The Gene *ncgl2918* Encodes a Novel Maleylpyruvate Isomerase That Needs Mycothiol as Cofactor and Links Mycothiol Biosynthesis and Gentisate Assimilation in *Corynebacterium glutamicum* 

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Data mining of the *Corynebacterium glutamicum* genome identified 4 genes analogous to the *mshA*, *mshB*, *mshC*, and *mshD* genes that are involved in biosynthesis of mycothiol in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. Individual deletion of these genes was carried out in this study. Mutants *mshC*<sup>−</sup> and *mshD*<sup>−</sup> lost the ability to produce mycothiol, but mutant *mshB*<sup>−</sup> produced mycothiol as the wild type did. The phenotypes of mutants *mshC*<sup>−</sup> and *mshD*<sup>−</sup> were the same as the wild type when grown in LB or BHIS media, but mutants *mshC*<sup>−</sup> and *mshD*<sup>−</sup> were not able to grow in mineral medium with gentisate or 3-hydroxybenzoate as carbon sources. *C. glutamicum* assimilated gentisate and 3-hydroxybenzoate via a glutathione-independent gentisate pathway. In this study it was found that the maleylpyruvate isomerase, which catalyzes the conversion of maleylpyruvate into fumarylpyruvate in the glutathione-independent gentisate pathway, needed mycothiol as a cofactor. This mycothiol-dependent maleylpyruvate isomerase gene (*ncgl2918*) was cloned, actively expressed, and purified from *Escherichia coli*. The purified mycothiol-dependent isomerase is a monomer of 34 kDa. The apparent *K<sub>m</sub>* and *V<sub>max</sub>* values for maleylpyruvate were determined to be 148.4 ± 11.9 μM and 1520 ± 57.4 μmol/min/mg, respectively (mycothiol concentration, 2.5 μM). Previous studies had shown that mycothiol played roles in detoxification of oxidative chemicals and antibiotics in streptomyces and mycobacteria. To our knowledge, this is the first demonstration that mycothiol is essential for growth of *C. glutamicum* with gentisate or 3-hydroxybenzoate as carbon sources and the first characterization of a mycothiol-dependent maleylpyruvate isomerase.

Mycothiol (1), also known as MSH and chemically 1D-myoo-inositol-2-(N-acetyl-L-cysteinylamido)-2-deoxy-D-glucopyranoside, is the major low-molecular mass thiol in mycobacteria and streptomyces (2). Some investigations showed that mycothiol is associated with protection of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* against antibiotics such as rifampin (3, 4) and also helped these pathogens to detoxify reactive oxygen species produced by host cells (5).

Thus, mycothiol is a potential target for medical treatment and has raised interest from both academia and industry. In addition, mycothiol also has the ability to protect cells against a range of toxic compounds. For example, in *Amycolatopsis methanolica* and *Rhodococcus erythropolis*, mycothiol detoxifies formaldehyde by acting as a cofactor for a formaldehyde dehydrogenase (6) and detoxifies alkylating agents such as monobromobimane by converting them to S-conjugates of mycothiol (7, 8). To date, the understanding of the physiological function of mycothiol is limited to detoxification and the protection of living cells (9), whereas essential metabolic roles for cell growth have not been reported.

A survey on the distribution of low-molecular mass thiols in microorganisms showed that *Corynebacterium diphtheriae* produced mycothiol (2). However, the occurrence of mycothiol in other *Corynebacterium* species has not been reported, and the physiological function of mycothiol in corynebacteria is still not well defined. The genus *Corynebacterium* covers both medical and industrial important species. For example, *Corynebacterium glutamicum* is commercially used for production of amino acids and vitamins (10), and *C. diphtheriae* is a pathogen for human beings. Recently, the genomes of *C. glutamicum* (11, 12), *Corynebacterium efficiens* (13), and *Corynebacterium jeikeium* (14) have been sequenced. This greatly stimulated the study of the physiology of corynebacteria. Consequently, *C. glutamicum* has been newly characterized for its robust ability to metabolize aromatic compounds (15–17), and a novel glutathione-independent gentisate pathway has been described (18).

The biosynthesis of mycothiol was characterized in *M. smegmatis* and *M. tuberculosis*, and the genes *mshB*, *mshC*, and *mshD* were found encoding for the enzymes that sequentially catalyze the formation of mycothiol from 1D-myoo-inositol-2-acetamido-2-deoxy-D-glucopyranoside (GlcNAC-Ins). A fourth gene, *mshA*, is involved in the production of GlcNAC-Ins, but the substrate of MshA has not been identified (9). Orthologs of these *msh* genes could be found in the genome data of the *Corynebacterium* species, but their functions in biosynthesis of mycothiol have not been proven experimentally.

During our studies on biosynthesis of mycothiol and assimilation of aromatic compounds with *C. glutamicum*, we found that mycothiol-negative mutants lost the ability to grow on several aromatic compounds. This surprising discovery raised the question of how mycothiol is involved in aromatic compound assimilation/degradation. In this report, our results indicate that mycothiol functions as an essential growth factor for *C. glutamicum* when gentisate and 3-hydroxybenzoate are provided as carbon sources. We further linked the biosynthesis...
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Table 1

Bacterial strains, plasmids, and primers used in this study

| Strains, plasmids, and primers | Relevant characteristics or sequences | Source/Ref. |
|---------------------------------|--------------------------------------|-------------|
| **E. coli**                     |                                       |             |
| XL1-Blue                        | supE44 leuR17 recA1 endA1 gyrA46 thi relA1 lac− F [proAB lacI] lacZΔM15 Tn10 (et1) | Stratagene (catalog No. 200249) |
| BL21(DE3)                       | lacI5 gal (λact857 ind-1 kam7 mini-5 lacIUV5-T7 gene1) | Novagen (catalog No. 69450-3) |
| **C. glutamicum**               |                                       |             |
| RES167                          | Restriction-deficient mutant of ATCC13032, Δ(cglLM-cglIR-cglII)R | Tauch et al. (21) |
| mshB−                           | A fragment of DNA of cgl1053 coding region was deleted. | This study |
| mshC+                           | A fragment of DNA of cgl1457 coding region was deleted. | This study |
| mshD−                           | A fragment of DNA of cgl2487 coding region was deleted. | This study |
| **S. clavuligerus**             |                                       |             |
| AS 4.1611T                      |                                       |             |
| **S. coelicolor**               |                                       |             |
| AS 4.1658T                      |                                       |             |
| **B. megaterium**               |                                       |             |
| AS 1.459                        |                                       |             |
| **B. subtilis**                 |                                       |             |
| AS 1.268                        |                                       |             |
| **Plasmids**                    |                                       |             |
| pK18mobsacBΔcgl1055             | Carrying cgl 1055 deletion            | This study |
| pK18mobsacBΔcgl1457             | Carrying cgl 1457 deletion            | This study |
| pK18mobsacBΔcgl2487             | Carrying cgl2487 deletion             | This study |
| pET28a                          | Expression vector with N-terminal hexahistidine affinity tag | Novagen |
| pET28a-ncg12918                 | pET28a derivative for expression of ncl12918 | This study |
| **Primers**                     |                                       |             |
| E2198F                          | AACACATATGACAACCTTCCCAGA (NdeI)       | To generate pET28a-ncg12918 |
| E2198R                          | GATATGTTCTAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl1055 |
| D1055R1                         | GATCTACATTTGATCTGAATGGGACGACACAGCACAGATGACCT | This study |
| D1055R2                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl2487 |
| D1055F1                         | CAGGGGTCCGCGATTTGAGGCTGCACTT | Novagen |
| D1055F2                         | CAGGGGTCCGCGATTTGAGGCTGCACTT | Novagen |
| D1457R1                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl1457 |
| D1457R2                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl2487 |
| D1457F1                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl2487 |
| D1457F2                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl2487 |
| D2847R1                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl2487 |
| D2847R2                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl2487 |
| D2847F1                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl2487 |
| D2847F2                         | GATCTATGTTCAATGGATTAGCTTAAAGA (HindIII) | To generate pK18mobsacBΔcgl2487 |

* China General Microbial Culture Collection.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—Bacterial strains used in this study are listed in Table 1. Escherichia coli strains were grown aerobically on a rotary shaker (150 rpm) at 37 °C in Luria-Bertani (LB) broth or on LB plate with 1.5% (w/v) agar. C. glutamicum, Streptomyces clavuligerus, Bacillus megaterium, Bacillus subtilis, and Streptomyces coelicolor were routinely grown in LB media on a rotary shaker (150 rpm) at 30 °C. To determine the growth with aromatic compounds, *C. glutamicum* strains were grown in mineral salts medium, pH 8.4, supplemented with 0.05 g liter−1 of yeast extract (15), on a rotary shaker (150 rpm) at 30 °C. Aromatic compounds were added at final concentrations of 2 mM. Cellular growth was monitored by measuring the turbidity at 600 nm. For generation of mutants and maintenance of *C. glutamicum*, BHIS (brain/heart broth with 0.5M sorbitol) medium was used. When needed, antibiotics were used at the following concentrations: kanamycin, 50 μg ml−1 for *E. coli* and 25 μg ml−1 for *C. glutamicum*.

Construction of Plasmids for Site-specific Gene Deletion—Plasmids pK18mobsacBΔcgl1055, pK18mobsacBΔcgl1457, and pK18mobsacBΔcgl2487 were constructed using the gene-SOEing method described by Horton (19). The primers used are listed in Table 1. The primary products were amplified using fusion DNA polymerase (New England Biolabs). The resulting products were purified using the PCR purification kit (Qiagen, Hilden, Germany) and then used as templates for the second round of PCR. The final products were digested with restriction enzymes corresponding to the cleavage sites introduced via PCR and ligated into appropriately digested pK18mobsacB. The ligation mixture was used to transform *E. coli* DH5αMCr, the transformants were selected on LB plates containing 50 μg ml−1 kanamycin and 40 μg ml−1 X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

To generate mutants in mycothiol biosynthesis from *C. glutamicum*, site-specific gene deletion was performed using the above generated pK18mobsacB derivatives that are not replicable in *C. glutamicum* and allow for marker-free deletion of the target genes (20). The resulting plasmids pK18mobsacBΔcgl1055, pK18mobsacBΔcgl1457, and pK18mobsacBΔcgl2487 were transformed into *C. glutamicum* ATCC 13032 by electroporation (21). Integration of the introduced plasmids into the chromosome by single crossover was tested by selection on BHIS plates containing 25 μg ml−1 kanamycin. For the deletion of the target gene, the kanamycin-resistant (KmR) cells were grown overnight in liquid BHIS and spread on BHIS plates containing 10% sucrose. Cells growing on this plate were tested for kanamycin sensitivity (KmS) by parallel picking on BHIS plates containing either kanamycin or sucrose. Sucrose-resistant and kanamycin-sensitive cells were then tested for deletion by PCR, using the corresponding DF1 and DR2 primer pair (Table 1).

 Extraction and Determination of Mycothiol in Wild Type and Mutants of *C. glutamicum*—The detection of mycothiol from *C. glutamicum* cells was carried out according to the procedures as described by Newton et al. (2) and was modified. One milliliter of 50% warm (60 °C) acetonitrile containing 20 mM Tris-HCl, pH 8.0, and 2 mM bromobimane (Sigma) was added to a tube that contained 200 mg of frozen cells, sonicated at 60 °C for 20 s, and maintained in a 60 °C water bath for 15 min in dark. After acidification with
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| Gene and position | Annotated function of gene products | Identified function of gene products | Sequence identity | Related gene products (organism, locus_tag) |
|-------------------|-------------------------------------|-------------------------------------|-------------------|-------------------------------------------|
| 423878–425134     | Predicted glycosyltransferase       | MshA glycosyltransferase            | 349/409 (85%)     | Conserved hypothetical protein (C. efficiens, CE0422) |
| 1147592–1148464   | Hypothetical protein                | MshB deacetylase                    | 291/405 (71%)     | Putative glycosyl transferase (C. diphtheriae, DIP0388) |
| 1596246–1597508   | Cysteinyl-tRNA synthetase           | MshC ligase                         | 232/407 (57%)     | Putative glycosyltransferase (N. farcinica, nfa51920) |
| 2737834–2738712   | Histone acetyltransferase           | HPA2-like protein                    | 213/421 (50%)     | MshA (M. tuberculosis, Rv0486) |
| 2573834–2738712   |                                    |                                     | 205/433 (47%)     | Putative glycosyltransferase (S. coelicolor, SCO2404) |

5 μl of 5 N methanesulfonic acid, the cellular debris was removed by centrifugation at 10,000 × g for 10 min. Supernatants were diluted 3-fold in aqueous 10 mM methanesulfonic acid prior to HPLC3 analysis. The bimane derivatives of various thiols were separated and detected with HPLC that was equipped with a C18 column (ZORBAX, 250 × 4.6 mm) and was operated at the following conditions. The column was first eluted with 10% methanol (in water) for 5 min, and then the methanol content was increased to 100% in 10 min. The bimane derivative of mycothiol was eluted at 11.79 min in this system.

Heterologous Expression of the Maleylpyruvate Isomerase Gene in E. coli Purification of Recombinant Protein—The expression plasmid pET28a-ncgl2918 was described previously (18). The plasmid was electroporated into E. coli BL21(DE3). Synthesis of recombinant protein in E. coli BL21(DE3) cells was initiated by addition of 0.5 mM isopropyl-β-D-galactopyranoside when the culture reached A600 of 0.6–0.8 and continued cultivation for an additional 8 h at 25 °C. Cells were harvested by centrifugation and were disrupted by sonification at 4 °C (160 W, 3 s sonifying vs 5 s break, 50 cycles) in Tris-HCl buffer (20 mM Tris-HCl, pH 8.0). Cellular lysate was centrifuged and the supernatant was used for protein purification. Recombinant protein was purified with the His-Bind protein purification kit (Novagen, Madison, WI) according to the manufacturer’s instructions. To remove any residue proteins, imidazole, and salts in the collected fractions, fractions were pooled and were further separated by Superdex™ 200 gel chromatography with Tris- HCl buffer (20 mM Tris-HCl, pH 8.0). All steps of chromatography were controlled by the fast protein liquid chromatography system (Äkta FPLC, Amersham Biosciences). The purified protein was concentrated by ultrafiltration through Millipore Ultra-15 (10 kDa) and stored at −70 °C.

Determination of Molecular Mass of the Purified Proteins—The native molecular mass of the maleylpyruvate isomerase was estimated by gel filtration chromatography on a prepacked Superdex 200 column (Amersham Biosciences). The column was equilibrated and eluted with 50 mM Tris-HCl (pH 7.6) containing 100 mM NaCl at a flow rate of 0.4 ml min⁻¹. Molecular mass was calculated according to their elution volume and calibrated with the molecular mass standard kit (MW-GF-1000, Sigma). The subunit molecular mass was determined with SDS-PAGE, which was conducted with a 5% stacking gel and 12% resolving gel and run in a Mini-PROTEIN II Electrophoresis Cell (Bio-Rad) according to the manufacturer’s instructions. Apparent molecular mass was estimated according to the relative mobility to protein standards with molecular mass ranging from 14 to 97 kDa.

Enzymatic Preparation of Maleylpyruvate and Determination of Maleylpyruvate Isomerase Activity—Maleylpyruvate was prepared from gentisate by gentisate 1,2-dioxygenase digestion (18) in a system containing (total volume 3 ml): 0.1 mM gentisate, 5 μl of recombinant 1,2-dioxygenase from E. coli, 50 mM Tris-HCl buffer, pH 8.0. After incubation at room temperature for 3 min (complete conversion of gentisate to maleylpyruvate), this mixture was used as the crude maleylpyruvate preparation without any further purification. For determination of maleylpyruvate isomerase activity, 10 μl of boiled cellular lysate of C. glutamicum and 5–10 μl of maleylpyruvate isomerase preparations were added to the above crude maleylpyruvate preparation. The maleylpyruvate isomerase activity was qualitatively monitored by scanning the spectral absorption changes at 250–400 nm with a UV visible spectrophotometer (Beckman Coulter DU800) at a wavelength interval of 1 nm, and was quantitatively determined by measuring the decrease of absorbance at 330 nm due to maleylpyruvate disappearance. 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 (22). To test if coenzyme A and cysteine support maleylpyruvate isomerase activity, coenzyme A and cysteine (each 0.01 mM) were included in the assay broth. Protein concentration was determined according to the method of Bradford (23), with bovine serum albumin as the standard.

The abbreviation used is: HPLC, high performance liquid chromatography.
Preparation and Treatment of Various Bacterial Cellular Lysates—To evaluate if the following bacteria contained the factor that supported maleylpyruvate isomerase from C. glutamicum, cells of about 0.15 g from each of S. clavuligerus, B. megaterium, B. subtilis, S. coelicolor, and mutants of mshB, mshC, and mshD of C. glutamicum were disrupted by sonication at 4°C (160 watts, 3 s sonifying versus 5 s break, 50 cycles) in Tris-HCl buffer (20 mM Tris-HCl, pH 8.0). Cellular debris was separated by centrifugation and the resulting supernatants were boiled for 5 min to eliminate any enzyme activities. The boiled supernatants (10 μl) were added to the maleylpyruvate isomerase activity assay mixtures, as described above.

Isolation of Mycothiol from C. glutamicum—Isolation and purification of mycothiol from C. glutamicum was carried out by integration of Sephadex LH20 chromatography and the methods of Newton et al. (24) with a thiol-affinity chromatography and Steenkamp et al. (25) with HPLC. Frozen cells (37 g) of C. glutamicum were thawed in 74 ml of 0.75 M perchloric acid. Acid-insoluble cellular debris was removed by centrifugation at 12,000 × g for 10 min. The supernatant was adjusted to pH 4.5 with 4 M KOH and then stored at 0°C for 30 min. Precipitated potassium perchlorate was removed by centrifugation at 10,000 × g for 10 min. The supernatant (81 ml) was mixed with 27 ml of buffer A (0.4 M Tris-HCl, 2 M NaCl, 4 mM EDTA, pH 7.5). The mixture was applied onto a thiopropyl-Sepharose6B column (Amersham Biosciences). The column was washed using 80 ml of buffer B (0.1 M Tris-HCl, pH 8.0). Cellular debris was separated by centrifugation and the resulting supernatants were boiled for 5 min to eliminate any enzyme activities. The boiled supernatants (10 μl) were added to the maleylpyruvate isomerase activity assay mixtures, as described above.

Mass Spectrometry—Positive-ion electrospray mass spectrometry analyses were performed on an Agilent 1100 LC/MSD Trap XCT instrument (Agilent Technologies, Palo Alto, CA). Instrument control, data acquisition, and data processing were done using the Agilent 1100 Chemstation (version 10.02) and Data Analysis (version 5.1) software. The instrument was operated in full-scan mode. Acquisition was performed in the continuum mode, and the acquisition parameters were as follows: tune source: trape drive (39.9), octopole RF amplitude (187.1 vpp), capillary exit (94.8 V), skim 1 (40.0 V), skim 2 (−5.0 V), dry temperature (325°C), nebulizer (50.00 p.s.i.), nitrogen gas (10.00 liters/min), HV capillary (2680 V), HV end-plate offset (−500 V). The scan (average of three spectra) was between m/z 100–550 with maximal Accu time of 200,000 μs and ICC target of 200,000. Fragmentation was set with SmartFrag Ampl followed: tune source: trape drive (39.9), octopole RF amplitude (187.1 vpp), capillary exit (94.8 V), skim 1 (40.0 V), skim 2 (−5.0 V), dry temperature (325°C), nebulizer (50.00 p.s.i.), nitrogen gas (10.00 liters/min), HV capillary (2680 V), HV end-plate offset (−500 V). The scan (average of three spectra) was between m/z 100–550 with maximal Accu time of 200,000 μs and ICC target of 200,000. Fragmentation was set with SmartFrag Ampl between 30 and 200%, fragmentation width, 10.00 m/z; fragmentation time, 40,000 μs; and fragmentation delay, 0 μs.

Kinetic Measurements—Michaelis-Menten kinetics of the maleylpyruvate isomerization catalyzed with isomerase from C. glutamicum was identified by plotting reaction rates against substrate concentrations (ranging from 66 to 380 μM) at a mycothiol concentration of 2.5 μM. The apparent Km and Vmax values were determined by nonlinear regression analysis of the plots of the initial isomerization rates and substrate concentrations.

Effects of Metal Ions and Chemicals on Maleylpyruvate Isomerase Activity—To test the effects of various metal ions and chemicals on the mycothiol-dependent maleylpyruvate isomerase, Mg2+, Ca2+, Cu2+, Ni2+, Mn2+, Fe3+, Zn2+ (each 1 mM), GSH (0.05 mM), dithiothreitol (0.05 mM), EDTA (1 and 10 mM), and bromobimane (1 mM) were incubated in 20 mM Tris-HCl, pH 8.0, with the enzyme for 15 min at room temperature. The activities of the treated enzymes were assayed as described above. The enzyme without treatment was run in parallel as control.

RESULTS

In M. smegmatis and M. tuberculosis, mycothiol is synthesized through the catalysis by the MshA, MshB, MshC, and MshD proteins (9). Putative genes homologous to those encoding for MshA, MshB, MshC, and MshD were identified in the genomes of C. glutamicum and other Corynebacterium species (Table 2). To identify the functions of these genes, efforts on generation of mutations within these genes were made. C. glutamicum deletion mutants in mshB, mshC, and mshD were successfully constructed, and the mutation of each gene was subsequently confirmed by PCR. HPLC analysis showed that wild type and the mshB− mutant produced mycothiol, and mshB− produced about 5% of the wild type, but the mutants mshC− and mshD− did not produce detectable levels of Msh (Fig. 1, A–D).

All the mutants mshB−, mshC−, and mshD− grew as well as the wild type strain of C. glutamicum, and there were no significant phenotypic differences observed, when cultivated in LB and BHIS media. How-
ever, we found that the mutants mshC and mshD lost the ability to grow on gentisate as the sole carbon source (Fig. 2). To see if their abilities to grow on other aromatic compounds were disturbed, these mutants were tested for growth on various aromatic compounds as carbon sources. The results obtained showed that mutants mshC and mshD also lost the ability to grow on 3-hydroxybenzoate, but their ability to assimilate 4-hydroxybenzoate, benzoate, phenol, and resorcinol was not disturbed (Table 3). Previous studies revealed that (i) benzoate was assimilated through the catechol 1,2-dioxygenase pathway (17), (ii) 4-hydroxybenzoate was assimilated through the protocatechuate 3,4-dioxygenase and the \(/\)-ketoadipate pathway (15), (iii) resorcinol was assimilated through the hydroxyquinol 1,2-dioxygenase pathway (16), and (iv) 3-hydroxybenzoate as well as gentisate were assimilated through a glutathione-independent gentisate pathway (18). Thus, we deduced that there might be a link between the glutathione-independent gentisate pathway and mycothiol biosynthesis in C. glutamicum.

Table 3

| Strains    | Gentisate | 3-Hydroxybenzoate | 4-Hydroxybenzoate | Benzoate | Phenol | Resorcinol |
|------------|-----------|-------------------|-------------------|----------|--------|------------|
| Wild type  | 0.57      | 0.54              | 0.62              | 0.62     | 0.41   | 0.54       |
| mshB<sup>+</sup> | 0.65      | 0.54              | 0.53              | 0.47     | 0.48   | 0.57       |
| mshC<sup>+</sup> | 0.07      | 0.04              | 0.59              | 0.59     | 0.48   | 0.56       |
| mshD<sup>+</sup> | 0.07      | 0.04              | 0.65              | 0.54     | 0.52   | 0.62       |

Table 4

| Bacterial species | Supportive (+/−) to maleylpyruvate isomerase | Mycothiol occurrence (+/−) and references |
|-------------------|---------------------------------------------|------------------------------------------|
| C. glutamicum     | +                                           | +, This study; Mycothiol is modified      |
| Treated with      | −                                           | −                                        |
| bromohibin        |                                            |                                          |
| mshB              | +                                           | +, This study                            |
| mshC<sup>+</sup>  |                                            | −                                        |
| mshD<sup>+</sup>  |                                            | −                                        |
| S. clavuligerus   | +                                           | +, Newton et al. (2)                      |
| S. coelicolor     | +                                           | +, Newton et al. (2)                      |
| B. megaterium     | −                                           | No data available                        |
| B. subtilis       | −                                           | −, Newton et al. (2)                      |

Genes encoding for the gentisate 1,2-dioxigenase (ncl2920), maleylpyruvate isomerase (ncl2918), and fumarylpyruvate hydrolase (ncl2919) in the glutathione-independent gentisate pathway were functionally identified in C. glutamicum (18). The gene ncl2918 encoding for the maleylpyruvate isomerase in C. glutamicum was not homologous to the known maleylpyruvate isomerase genes mhbI from Klebsiella pneumoniae (26) and nagL from Ratstonia strain U2 (27), which encode a glutathione-dependent maleylpyruvate isomerase. Rather, ncl2918 is homologous to a group of genes encoding for hypothetical proteins in C. efficiens
Streptomyces strain WA46 (sdgF), S. coelicolor (SCO1959), Streptomyces avermitilis (SAV6285), and Nocardia farcinica (nfa2300) (Fig. 3). Ncgl2918 was identified to be a maleylpyruvate isomerase that did not rely on glutathione (18), or coenzyme A, or cysteine as cofactor (this study). However, results from this study showed that when this C. glutamicum isomerase was expressed in E. coli its activity was dependent on some cofactor occurring in boiled cellular lysates of C. glutamicum (Table 4). It was further found that such a cofactor also existed in cellular lysates of S. coelicolor and S. clavuligerus, but not in B. subtilis and B. megaterium that was reported to have a glutathione-independent maleylpyruvate isomerase activity (Table 4) (22). This cofactor disappeared when bromobimane, a thiol-binding agent, was added to the boiled cellular lysate of C. glutamicum (Table 4), indicating that this cofactor probably has a thiol group.

The cofactor that supported maleylpyruvate isomerase activity in C. glutamicum was purified (Fig. 4A) by following the procedures described under "Experimental Procedures." The purified cofactor was subjected to ESI and tandem MS analyses (Fig. 4, B–D). Positive-ion ESI-MS analysis of this compound produced pseudo-molecular ions at \( m/z 487.1 (\text{[M} + \text{H}]^+) \) and \( m/z 509.2 (\text{[M} + \text{Na}]^+) \). The prominent fragment ion at \( m/z 307.1 \) resulted from the loss of an inositol-like unit (C₆H₁₂O₆ (m/z 180)). The fragments at \( m/z 307.1 \) and \( m/z 273.1 \) of the
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![Diagram of maleylpyruvate isomerase]

**FIGURE 5.** Isomerization of maleylpyruvate to fumarylpyruvate catalyzed by the mycothiol-dependent maleylpyruvate isomerase (A) and absorption spectral changes during catalysis (B). The spectral changes were recorded at time intervals of 30 s.

The MS/MS spectrum are corresponding to \([M + H] - C_6H_{12}O_6\)^+ and \([M + H] - C_6H_{12}O_6 - H_2S\)^+ , respectively. Upon MS^3 analysis on \(m/z\) 307.1 (Fig. 3C), fragment ions at \(m/z\) 289.1 \([M + H] - C_6H_{12}O_6 - H_2O\)^+, \(m/z\) 273.1 \([M + H] - C_6H_{12}O_6 - H_2S\)^+, and \(m/z\) 255.0 \([M + H] - C_6H_{12}O_6 - H_2O - H_2S\)^+ were observed. In addition, the observed signal at \(m/z\) 162.0 was rationalized as the \([N\text{-acyethylcysteine}]^+\) fragment. These spectral evidence indicated that the thiol-containing compound is mycothiol.

Previously, mycothiol was detected in *C. diphtheriae* (2). Thus, the identification of mycothiol in *C. glutamicum* might indicate that mycothiol is common in corynebacteria. When the purified mycothiol was added to the maleylpyruvate isomerase from *C. glutamicum* produced in recombinant *E. coli*, significant isomerase activity was observed in the enzyme assay (Fig. 5), indicating that the maleylpyruvate isomerization of *C. glutamicum* is mycothiol-dependent. Very recently, Newton et al. (28) have reported that the mshD^− mutant of *M. smegmatis* produced altered thiols of N-formyl/succinyl-Cys-GlcN-Ins. The fact that the mshD^− mutant of *C. glutamicum* did not produce remarkable amounts of altered thiols (Fig. 1D) indicated that there might be differences in mycothiol biosynthesis between corynebacteria and mycobacteria.

This mycothiol-dependent maleylpyruvate isomerase from recombinant *E. coli* was analyzed by SDS-PAGE and gel filtration chromatography. Results showed a single protein band at 33 kDa in the SDS-PAGE and a molecular mass of 34 kDa in gel filtration chromatography. Thus, this mycothiol-dependent maleylpyruvate isomerase of *C. glutamicum* is a monomeric enzyme. Amino acid sequence analysis revealed a cysteine residue at position 61 (Cys^61). Treatment of the enzyme with thiol-modifying agents such as bromobimane, N-ethylmaleimide, p-chloromercuribenzoate, and iodoacetamide did not significantly inhibit the activity of the enzyme (18), indicating that the cysteine residue in this protein is not important for enzyme activity. Furthermore, alignment of several *Ncgl2918* orthologs showed that the cysteine residue is not conserved (Fig. 3). In addition, site-directed mutation of this cysteine residue into the alanine residue did not cause a loss in enzymatic activity (data not shown). Divalent metal ions including Mg^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, and Mn^{2+} (each 1 mM) did not affect the activity of the mycothiol-dependent maleylpyruvate isomerase. Fe^{3+} (1 mM) showed a moderate inhibition with a residual activity of 85%. Zn^{2+} (1 mM) showed a moderate stimulation of activity to 121%. EDTA at 1 mM slightly increased activity, but when its concentration reached 10 mM, the activity was inhibited completely. The apparent \(K_m\) and \(V_{max}\) values for maleylpyruvate were determined to be 148.4 ± 11.9 \(\mu\)M and 1520 ± 57.4 \(\mu\)mol/min/mg, respectively.

**DISCUSSION**

In this study, the biosynthesis of mycothiol in *C. glutamicum* and two essential genes, *mshC* (*nclg1457*) and *mshD* (*nclg2487*), involved in the biosynthetic pathway have been identified. Although the biosynthesis of mycothiol and the genes involved in the biosynthetic pathway in *M. smegmatis* and *M. tuberculosis* are well understood (4, 29–32), the occurrence of mycothiol and its biosynthetic pathway had not been identified in corynebacteria. Mycothiol was detected in *C. diphtheriae* (2), as well as in *C. glutamicum* cells (this study). By genome data mining, genes orthologous to the *Mycobacterium mshA, mshB, mshC, and mshD* genes were identified in the genomes of *C. glutamicum, C. effi-
it is reasonable to deduce that biosynthesis of mycothiol is common to many mycobacteria. Based on the results obtained in this study, we believe that the mycothiol-dependent maleylpyruvate isomerase catalyzes the key step that couples the mycothiol biosynthesis and the gentisate/3-hydroxybenzoate degradation. Considering the many similarities between mycothiol and glutathione metabolism, we believe that the mycothiol-dependent maleylpyruvate isomerase is analogous to glutathione-dependent glyoxylases, S-transferases, thiol-transferases, etc., and that more mycothiol-dependent enzymes will be identified in the near future.

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