

## Cloning and Characterization of Uronate Dehydrogenases from Two Pseudomonads and *Agrobacterium tumefaciens* Strain C58<sup>∇‡</sup>

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Uronate dehydrogenase has been cloned from *Pseudomonas syringae* pv. tomato strain DC3000, *Pseudomonas putida* KT2440, and *Agrobacterium tumefaciens* strain C58. The genes were identified by using a novel complementation assay employing an *Escherichia coli* mutant incapable of consuming glucuronate as the sole carbon source but capable of growth on glucarate. A shotgun library of *P. syringae* was screened in the mutant *E. coli* by growing transformed cells on minimal medium containing glucuronic acid. Colonies that survived were evaluated for uronate dehydrogenase, which is capable of converting glucuronic acid to glucaric acid. In this manner, a 0.8-kb open reading frame was identified and subsequently verified to be *udh*. Homologous enzymes in *P. putida* and *A. tumefaciens* were identified based on a similarity search of the sequenced genomes. Recombinant proteins from each of the three organisms expressed in *E. coli* were purified and characterized. For all three enzymes, the turnover number ( $k_{\text{cat}}$ ) with glucuronate as a substrate was higher than that with galacturonate; however, the Michaelis constant ( $K_m$ ) for galacturonate was lower than that for glucuronate. The *A. tumefaciens* enzyme was found to have the highest rate constant ( $k_{\text{cat}} = 1.9 \times 10^2 \text{ s}^{-1}$  on glucuronate), which was more than twofold higher than those of both of the pseudomonad enzymes.

Aldohexuronate catabolism in bacteria is reported to involve two different pathways, one initiating with an isomerization step and the other with an oxidation step. In the isomerization pathway, aldohexuronate (glucuronate and galacturonate) is isomerized to ketohexuronate by uronate isomerase and ultimately degraded to pyruvate and 3-phosphoglyceraldehyde. The isomerization pathway has been previously reported to occur in bacteria, including *Escherichia coli* (7), *Erwinia carotovora* (18), *Erwinia chrysanthemi* (15), *Klebsiella pneumoniae* (9, 23), and *Serratia marcescens* (28). In the oxidation pathway, aldohexuronate is oxidized to aldohexarate by uronate dehydrogenase (Udh) and further catabolized to pyruvate (2, 5, 7, 9, 18, 19, 24). Uronate dehydrogenase, the key enzyme of this pathway, has been investigated in two plant pathogen bacteria, *Pseudomonas syringae* and *Agrobacterium tumefaciens*. To date, only limited studies pertaining to the properties of Udh have been reported in the literature (3, 6, 38, 43), and no sequence has yet been identified. Udh is classified as an NAD-linked oxidoreductase (EC 1.1.1.203), with a total molecular weight of about 60,000. It is a homodimer composed of two subunits with molecular weights of about 30,000 each (38). Udh is a thermally unstable, reversible enzyme, with an optimum pH of about 8.0 (3, 6, 38).

In *E. coli* MG1655 that has the isomerization pathway for aldohexuronate catabolism, glucuronate is transported by an aldohexuronate transporter encoded by *exuT* and converted to

fructuronate by uronate isomerase, encoded by *uxaC* (22, 30) (Fig. 1). Fructuronate is transferred to the Entner-Doudoroff pathway to be utilized as an energy source via 2-keto-3-deoxy-6-phospho-gluconate (7, 27, 31, 32). Therefore, *E. coli* MG1655 with a *uxaC* deletion cannot use glucuronate as a carbon source. In this strain, glucarate is converted to 5-keto-4-deoxy-D-glucarate by D-glucarate dehydratase, encoded by *gudD*, and then transferred to glycolysis via pyruvate or 2-phosphoglycerate (27, 33). Recently, a number of bacterial genome sequences have been published, including those of the Udh-containing *P. syringae* pv. tomato strain DC3000 and *A. tumefaciens* strain C58 (4, 10). A shotgun library of *P. syringae* was constructed to identify the gene encoding Udh. Screening for Udh was conducted in *E. coli* MG1655  $\Delta\text{uxaC}$ . Since uronate dehydrogenase converts glucuronate to glucarate, *uxaC* deletion strains of *E. coli* harboring the shotgun library of *P. syringae* that can grow in a minimal medium containing glucuronate as a sole carbon source may carry the gene encoding Udh (Fig. 1). Once an initial Udh is identified from *P. syringae*, a BLAST homology search may lead to the identification of Udh from other bacteria.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Strains, plasmids, and primer sequences used in this study are indicated in Table 1. Media and chemical reagents were purchased from Sigma (St. Louis, MO) or BD Biosciences (San Jose, CA). *P. syringae* pv. tomato strain DC3000 was used as the source of the genomic library and was donated by Frederick Ausubel of Massachusetts General Hospital. *P. syringae* was grown in Luria-Bertani (LB) medium with 50  $\mu\text{g}/\text{ml}$  rifampin (rifampicin) at 30°C. *Pseudomonas putida* KT2440 (ATCC 47054) was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown in LB medium at 30°C. *E. coli* strains were grown in 2YT medium (16 g tryptone, 10 g yeast extract, and 10 g sodium chloride per liter) at 37°C. As required, ampicillin and kanamycin were added to the medium at 100 and 25  $\mu\text{g}/\text{ml}$ , respectively. *Escherichia coli* DH10B ( $F^- \text{mcrA} \Delta(\text{mrr-hsdRMS-mcrBC}) \phi 80\text{lacZ}\Delta\text{M15} \Delta\text{lacX74} \text{recA1} \text{endA1} \text{araD139} \Delta(\text{ara-leu})7697 \text{galU} \text{galK} \lambda^- \text{rpsL} \text{nupG}$ ) was used as the host strain for the genomic library as well as for subcloning of screened genes (Invitrogen Corp., Carlsbad, CA). *E. coli* MG1655  $\Delta\text{uxaC}$

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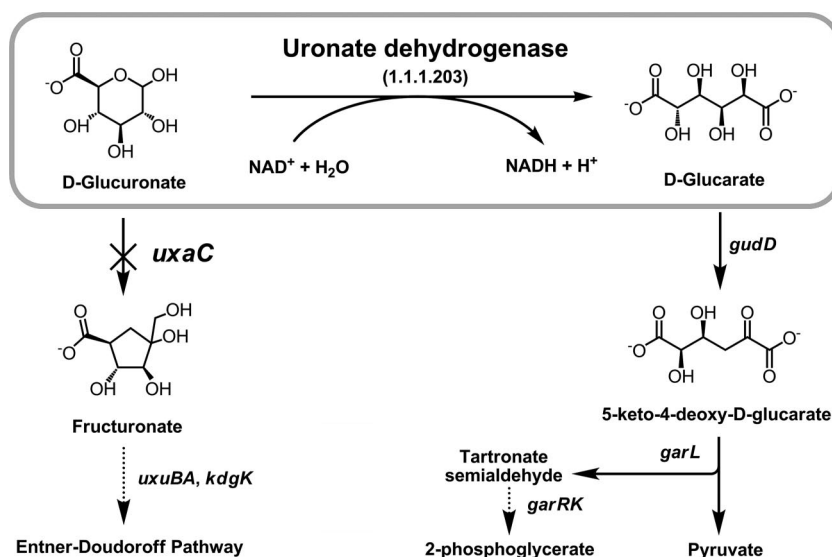


FIG. 1. Catabolism of glucuronate and glucarate in bacteria. Glucuronate consumption is prevented by knockout of the *uxaC* gene. The presence of uronate dehydrogenase in a *uxaC* knockout enables growth of *E. coli* on glucuronate.

was provided by F. R. Blattner of the *E. coli* Genome Project at University of Wisconsin—Madison. For M9 minimal agar, 22 mM glucose, glucuronate, or glucarate was used as a carbon source. Plasmid vectors pTrc99A and pTrc99SE were used for construction of the genomic library and as an expression vector for candidate genes, respectively (Table 1). The plasmid pTrc99SE was donated by Seon-Won Kim at Gyeongsang National University, Korea. pBluescript (Invitrogen, Carlsbad, CA) was used as a general cloning vector.

**Genomic DNA preparation and construction and screening of *P. syringae* genomic library.** Genomic DNA preparation and general cloning procedures were carried out as described by Sambrook and Russell (35). The genomic DNA of *A. tumefaciens* strain C58 was purchased from the ATCC (ATCC number 33970D). Restriction enzymes and T4 ligase were purchased from New England Biolabs (Beverly, MA). *P. syringae* genomic DNA was partially digested with BfuCI and then loaded onto a 0.8% agarose gel. Fragments of 2 to 6 kb were purified from the gel and then ligated to pTrc99A with dephosphorylated BamHI ends. After ligation for 2 days at 4°C, the reaction mixtures were used to transform *E. coli* DH10B. Successful transformant clones were collected and pooled from agar plates, followed by storage at -80°C. Plasmid pools isolated from the colony pools were used to transform *E. coli* MG1655  $\Delta$ *uxaC* to screen for Udh activity. Transformed strains were cultured on M9 minimal agar plates with 22 mM glucuronate for 4 days at 30°C. Surviving clones from plates were screened by purifying and sequencing their plasmids. The sequencing results were compared with the genome sequence of *P. syringae* pv. tomato strain DC3000, as reported in GenBank (accession number NC\_004578 [http://www.ncbi.nlm.nih.gov/]).

**Construction of expression plasmid vectors containing *udh* genes.** PCR amplification was carried out using *Pfu* Turbo AD as described by the manufacturer (Stratagene, La Jolla, CA). The three candidate genes of *iolE*, *iolB*, and PSPTO\_1053 were each amplified from the genomic DNA using primers as listed in Table 1. PCR products were blunt-end ligated to EcoRV-digested pBluescriptII, resulting in pBioIE, pBioIB, pBioIEB, and pB1053, which were each sequenced to confirm their identities. *iolE*, *iolB*, and *iolEB* were each cleaved by digestion with EcoRI and SalI and then ligated to pTrc99A digested by the same enzymes to construct pTioIE, pTioIB, and pTioIEB, respectively. PSPTO\_1053 from pB1053 was cleaved by digestion with NcoI and SacI and then ligated to pTrc99A digested by the same enzymes, resulting in pT1053.

Putative *udh* genes from genomic DNA of *A. tumefaciens*, *P. putida*, and *P. syringae* were amplified using the primer pairs ATudh2-F/ATudh-R, PPudh-F/PPudh-R, and PSudh-F/1053-R, respectively (Table 1). PCR products were blunt-end ligated to pBluescriptII digested with EcoRV, resulting in plasmids pBATudh2, pBPudh, and pBPSudh. To construct plasmids pTATudh2, pTPPudh, and pTPSudh, the corresponding genes were excised with EcoRI and SacI from pBATudh2, pBPudh, and pBPSudh, respectively, and were inserted into the same sites of pTrc99SE.

**Protein purification and determination of kinetic parameters.** The *udh* genes from genomic DNA of *A. tumefaciens*, *P. putida*, and *P. syringae* were amplified using primers ATuEQ-F/R, PPuEQ-F/R, and PSuEQ-F/R (Table 1), and the PCR products were digested with SacI and HindIII and inserted into the same sites of pET21b containing a six-His tag to construct pETATu, pETPPu, and pETPSu, respectively (Table 1). These plasmids were used to transform *E. coli* BL21(DE3) for use for protein expression. The recombinant *E. coli* BL21 strains were cultivated at 30°C and 250 rpm for 6 h after IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) induction. Protein purification was carried out using the Pro-Bond purification system as described by the manufacturer (Invitrogen Corp., Carlsbad, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Sambrook and Russell (35). Enzyme activities on substrates of purified proteins were measured by monitoring initial NADH generation at 340 nm and room temperature. Kinetic analysis on glucuronate and galacturonate was carried out using 0 to 10 mM glucuronate or galacturonate and 1.2 mM  $\text{NAD}^+$  in 100 mM Tris-HCl, pH 8.0. Kinetic analysis on  $\text{NAD}^+$  was performed using 0 to 2 mM  $\text{NAD}^+$  and 10 mM glucuronate in 100 mM Tris-HCl, pH 8.0. A series of enzymatic assays were conducted to estimate the initial activity as a function of starting substrate concentration. These data were used to fit the parameters of the Michaelis-Menten kinetic model,  $k_{\text{cat}}$  and  $K_m$ , by nonlinear least-squares regression. Nonlinear least-squares regression analyses were performed via the Gauss-Newton method as implemented using the intrinsic *nlinfit* function of the Matlab software program.

**LC-MS and circular dichroism (CD) analysis for determination of glucarate produced from glucuronate by Udh.** The reaction mixture for the production of glucarate from glucuronate by Udh consisted of 20 mM glucuronate, 21.6 mM  $\text{NAD}^+$ , 40 mM sodium phosphate buffer, pH 8.0, and bacterial lysate prepared as described above. The enzyme reaction was performed by the addition of either crude lysate or purified proteins to the reaction mixture and incubation at room temperature for 60 min, and this was stopped by the addition of 1 M sodium hydroxide. Glucarate was separated from the reaction mixture by using a column packed with boronic acid affinity gel (Affi-Gel boronate gel; Bio-Rad Laboratories, Hercules, CA) which is able to bind to the coplanar adjacent *cis*-hydroxyl groups of glucarate (29). Glucuronate cannot bind to the gel due to its *trans*-diol groups. After the Affi-Gel column was loaded with reaction mixture, the column was washed with 80 mM potassium phosphate-20 mM boric acid buffer (pH 7.0), and then glucarate was eluted by the addition of 0.1 M HCl. The eluent was neutralized by the addition of 5 M NaOH then analyzed by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1100 series LC/MSD instrument (Agilent Technologies) equipped with an Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad Laboratories, Hercules, CA) and an electron spray ionization detector. Mass spectra were obtained in both the positive and negative ion detection modes. Trifluoroacetic acid (0.1% [vol/vol]), pH 2.0, was used as the mobile phase at a flow rate of 0.5 ml/min at room temperature.

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description <sup>b</sup>	Reference or source
<b>Strains</b>		
<i>Pseudomonas syringae</i> pv. tomato strain DC3000	Wild type	Frederick Ausubel
<i>Pseudomonas putida</i> KT2440	Wild type	ATCC 47504
<i>Escherichia coli</i> DH10B	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 endA1 araD139</i> Δ( <i>ara-leu</i> )7697 <i>galU galK</i> λ <sup>-</sup> <i>rpsL nupG</i>	Invitrogen Corp., Carlsbad, CA
<i>Escherichia coli</i> MG1655 Δ <i>uxaC</i>	Wild type with deletion of the <i>uxaC</i> gene, encodes D-glucuronate isomerase	17
<i>Escherichia coli</i> BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>gal dcm</i> (DE3)	Invitrogen Corp., Carlsbad, CA
<b>Plasmids</b>		
pBluescriptII	<i>lac</i> promoter, ColE1 origin, ampicillin resistant, <i>lacZ</i>	Stratagene, La Jolla, CA
pTrc99A	<i>trc</i> promoter, pBR322 origin, ampicillin resistant, <i>lacI<sup>q</sup></i>	1
pET21b	T7 promoter, ColE1 origin, ampicillin resistant, <i>lacI</i>	Novagen, Darmstadt, Germany
pTrc99SE	pTrc99A containing RBS sequence of AGGAGGTAATAAAT	Seon-Won Kim
pTiolE	pTrc99A with <i>iolE</i> of <i>P. syringae</i>	This study
pTiolB	pTrc99A with <i>iolB</i> of <i>P. syringae</i>	This study
pTiolEB	pTrc99A with <i>iolE</i> and <i>iolB</i> of <i>P. syringae</i>	This study
pT1053	pTrc99A with PSPTO_1053 of <i>P. syringae</i>	This study
pTATudh2	pTrc99SE with <i>udh</i> of <i>A. tumefaciens</i>	This study
pTPPudh	pTrc99SE with <i>udh</i> of <i>P. putida</i>	This study
pTPSudh	pTrc99SE with <i>udh</i> of <i>P. syringae</i>	This study
pETATu	pET21b with <i>udh</i> of <i>A. tumefaciens</i>	This study
pETPPu	pET21b with <i>udh</i> of <i>P. putida</i>	This study
pETPSu	pET21b with <i>udh</i> of <i>P. syringae</i>	This study
<b>Primers<sup>a</sup></b>		
<i>iolE</i> -F	5'-CGAATTCAGGAGGTACAACCATGCCTGTTTCAG-3'	
<i>iolE</i> -R	5'-CGTCGACTTATCGCGCATCGCCAGCAGTTG-3'	
<i>iolB</i> -F	5'-CGAATTCAGGAGGATTGAATCATGAGTC-3'	
<i>iolB</i> -R	5'-CGTCGACTTAAAGATCCAGCAGCCAGC-3'	
1053-F	5'-GCCATGGCATCGGCTCATACCAC-3'	
1053-R	5'-CGAGCTCTTATTATCGCCGAACGGTCC-3'	
ATudh2-F	5'-CTAGAATTCATGAAACGGCTTCTTGTTACC-3'	
ATudh-R	5'-CTAGAGCTCTTAGCTCTGTTTGAAGATCGGGTTG-3'	
PPudh-F	5'-GTCGAATTCATGACCACTACCCCTTCAATC-3'	
PPudh-R	5'-CTAGAGCTCCGTGGGGTTAGTTGAACGGGC-3'	
PSudh-F	5'-CTAGAATTCATGGCATCGGCTCATACCACTC-3'	
ATuEQ-F	5'-TCAGAGCTCGAAACGGCTTCTTGTTACCGGTGC-3'	
ATuEQ-R	5'-CTGAAGCTTGCTCTGTTTGAAGATCGGGTTGTCG-3'	
PPuEQ-F	5'-TCAGAGCTCGACCACTACCCCTTCAATCGCC-3'	
PPuEQ-R	5'-CTGAAGCTTGTTGAACGGGCCCGCCACGGCG-3'	
PSuEQ-F	5'-TCAGAGCTCGGCATCGGCTCATACCACTCAAATCC-3'	
PSuEQ-R	5'-CTGAAGCTTTTATCGCCGAACGGTCCGGACGC-3'	

<sup>a</sup> Primer binding sites, restriction sites, and start or stop codons are indicated as letters with boldface, double underline, and single underline, respectively.

<sup>b</sup> RBS, ribosome binding site.

The stereochemistry of glucarate formed from glucuronate was confirmed by comparing its CD spectrum with that of an authentic glucarate standard. CD was performed on a model 202 CD spectrometer (Aviv Biomedical, Lakewood, NJ). Reaction mixtures contained 20 mM glucuronic acid, 7 mM NAD<sup>+</sup>, 100 mM potassium phosphate buffer (pH 8.0), and the purified enzymes prepared as described above. Glucarate was separated from glucuronate by using boronic acid affinity gel as described above.

**Computational analysis including sequence identification and alignment analysis.** Biocyc (<http://biocyc.org/>) was used to identify relevant metabolic pathways and metabolites. DNA sequences for *P. syringae*, *P. putida*, and *A. tumefaciens* were obtained from the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>), with accession numbers NC\_004578, NC\_002947, and NC\_003063, respectively. Homology and conserved domain searches were performed using the NCBI BLAST algorithm. Sequence management and alignment were carried out using Vector NTI software (Invitrogen, Carlsbad, CA). Alignment and phylogenetic analyses were performed using the AlignX module of Vector NTI.

**Nucleotide sequence accession numbers.** The *udh* gene sequence from *P. syringae* has been deposited in GenBank (accession number EU377538). The

corresponding genes from *A. tumefaciens* and *P. putida* were deposited with accession numbers BK006462 and BK006380, respectively.

## RESULTS

**Cloning of the *udh* gene from *Pseudomonas syringae*.** The screen established to identify the gene corresponding to Udh activity in *P. syringae* utilized a mutant strain of *E. coli* MG1655. The deletion of *uxaC* prevents growth on glucuronate while permitting the strain to retain the ability to grow on glucarate as a sole carbon source. Since Udh catalyzes the conversion of glucuronate to glucarate (3, 38), *E. coli* MG1655 Δ*uxaC* clones harboring *udh* genes from a *P. syringae* genomic library should grow on glucuronate as the sole carbon source. *E. coli* DH10B and pTrc99A were used as the host strain and

plasmid vector, respectively, for the initial construction of the *P. syringae* genomic library. A plasmid library pool was prepared from the *E. coli* DH10B clone pool and then used to transform the  $\Delta uxaC$  strain. Transformed  $\Delta uxaC$  clones were incubated on M9 minimal agar containing glucuronate for 4 days at 30°C.

From 10 agar plates, 28 clones were selected for further screening, each of which contained an inserted fragment of 2 to 5 kb. From these, eight clones with different-sized inserts were sequenced for comparison with the *P. syringae* genome sequence (GenBank accession number NC\_004578). Six of these clones included *iolE*, *iolB*, or both of them, while one clone contained the unassigned PSPTO\_1053 open reading frame. The final clone included a chimera of the *iolEB* and PSPTO\_1053 regions. The open reading frames from the library fragments were PCR amplified and inserted into expression vector pTrc99A, yielding plasmids pTiolE, pTiolB, pTiolEB, and pT1053. Clones containing these vectors were used to determine which gene corresponded to uronate dehydrogenase activity. *E. coli* MG1655, the  $\Delta uxaC$  derivative, and four  $\Delta uxaC$  clones transformed with the candidate genes were incubated on M9 minimal agar containing glucuronate as the sole carbon source. Wild-type MG1655, MG1655(pTiolB)  $\Delta uxaC$ , MG1655(pTiolEB)  $\Delta uxaC$ , and MG1655(pT1053)  $\Delta uxaC$  strains grew on M9-glucuronate agar, while the MG1655(pTrc99A)  $\Delta uxaC$  and MG1655(pTiolE)  $\Delta uxaC$  strains did not. Therefore, *iolB* and PSPTO\_1053 were responsible for growth on glucuronate as the sole carbon source, identifying them as candidate *udh* genes.

To further discriminate between the two candidate genes, plasmids pTiolB and pT1053 were used to transform *E. coli* DH10B to express the recombinant genes. The resulting clones were grown in LB medium with 0.1 mM IPTG. Analysis of Udh activity in crude lysates from these two clones suggested that the strain harboring pT1053, but not that harboring pTiolB, exhibits Udh activity. The assay employed glucuronate as a substrate and monitored production of NADH at 340 nm. Consequently, it was deduced that the 828-bp PSPTO\_1053 gene encoded uronate dehydrogenase. The gene is hereafter referred to as *udh* and was registered in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) under accession number EU377538.

**Cloning and identification of *udh* genes from *P. putida* and *A. tumefaciens*.** The translated protein sequence from *udh* from *P. syringae* was analyzed using BLASTP from NCBI (<http://www.ncbi.nlm.nih.gov/blast/>) to identify putative homologues. The Udh activity of *A. tumefaciens* has been studied previously (5, 6, 43). The translation of open reading frame Atu3143 of *A. tumefaciens* had the highest sequence identity from this organism (47.8%) and was considered a candidate for a homologous Udh. Another candidate open reading frame, PP1171 of *Pseudomonas putida* KT2440, was also found to have high similarity to *P. syringae* Udh, with a sequence identity of 75.6%. Atu3143 and PP1171 were PCR amplified from their respective genomes and, along with *udh* from *P. syringae*, were integrated into plasmid vector pTrc99SE to create plasmids pTATudh2, pTPPudh, and pTPSudh, respectively, for comparison of relative activities of the expressed recombinant proteins. Transformed DH10B clones were cultivated in LB medium with or without 0.1 mM IPTG before the preparation of crude lysates to carry out enzymatic analysis. These assays

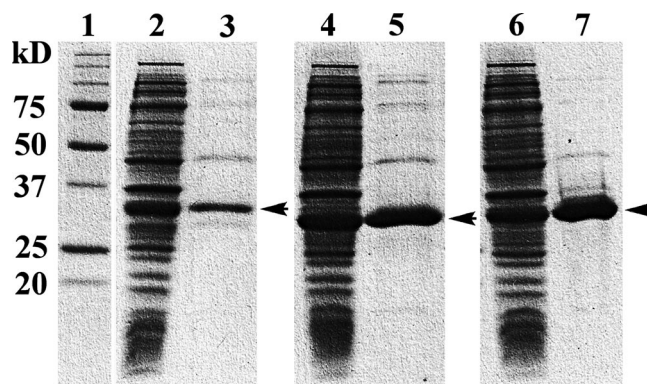


FIG. 2. SDS-PAGE analysis of purified Udhs. The purified Udhs were subjected to electrophoresis in a 12% SDS-polyacrylamide gel under denaturing conditions. Lane 1, molecular weight markers; lanes 2 and 3, crude extract and purified *A. tumefaciens* Udh of *E. coli* BL21(DE3) expressing pETATu; lanes 4 and 5, crude extract and purified *P. putida* Udh of *E. coli* BL21(DE3) expressing pETPPu; lanes 6 and 7, crude extract and purified *P. syringae* Udh of *E. coli* BL21(DE3) expressing pETPSu. Molecular masses (in kDa, equivalent to molecular weights in thousands) are shown to the left. The purified Udhs are indicated by the arrows.

confirmed a NAD<sup>+</sup>-consuming activity in the presence of glucuronate as a substrate for the recombinant proteins of *A. tumefaciens* and *P. putida*, similar to that previously obtained with *P. syringae*. The two *udh* genes from *A. tumefaciens* and *P. putida* were also deposited in GenBank under accession numbers BK006462 and BK006380, respectively.

**Purification and characterization of recombinant Udh and analysis of the reaction product.** Enzyme reactions using crude *E. coli* lysates containing the *P. syringae* *udh* gene confirmed the presence of an activity that utilized glucuronate as a substrate, with the reaction rate proportional to glucuronate concentration for low substrate loads (data not shown). The activity also utilized NAD<sup>+</sup> but not NADP<sup>+</sup> as a cofactor (data not shown). These results indicated that the substrate was oxidized. An examination of the structure of glucuronate suggests two possible points of oxidation: the conversion of an alcohol to a ketone or the conversion of the aldehyde to carboxylic acid, the latter reaction producing glucarate. The difference in these two products should be evident from mass spectra, as the former would result in a mass difference of -2 relative to the substrate, while the latter would produce a mass difference of +16. To confirm the product of the enzyme reaction as glucarate, a sample was analyzed by LC-MS. The spectra of the eluent separated from the enzyme reaction and a glucarate standard were in agreement, suggesting glucarate as the product of the Udh reaction (see Fig. S1 in the supplemental material).

Each of the three *udh* genes was expressed in *E. coli* with six-His tags and purified to determine the kinetic parameters of the corresponding enzymes. Purified enzymes were analyzed by SDS-PAGE to confirm the molecular weight of the monomer and estimate purity (Fig. 2). The Udh proteins of *P. syringae* and *P. putida* both had molecular weights of approximately 30,000, which are consistent with both the translation of the cloned gene and previous reports (38). The *A. tumefaciens* Udh is slightly larger, with a molecular weight of 32,000.

TABLE 2. Turnover numbers and Michaelis constants of uronate dehydrogenases from *A. tumefaciens*, *P. putida*, and *P. syringae*

Strain and substrate	Kinetics parameter		
	$k_{\text{cat}}$ ( $10^2 \text{ s}^{-1}$ )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ ( $10^2 \text{ s}^{-1} \text{ mM}^{-1}$ )
<i>A. tumefaciens</i>			
Glucuronate	1.9 ± 0.1	0.37 ± 0.12	5.2
Galacturonate	0.92 ± 0.14	0.16 ± 0.12	5.7
NAD <sup>+</sup>		0.18 ± 0.03	11
<i>P. putida</i>			
Glucuronate	0.55 ± 0.03	0.25 ± 0.07	2.2
Galacturonate	0.30 ± 0.03	0.10 ± 0.06	3.0
NAD <sup>+</sup>		0.21 ± 0.02	2.6
<i>P. syringae</i>			
Glucuronate	0.74 ± 0.03	0.28 ± 0.07	2.6
Galacturonate	0.24 ± 0.01	0.04 ± 0.01	6.0
NAD <sup>+</sup>		0.17 ± 0.07	4.3

The purified preparations were used to determine the kinetic parameters,  $k_{\text{cat}}$  and  $K_m$ , for each of the enzymes. Both glucuronate and galacturonate were used as substrates, and the NAD<sup>+</sup> cofactor concentration was also varied to determine the corresponding  $K_m$  (Table 2). Measurements of  $k_{\text{cat}}$  obtained by varying the cofactor concentration were within 20% of the values obtained using glucuronate as the substrate (data not shown). In all cases, the  $k_{\text{cat}}$  for glucuronate was higher than that for galacturonate. The highest rate constant was found for the *A. tumefaciens* enzyme utilizing glucuronate as the substrate ( $k_{\text{cat}} = 1.9 \times 10^2 \text{ s}^{-1}$ ), which was more than twofold higher than the rate for the *Pseudomonas* enzymes. However, the Michaelis (affinity) constant was lower for galacturonate in all cases, with the lowest  $K_m$ , 0.04 mM, found for the *P. syringae* enzyme utilizing galacturonate as the substrate. The first-order rate constants ( $k_{\text{cat}}/K_m$ ) were highest for galacturonate as substrate, with the largest difference between glucuronate and galacturonate observed for *P. syringae*.

The responses of the enzyme activities to changes in pH and temperature were also investigated (Fig. 3). A pH optimum of 8.0 was observed for both the *A. tumefaciens* and *P. syringae* enzymes, although the activities were relatively unchanged between pH ~7 and pH ~8 for *P. syringae* Udh (Fig. 3a). This pH

behavior is consistent with previous reports for *P. syringae* Udh (3). The *P. putida* enzyme exhibited highest activity at pH ~7.0. In general, enzyme activities varied approximately 10% between pH ~5 and pH ~8, with significant drops in activity observed for pH values greater than 8 for all three enzymes.

The impact of temperature was evaluated in two ways. First, the thermal stability was examined by exposing enzyme preparations to various temperatures for 30 min and then performing the enzyme assay under standard conditions. The *A. tumefaciens* Udh was found to exhibit a significantly higher thermal stability than either of the *Pseudomonas* enzymes (Fig. 3b). The activity remained near 80% of the maximum after exposure of the *A. tumefaciens* preparation to 37°C, while the corresponding activities for both of the other enzymes were below 20% of the maximum. The stability profiles for the two *Pseudomonas* enzymes were similar to one another. Finally, enzyme activity was evaluated for assays conducted under increasing temperatures. These activities followed a general trend of increasing with increasing temperatures between 4 and 42°C, which is consistent with an Arrhenius-type dependence of the catalytic rate constant on temperature (Fig. 3c).

For the final characterization of the products of these reactions, the boronic acid affinity gel was used to isolate the putative glucarate produced from all three enzymes in *in vitro* reactions using purified proteins. Samples of the three products were then subjected to CD analysis to examine the stereochemistry of the compounds. All three spectra were in agreement with a glucarate standard, confirming the identity of the product as glucaric acid and the identity of the three genes as those encoding uronate dehydrogenases (data not shown).

## DISCUSSION

Udh catalyzes the first step of an oxidation pathway for aldohexuronate catabolism in bacteria. For bacteria, only limited studies of Udh in *P. syringae* and *A. tumefaciens* have been reported. Moreover, Udh has been even more rarely studied in eukaryotes. A Udh sequence in the wine grape *Vitis vinifera* has been identified as galacturonate reductase (EC 1.1.1.203; BRENDA accession number A1Y2Z0, GenBank accession number DQ843600). We synthesized this gene with codon usage optimized for expression in *E. coli* (DNA 2.0, Menlo Park, CA) and expressed the recombinant protein. However, no

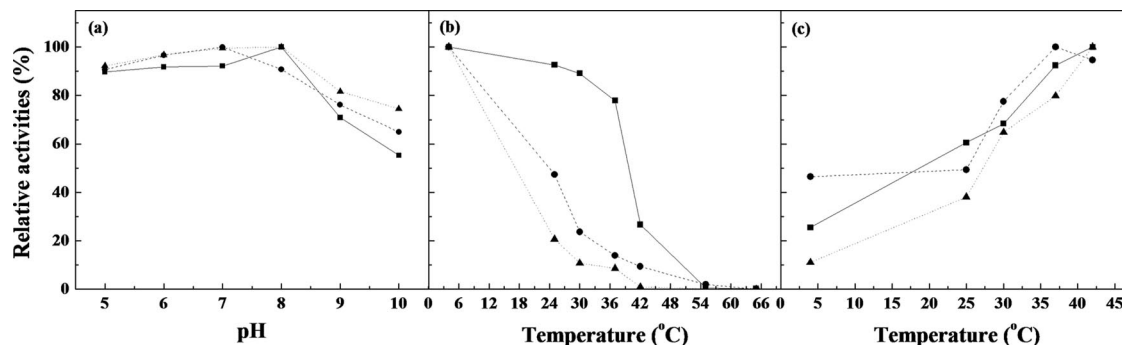


FIG. 3. Effects of pH and temperature on activities of Udh's from *A. tumefaciens*, *P. putida*, and *P. syringae* udh. (a) Relative activities as a function of pH. (b) Relative activities after incubation for 30 min at indicated temperatures. (c) Relative activities as a function of assay temperature. Squares, *A. tumefaciens* Udh; circles, *P. putida* Udh; triangles, *P. syringae* Udh.

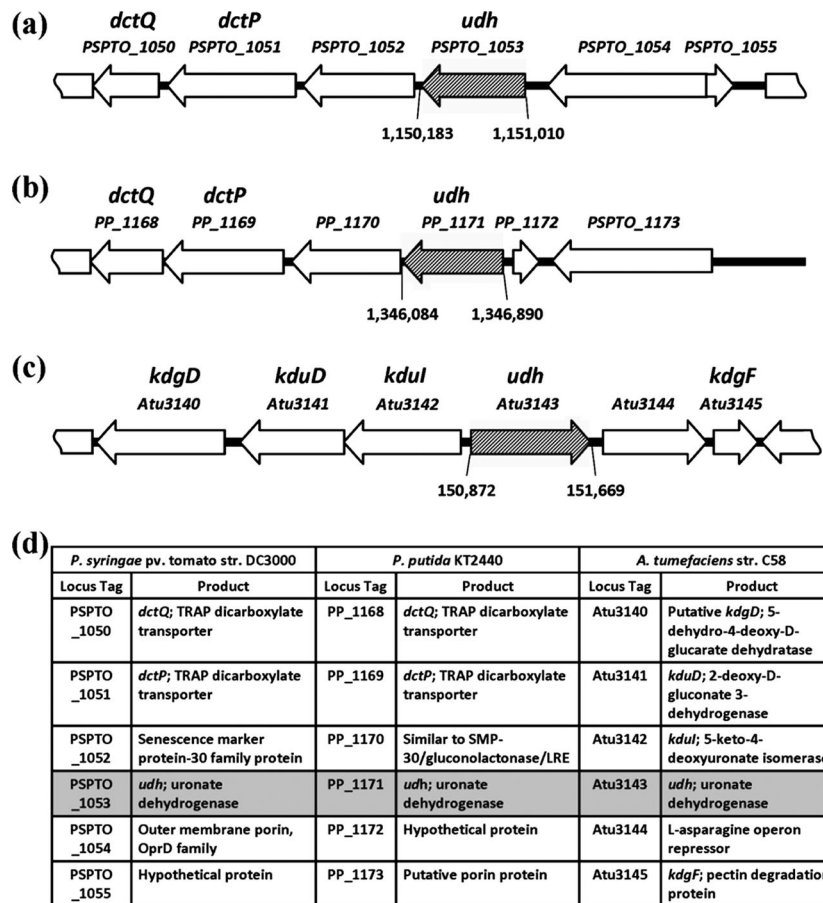


FIG. 4. Loci of *udh* genes on chromosomes of *P. syringae* pv. tomato strain (str.) DC3000 (a), *P. putida* KT2440 (b), and *A. tumefaciens* strain C58 (c). (d) Identities of adjacent genes. These loci and identities refer to the genome sequences of NC\_004578 (*P. syringae* pv. tomato strain DC3000), NC\_002947 (*P. putida* KT2440), and NC\_003063 (*A. tumefaciens* strain C58).

activity related to Udh was observed when using either NAD<sup>+</sup> or NADP<sup>+</sup> as a cofactor (data not shown). An alignment of this sequence with the *P. syringae* Udh identified in the current work reveals only 10% identity between them. We cannot rule out the possibility that the *V. vinifera* enzyme could not be functionally expressed in *E. coli*; however, based on the alignment, we conclude that the reported sequence from *V. vinifera* either is not uronate dehydrogenase or is a highly divergent version of the enzyme.

A shotgun library of *P. syringae* was introduced into *uxaC* deletion strains of *E. coli* to screen for the *udh* gene encoding uronate dehydrogenase, and PSPTO\_1053 and *iolB* were identified and screened as possible Udh gene candidates. By enzymatic analysis, PSPTO\_1053 was ultimately identified as the *udh* gene encoding uronate dehydrogenase. In a *uxaC* deletion mutant of *E. coli*, in which glucuronate catabolism is abolished, glucuronate was converted to glucarate by uronate dehydrogenase and then degraded to pyruvate or 2-phosphoglycerate, from which it can be used as an energy source (27, 33). In *uxaC* deletion strains of *E. coli*, introduction of the *iolB* gene allowed for growth on M9 agar containing glucuronate as a sole carbon source as well, but this gene did not possess Udh activity. *IolB* has previously been reported as a protein related to myo-inositol catabolism in *Bacillus subtilis* and *Lactobacillus casei*

(41, 42). *IolB* belongs to the *iol* operon used for myo-inositol degradation in *Bacillus subtilis* and converts 5-deoxy-glucuronate to 2-deoxy-5-keto-D-gluconate (42). *IolB* of *P. syringae* has about 48% homology with that of *B. subtilis*. The precise mechanism of glucuronate consumption in cells harboring *IolB* in our screen is unclear. Presumably, this protein is able to convert glucuronate to an analogous compound that is compatible with *E. coli* metabolism.

The *udh* gene loci in the genomes of *P. syringae*, *P. putida*, and *A. tumefaciens* are illustrated in Fig. 4. The *udh* loci of *P. syringae* and *P. putida* are at about 1,150 and 1,346 kb, respectively, while the *udh* locus in *A. tumefaciens* is at about 150 kb. In *A. tumefaciens*, the genes *Atu3140*, *Atu3141*, *Atu3142*, and *Atu3145* adjacent to *udh* are *kdgD*, *kduD*, *kduI*, and *kdgF*, respectively, and are related to pectin degradation. Pectin is a heteropolysaccharide, consisting of  $\alpha$ -1,4-linked D-galacturonate residues, which is derived from plant cell walls. Pectin degradation and uptake by bacteria have been well researched in studies of phytopathogenic pectobacteria, including *Erwinia chrysanthemi* and *Erwinia carotovora* by Hugouvieux-Cotte-Pattat et al. (12–14). In *E. chrysanthemi*, pectin is degraded by genes of the *kdu* or *kdg* operon to use as an energy source. In *P. syringae* and *P. putida*, the genes adjacent to *udh* are identified as TRAP (tripartite ATP-independent periplasmic) di-

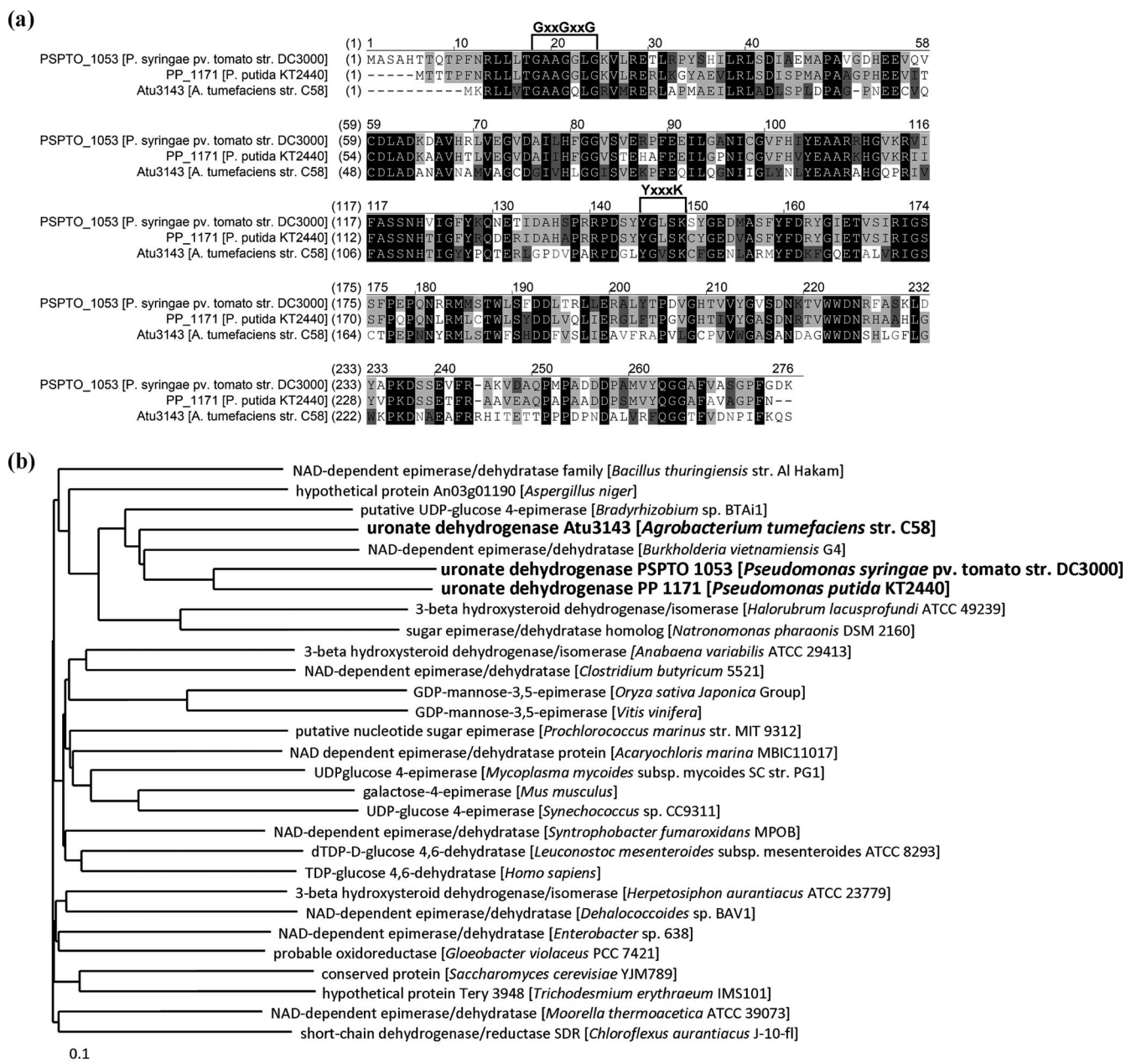


FIG. 5. (a) Alignment of uronate dehydrogenase from *P. syringae* pv. tomato strain (str.) DC3000, *P. putida* KT2440, and *A. tumefaciens* strain C58. For alignment, identical, conservative, and similar amino acid residues are represented as black, dark gray, and light gray blocks, respectively. Primary sequence motifs are indicated as GxxGxxG and YxxxK. (b) Phylogenetic analysis of the uronate dehydrogenase homologues from diverse prokaryotic and eukaryotic species. Phylogenetic analysis was performed using homologues of PSPTO\_1053 of *P. syringae* pv. tomato strain DC3000. Uronate dehydrogenases are indicated in bold.

carboxylate transporters and porin. Among these genes, the porin protein gene (PSPTO\_1054 and PP\_1173) is known to be related to uptake of oligogalacturonate derived from pectin degradation (34). Uronate dehydrogenase in plant pathogen bacteria might therefore function in the utilization of a hexuronate, derived from host plant cell wall pectin, which is subsequently converted to hexarate.

Alignment of the three uronate dehydrogenases from *P. syringae*, *P. putida*, and *A. tumefaciens* and phylogenetic analysis of their homologs were performed (Fig. 5). The sequences

of the enzymes show two primary sequence motifs, YxxxK and GxxGxxG, related to conserved domains (Fig. 5a). The YxxxK motif is located between Tyr<sub>145</sub> and Lys<sub>149</sub> of *P. syringae* Udh and is the primary motif of the 3- $\alpha$ /3- $\beta$  hydroxysteroid dehydrogenase domain (11, 37). The GxxGxxG motif located in the Gly<sub>18</sub>-to-Gly<sub>24</sub> region of *P. syringae* Udh is similar to Rossmann folds, GxxxG or Gx<sub>1-2</sub>GxxG, which have been discovered in NAD<sup>+</sup> binding domains (20). In the phylogenetic analysis, the uronate dehydrogenase showed homologies with NAD-dependent epimerase/dehydratase, nucleotide sugar epi-

merase, 3-beta hydroxysteroid dehydrogenase/isomerase, and short-chain dehydrogenase/reductase in archaea and bacteria, including proteobacteria, cyanobacteria, green nonsulfur bacteria, and gram-positive bacteria, as well as homology with nucleotide sugar epimerase in a few eukaryotes, including fungi, plants, and humans (Fig. 5b). The three uronate dehydrogenases screened in this study are present in alpha-proteobacteria and gammaproteobacteria, and their homologies are relatively close to those in the *Archaea Halorubrum lacusprofundi* and *Natronomonas pharaonis* and the fungus *Aspergillus niger*.

We have screened and sequenced three uronate dehydrogenases from *A. tumefaciens*, *P. putida*, and *P. syringae* that can effectively convert glucuronate to glucarate. While this enzyme is important for the catabolism of uronic acids in several types of bacteria, it may also be useful in the development of biosynthetic pathways for the production of aldaric acids, such as glucaric acid. Glucarate is the end product of nucleotide sugar metabolism and is found naturally in mammals and plants (21, 39). Glucarate and its derivatives, such as glucaro-1,4-lactone, have been studied previously as detoxifying and natural anticarcinogenic compounds (8, 21, 36, 39), as well as building blocks for polymer synthesis (16). Glucarate has also been designated as a potential "top value-added" chemical to be produced from biomass (40). Presently, glucarate is synthesized from glucose by chemical oxidation using a strong oxidant such as nitric acid or nitric oxide (25). We used the *udh* of *P. syringae* identified in this study to successfully produce glucaric acid from a synthetic pathway in *E. coli* (26).

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#### REFERENCES

- Amann, E., B. Ochs, and K. J. Abel. 1988. Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* **69**:301–315.
- Ashwell, A., A. J. Wahba, and J. Hickman. 1958. A new pathway of uronic acid metabolism. *Biochim. Biophys. Acta* **30**:186–187.
- Bateman, D. F., T. Kosuge, and W. W. Kilgore. 1970. Purification and properties of uronate dehydrogenase from *Pseudomonas syringae*. *Arch. Biochem. Biophys.* **136**:97–105.
- Buell, C. R., V. Joardar, M. Lindeberg, J. Selengut, I. T. Paulsen, M. L. Gwinn, R. J. Dodson, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, S. Daugherty, L. Brinkac, M. J. Beanan, D. H. Haft, W. C. Nelson, T. Davidsen, N. Zafar, L. Zhou, J. Liu, Q. Yuan, H. Khouri, N. Fedorova, B. Tran, D. Russell, K. Berry, T. Utterback, S. E. Van Aken, T. V. Feldblyum, M. D'Ascenzo, W. L. Deng, A. R. Ramos, J. R. Alfano, S. Cartinhour, A. K. Chatterjee, T. P. Delaney, S. G. Lazarowitz, G. B. Martin, D. J. Schneider, X. Tang, C. L. Bender, O. White, C. M. Fraser, and A. Collmer. 2003. The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. USA* **100**:10181–10186.
- Chang, Y. F., and D. S. Feingold. 1970. D-Glucaric acid and galactaric acid catabolism by *Agrobacterium tumefaciens*. *J. Bacteriol.* **102**:85–96.
- Chang, Y. F., and D. S. Feingold. 1969. Hexuronic acid dehydrogenase of *Agrobacterium tumefaciens*. *J. Bacteriol.* **99**:667–673.
- Cynkin, M. A., and G. Ashwell. 1960. Uronic acid metabolism in bacteria. IV. Purification and properties of 2-keto-3-deoxy-D-glucuronokinase in *Escherichia coli*. *J. Biol. Chem.* **235**:1576–1579.
- Duff, K. 2002. Calcium-D-glucarate. *Altern. Med. Rev.* **7**:336–339.
- Farmer, J. J., III, and R. G. Eagon. 1969. Aldohexuronic acid catabolism by a soil *Aeromonas*. *J. Bacteriol.* **97**:97–106.
- Goodner, B., G. Hinkle, S. Gattung, N. Miller, M. Blanchard, B. Qurollo, B. S. Goldman, Y. Cao, M. Askenazi, C. Halling, L. Mullin, K. Houmiel, J. Gordon, M. Vaudin, O. Iartchouk, A. Epp, F. Liu, C. Wollam, M. Allinger, D. Doughty, C. Scott, C. Lappas, B. Markelz, C. Flanagan, C. Crowell, J. Gurson, C. Lomo, C. Sear, G. Strub, C. Cielo, and S. Slater. 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* **294**:2323–2328.
- Hoffmann, F., C. Sotriffer, A. Evers, G. Xiong, and E. Maser. 2007. Understanding oligomerization in 3alpha-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni*: an in silico approach and evidence for an active protein. *J. Biotechnol.* **129**:131–139.
- Hugouvioux-Cotte-Pattat, N., G. Condemine, W. Nasser, and S. Reverchon. 1996. Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annu. Rev. Microbiol.* **50**:213–257.
- Hugouvioux-Cotte-Pattat, N., W. Nasser, and J. Robert-Baudouy. 1994. Molecular characterization of the *Erwinia chrysanthemi* *kdgK* gene involved in pectin degradation. *J. Bacteriol.* **176**:2386–2392.
- Hugouvioux-Cotte-Pattat, N., and S. Reverchon. 2001. Two transporters, TogT and TogMNAB, are responsible for oligogalacturonide uptake in *Erwinia chrysanthemi* 3937. *Mol. Microbiol.* **41**:1125–1132.
- Hugouvioux-Cotte-Pattat, N., and J. Robert-Baudouy. 1987. Hexuronate catabolism in *Erwinia chrysanthemi*. *J. Bacteriol.* **169**:1223–1231.
- Ibert, M., F. Marsais, N. Merbouh, and C. Bruckner. 2002. Determination of the side-products formed during the nitroxide-mediated bleach oxidation of glucose to glucaric acid. *Carbohydr. Res.* **337**:1059–1063.
- Kang, Y., T. Durfee, J. D. Glasner, Y. Qiu, D. Frisch, K. M. Winterberg, and F. R. Blattner. 2004. Systematic mutagenesis of the *Escherichia coli* genome. *J. Bacteriol.* **186**:4921–4930.
- Kilgore, W. W., and M. P. Starr. 1959. Catabolism of galacturonic and glucuronic acids by *Erwinia carotovora*. *J. Biol. Chem.* **234**:2227–2235.
- Kilgore, W. W., and M. P. Starr. 1959. Uronate oxidation by phytopathogenic pseudomonads. *Nature* **183**:1412–1413.
- Kleiger, G., and D. Eisenberg. 2002. GXXXG and GXXXA motifs stabilize FAD and NAD(P)-binding Rossmann folds through C(alpha)-H...O hydrogen bonds and van der Waals interactions. *J. Mol. Biol.* **323**:69–76.
- Marsh, C. A. 1986. Biosynthesis of D-glucaric acid in mammals: a free-radical mechanism? *Carbohydr. Res.* **153**:119–131.
- Mata-Gilsinger, M., and P. Ritzenthaler. 1983. Physical mapping of the *exuT* and *uxaC* operators by use of *exu* plasmids and generation of deletion mutants in vitro. *J. Bacteriol.* **155**:973–982.
- McRorie, R. A., and G. D. Novelli. 1958. Glucuronate metabolism by *Aerobacter aerogenes*. *Nature* **182**:1504–1505.
- McRorie, R. A., A. K. Williams, and W. J. Payne. 1959. Alduronic acid metabolism by bacteria. *J. Bacteriol.* **77**:212–216.
- Merbouh, N., J. Francois Thaburet, M. Ibert, F. Marsais, and J. M. Bobbitt. 2001. Facile nitroxide-mediated oxidations of D-glucose to D-glucaric acid. *Carbohydr. Res.* **336**:75–78.
- Moon, T. S., S. H. Yoon, A. M. Lanza, J. D. Roy-Mayhew, and K. J. Prather. 5 December 2008. Production of glucaric acid from a synthetic pathway in recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* doi:10.1128/AEM.00973-08.
- Neidhardt, F. C., and R. Curtiss. 1996. *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
- Payne, W. J., and R. McRorie. 1958. Glucuronate isomerase from *Serratia marcescens*. *Biochim. Biophys. Acta* **29**:466–467.
- Poon, R., D. C. Villeneuve, I. Chu, and R. Kinach. 1993. HPLC determination of D-glucaric acid in human urine. *J. Anal. Toxicol.* **17**:146–150.
- Portalier, R. C., J. M. Robert-Baudouy, and G. M. Nemoz. 1974. Studies of mutations in the uronic isomerase and altronic oxidoreductase structural genes of *Escherichia coli* K 12. *Mol. Gen. Genet.* **128**:301–319. (In French.)
- Robert-Baudouy, J., J. Jimeno-Abendano, and F. Stoeber. 1982. D-Mannonate and D-altrionate dehydratases of *Escherichia coli* K12. *Methods Enzymol.* **90**(Part E):288–294.
- Robert-Baudouy, J. M., and F. R. Stoeber. 1973. Purification and properties of D-mannonate hydrolyase from *Escherichia coli* K12. *Biochim. Biophys. Acta* **309**:473–485. (In French.)
- Robertson, A. M., P. A. Sullivan, M. C. Jones-Mortimer, and H. L. Kornberg. 1980. Two genes affecting glucarate utilization in *Escherichia coli* K12. *J. Gen. Microbiol.* **117**:377–382.
- Rodionov, D. A., M. S. Gelfand, and N. Hugouvioux-Cotte-Pattat. 2004. Comparative genomics of the KdgR regulon in *Erwinia chrysanthemi* 3937 and other gamma-proteobacteria. *Microbiology* **150**:3571–3590.
- Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.



36. **Singh, J., and K. P. Gupta.** 2003. Calcium glucarate prevents tumor formation in mouse skin. *Biomed. Environ. Sci.* **16**:9–16.
37. **Thomas, J. L., J. I. Mason, S. Brandt, B. R. Spencer, Jr., and W. Norris.** 2002. Structure/function relationships responsible for the kinetic differences between human type 1 and type 2 3 $\beta$ -hydroxysteroid dehydrogenase and for the catalysis of the type 1 activity. *J. Biol. Chem.* **277**:42795–42801.
38. **Wagner, G., and S. Hollmann.** 1976. Uronic acid dehydrogenase from *Pseudomonas syringae*. Purification and properties. *Eur. J. Biochem.* **61**:589–596.
39. **Walaszek, Z.** 1990. Potential use of D-glucaric acid derivatives in cancer prevention. *Cancer Lett.* **54**:1–8.
40. **Werpy, T. A., and G. Petersen.** 2004. Top value added chemicals from biomass, vol. 1. Pacific Northwest National Laboratory, Richland, WA.
41. **Yebra, M. J., M. Zuniga, S. Beaufils, G. Perez-Martinez, J. Deutscher, and V. Monedero.** 2007. Identification of a gene cluster enabling *Lactobacillus casei* BL23 to utilize *myo*-inositol. *Appl. Environ. Microbiol.* **73**:3850–3858.
42. **Yoshida, K., M. Yamaguchi, T. Morinaga, M. Kinohara, M. Ikeuchi, H. Ashida, and Y. Fujita.** 2008. *myo*-Inositol catabolism in *Bacillus subtilis*. *J. Biol. Chem.* **283**:10415–10424.
43. **Zajic, J. E.** 1959. Hexuronic dehydrogenase of *Agrobacterium tumefaciens*. *J. Bacteriol.* **78**:734–735.