophotometric assays when attempting to distinguish between hydrolysis and isomerization of maleylpyruvate. An organism which possesses a glutathione-independent isomerase at a lower specific activity than fumarylpyruvate hydrolase will appear to be similar to an organism which possesses a maleylpyruvate hydrolase by the rapid spectrophotometric assay described by Crawford (5). Under these conditions, a transient increase in maleylpyruvate would occur without the accumulation of fumarylpyruvate since hydrolysis would occur faster than the rate of production of fumarylpyruvate. This situation appears to account for the observations of Clark and Buswell (3) in their studies of gentisate catabolism in *Bacillus stearothermophilus*. Therefore, the identification of maleic acid as a product of gentisate catabolism is required to distinguish unambiguously between a glutathione-independent maleylpyruvate isomerase and a maleylpyruvate hydrolase. Although the spectrophotometric assay at pH 8 described here can be used to demonstrate a glutathi-

one-independent maleylpyruvate isomerase, it cannot be used to prove the presence of maleylpyruvate hydrolase.

Crawford and co-workers have reported that maleylpyruvate hydrolases are common to bacteria of the genus *Bacillus* (4, 5, 7). Maleylpyruvate hydrolase was apparently demonstrated by these investigators by a spectrophotometric assay to follow gentisate oxidation which was not affected by the addition of either glutathione or NEM. In only two of the *Bacillus* strains examined by these investigators was maleic acid reported to be identified as a product of gentisate catabolism. Since their spectrophotometric assay is ambiguous and inconclusive in distinguishing between a maleylpyruvate hydrolase and a glutathione-independent maleylpyruvate isomerase, additional evidence should be obtained to firmly establish the presence of maleylpyruvate hydrolases in all of the strains of *Bacillus* previously reported to possess this enzyme.

Maleylpyruvate hydrolases have been reported in other bacteria which were selected for growth on substrates metabolized via substituted gentisates. A substituted maleylpyruvate hydrolase was first shown to be involved in the catabolism of 2,5-xylenol via 4-methylgentisate in *Pseudomonas alcaligenes* 25X (13). Two different maleylpyruvate hydrolases have been shown in this organism (1). Their role in gentisate metabolism has been reconfirmed in this report by the identification of maleic acid as a product of gentisate catabolism. A substituted maleylpyruvate hydrolase has also been implicated in the catabolism of 4-methoxygentisate, resulting in the oxidation of 2,4,5-trimethoxybenzoate metabolism in *Arthrobacter* sp. strain 245T (19). In both of these bacteria, a hydrolytic step is employed without prior isomerization of the substituted maleylpyruvate. In addition, it is of note that the glutathione-dependent isomerase of *Moraxella* sp. strain OA3 fails to isomerize substituted maleylpyruvate (6).

The inhibitory effect of the sulfhydryl inhibitors, *p*-chloromercuribenzoate and iodoacetate, suggests that a sulfhydryl group is closely associated with the glutathione-independent maleylpyruvate isomerase but is not a freely dissociable cofactor such as glutathione. NEM and iodoacetamide have little effect on the maleylpyruvate isomerase from *B. megaterium*, which demonstrates that a freely dissociable cofactor such as glutathione does not function in this enzyme. However, the potent irreversible inhibition demonstrated by *p*-chloromercuribenzoate and iodoacetate suggests that a less accessible sulfhydryl group in this enzyme plays a crucial role in the isomerization of maleylpyruvate. This sensitive sulfhydryl group probably plays a role similar to that played by the sulfhydryl of glutathione in the glutathione-dependent maleylpyruvate isomerases.

The mechanism of the glutathione-independent maleylpyruvate isomerase from *B. megaterium* appears to be similar to that of other glutathione-dependent isomerases with respect to the participation of solvent protons (18, 24). Fumaric acid recovered from the oxidation of gentisic acid by cell extracts of *B. megaterium* in deuterated solvents was found not to be enriched with deuterium. This finding is identical to the results of Lack (18) obtained with a glutathione-dependent maleylpyruvate isomerase from a soil pseudomonad and the results of Seltzer and Lin (24) with the glutathione-dependent maleylacetoacetate isomerase from *Vibrio* sp. strain O1. Both of these reports demonstrated no deuterium enrichment resulting from the isomerization reaction. However, the isotope effect observed here for the rate of isomerization of maleylpyruvate by cell extracts of *B. megaterium* suggests that an exchangeable proton does play some role in the isomerization reaction. This observation could be accounted for by an exchangeable proton on a sulfhydryl group, which may play a role in the isomerization reaction.

TABLE 4. Glutathione content

Organism	Growth substrate	GSH + 1/2 GSSG[a] (μmol/g [dry wt])
Pseudomonas alcaligenes 25X	2,5-Xylenol	0.33
Pseudomonas alcaligenes 25X	*m*-Hydroxybenzoate	4.98
Moraxella sp. strain OA3	*m*-Hydroxybenzoate	1.65
Bacillus megaterium 410	*m*-Hydroxybenzoate	<0.005
Bacillus sp. strain B4	*m*-Hydroxybenzoate	<0.003
Corynebacterium sp.	*m*-Hydroxybenzoate	<0.003

[a] Total glutathione as determined by the glutathione reductase of Fahey et al. (8).

The results reported in this paper raise the question of how gram-positive bacteria perform other metabolic functions previously described as requiring glutathione. Glutathione has been reported to be required for the maintenance of sulfhydryl groups in the appropriate oxidation state and for the transport of amino acids (21). In addition, glutathione has been shown to be a cofactor for a variety of enzymes such as ribonucleoside reductase (11), glyoxylase (23), formaldehyde dehydrogenase (25), hydroperoxidase (2), and maleylacetoacetate isomerase (16). The presence of glutathione-independent isomerases in *B. megaterium* and other gram-positive bacteria demonstrated in this report reveals how these bacteria have evolved an alternative catabolic pathway for gentisate metabolism in the absence of glutathione.

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