Cloning and Characterization of Uronate Dehydrogenases from Two Pseudomonads and Agrobacterium tumefaciens Strain C58\textsuperscript{\textdagger}\textdaggerdbl

Sang-Hwal Yoon, Tae Seok Moon, Pooya Iranpour,\textsuperscript{†} Amanda M. Lanza, and Kristala Jones Prather\textsuperscript{∗}

Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139

Received 28 April 2008/Accepted 30 November 2008

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\textsubscript{cat}) with glucuronate as a substrate was higher than that with galacturonate; however, the Michaelis constant (K\textsubscript{m}) for galacturonate was lower than that for glucuronate. The A. tumefaciens enzyme was found to have the highest rate constant (k\textsubscript{cat} = 1.9 \times 10^{5} \text{ s}^{-1} \text{ 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-phosphoglycerate. 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 approximately 30,000 each (38). Udh is a ther- mally 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-6-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 ΔuxaC. 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 Udhs 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 μg/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 μg/ml, respectively. Escherichia coli DH10B (E. coli mcrA Δ(mcrA-mcrB) p80lacZ23M15 ΔuxaC74 recA1 endA1 araD139 (ara-leu)7697 galU galK Δ(pslL 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 ΔuxaC

\textsuperscript{a} Corresponding author. Mailing address: Department of Chemical Engineering, Room 66-458, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139. Phone: (617) 253-1950. Fax: (617) 258-5042. E-mail: kljp@mit.edu.

\textsuperscript{†} Present address: The University of Texas Health Science Center at San Antonio, San Antonio, TX.

\textsuperscript{‡} Supplemental material for this article may be found at http://jb.asm.org/.

\textsuperscript{†} Published ahead of print on 5 December 2008.
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 ΔuxaC to screen for Udh activity. Transformed strains were cultivated 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 pBioE, pBioIB, and pPS1053, which were each sequenced to confirm their identities. iolE, iolB, and isolE were each cleaved by digestion with EcoRI and Sall and then ligated to pTrc99A digested by the same enzymes to construct pTioE, pTioIB, and pTioSA, respectively. PSPTO_1053 from pPS1053 was cleaved by digestion with NcoI and SacI and then ligated to pTrc99A digested by the same enzymes, resulting in pTrc1053.

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/PSudh-R, respectively (Table 1). PCR products were blunt-end ligated to pBlueScriptII digested with EcoRV, resulting in plasmids pBATUdh2, pBPudh, and pBPpsudh. To construct plasmids pLATUdh2, pPPTudp, and pTPSudh, the corresponding genes were excised with EcoRI and SacI from pBATUdh2, pBPudp, and pBPpsudh, 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 ATUdhQ/F/R, PPudhQ/F/R, and PSudhQ/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-β-D-thiogalactopyranoside) induction. Protein purification was carried out using the ProBond 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 NADH in 100 mM Tris-HCl, pH 8.0. Kinetic analysis on NADH was performed using 0 to 2 mM NADH and 10 mM glucarate 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, Kcat and Km, 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 NADH, 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 co-hydroxyl groups of glucarate (29). Glucarate cannot bind to the gel due to its triol-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.

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.

[Diagram of Uronate dehydrogenase pathway]
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).

Wild type with deletion of the udhC gene, encodes O-glucuronate isomerase.

Wild type

Plasmids
- pBluescriptII: lac promoter, ColE1 origin, ampicillin resistant, lacZ
- pTrc99A: trc promoter, pBR322 origin, ampicillin resistant, lacP
- pET21b: T7 promoter, ColE1 origin, ampicillin resistant, lacI
- pTrc99SE: pTrc99A containing RBS sequence of AGGAGGTAAATAATT
- pTiOE: pTrc99A with iOE of P. syringae
- pTiOB: pTrc99A with iOBl of P. syringae
- pTiOEB: pTrc99A with iOEl and oBl of P. syringae
- pTATudh2: pTrc99SE with odh of A. tumefaciens
- pTPudh: pTrc99SE with udh of P. putida
- pTPSudh: pTrc99SE with udh of P. syringae
- pETATu: pET21b with iodh of A. tumefaciens
- pETPPu: pET21b with udh of P. putida
- pETPSu: pET21b with udh of P. syringae

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

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas syringae</em> pv. tomato strain DC3000</td>
<td>Wild type</td>
<td>Frederick Ausubel</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> KT2440</td>
<td>F' mcrA Δ(mrr-hsdRMSc-mcrBC) q80lacZΔM15 ΔlacX74 recA1 endA1 araD139 (ara-leu)7697 galU galK λ- rpsL supG</td>
<td>ATCC 47504</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH10B</td>
<td>F- lac lacY1 Δ(lac-proAB) thi-1 galK galE rpsL mcrB</td>
<td>Invitrogen Corp., Carlsbad, CA</td>
</tr>
<tr>
<td><em>Escherichia coli</em> MG1655 ΔuxaC</td>
<td>F- ompT hsdR (rB mB) gal dcm (DE3)</td>
<td>17</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BL21(DE3)</td>
<td></td>
<td>Invitrogen Corp., Carlsbad, CA</td>
</tr>
</tbody>
</table>

Plasmids
- pBluescriptII: lac promoter, ColE1 origin, ampicillin resistant, lacZ
- pTrc99A: trc promoter, pBR322 origin, ampicillin resistant, lacP
- pET21b: T7 promoter, ColE1 origin, ampicillin resistant, lacI
- pTrc99SE: pTrc99A containing RBS sequence of AGGAGGTAAATAATT
- pTiOE: pTrc99A with iOE of P. syringae
- pTiOB: pTrc99A with iOBl of P. syringae
- pTiOEB: pTrc99A with iOEl and oBl of P. syringae
- pTATudh2: pTrc99SE with odh of A. tumefaciens
- pTPudh: pTrc99SE with udh of P. putida
- pTPSudh: pTrc99SE with udh of P. syringae
- pETATu: pET21b with iodh of A. tumefaciens
- pETPPu: pET21b with udh of P. putida
- pETPSu: pET21b with udh of P. syringae

Primers
- iOE-F: 5’-CGAATTCAGGAGGTACACCATGGCTTTCGACGAC-3’
- iOE-R: 5’-CTAAGTTCACGCGCACTCC-3’
- iOB-F: 5’-GAATTCGGAGGATCCATGCTGACGCTTCT-3’
- iOB-R: 5’-TGCATCTGATCAGTACGTTCTA-3’
- 1053-F: 5’-CTGCACTGATCGTACGTTCTA-3’
- 1053-R: 5’-GCATCTGATCAGTACGTTCTA-3’
- ATudh2-F: 5’-CTAAGTTCACGCGCACTCC-3’
- ATudh-R: 5’-GAATTCGGAGGATCCATGCTGACGACGAGTGCAG-3’
- PPudh-F: 5’-GTCATCTGATCAGTACGTTCTA-3’
- PPudh-R: 5’-TGCATCTGATCAGTACGTTCTA-3’
- PSudh-F: 5’-CTAAGTTCACGCGCACTCC-3’
- PSudh-R: 5’-GAATTCGGAGGATCCATGCTGACGACGAGTGCAG-3’
- ATuEO-F: 5’-GCATCTGATCAGTACGTTCTA-3’
- ATuEO-R: 5’-CTAAGTTCACGCGCACTCC-3’
- PPuEO-F: 5’-CTAAGTTCACGCGCACTCC-3’
- PPuEO-R: 5’-CTAAGTTCACGCGCACTCC-3’
- SuEO-F: 5’-CTAAGTTCACGCGCACTCC-3’
- SuEO-R: 5’-CTAAGTTCACGCGCACTCC-3’

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, performed on a model 202 CD spectrometer (Aviv Biomedical, Lakewood, NJ). The deletion of the udhC gene, encodes O-glucuronate isomerase.

Computational analysis including sequence identification and alignment analysis. Biozize (http://biozize.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 the *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 *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 ΔuxaC strain. Transformed ΔuxaC 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 ΔuxaC derivative, and four ΔuxaC clones transformed with the candidate genes were incubated on M9 minimal agar containing glucuronate as the sole carbon source. Wild-type MG1655, MG1655(pTiolB) ΔuxaC, MG1655(pTiolEB) ΔuxaC, and MG1655(pT1053) ΔuxaC strains grew on M9-glucuronate agar, while the MG1655(pTrc99A) ΔuxaC and MG1655(pTiolE) ΔuxaC strains did not. Therefore, *iolB* and PSPTO_1053 were selected 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 pTPTSu, 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 confirmed a NAD⁺-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⁺ but not NADP⁺ 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.

**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.
The purified preparations were used to determine the kinetic parameters, $k_{cat}$ and $K_m$, for each of the enzymes. Both glucuronate and galacturonate were used as substrates, and the NAD$^+$ cofactor concentration was also varied to determine the corresponding $K_m$ (Table 2). Measurements of $k_{cat}$ 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 $k_{cat}$ for glucuronate was higher than that for galacturonate. The highest rate constant was found for the A. tumefaciens enzyme utilizing glucuronate as the substrate ($k_{cat} = 1.9 \times 10^5 \text{ s}^{-1}$), 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 ($k_{cat}/K_m$) 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).

The purified preparations were used to determine the kinetic parameters, $k_{cat}$ and $K_m$, for each of the enzymes. Both glucuronate and galacturonate were used as substrates, and the NAD$^+$ cofactor concentration was also varied to determine the corresponding $K_m$ (Table 2). Measurements of $k_{cat}$ 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 $k_{cat}$ for glucuronate was higher than that for galacturonate. The highest rate constant was found for the A. tumefaciens enzyme utilizing glucuronate as the substrate ($k_{cat} = 1.9 \times 10^5 \text{ s}^{-1}$), 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 ($k_{cat}/K_m$) 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 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 aldohexurionate 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

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

| Strain and substrate | Kinetics parameter |  |
|----------------------|--------------------|  |
|                      | $k_{cat}$ (10^5 s^-1) | $K_m$ (mM) | $k_{cat}/K_m$ (10^5 s^-1 mM^-1) |
| A. tumefaciens        | Glucuronate        | 1.9 ± 0.1  | 0.37 ± 0.12 | 5.2 |
|                      | Galacturonate      | 0.92 ± 0.14 | 0.16 ± 0.12 | 5.7 |
|                      | NAD$^+$            | 0.18 ± 0.03 | 11 |
| P. putida             | Glucuronate        | 0.55 ± 0.03 | 0.25 ± 0.07 | 2.2 |
|                      | Galacturonate      | 0.30 ± 0.03 | 0.10 ± 0.06 | 3.0 |
|                      | NAD$^+$            | 0.21 ± 0.02 | 2.6 |
| P. syringae           | Glucuronate        | 0.74 ± 0.03 | 0.28 ± 0.07 | 2.6 |
|                      | Galacturonate      | 0.24 ± 0.01 | 0.04 ± 0.01 | 6.0 |
|                      | NAD$^+$            | 0.17 ± 0.07 | 4.3 |

**FIG. 3.** Effects of pH and temperature on activities of Udh from A. tumefaciens, P. putida, and P. syringae. (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.
activity related to Udh was observed when using either NAD$^+$ or NADP$^+$ 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 adjacent to *udh* are identified as TRAP (tripartite ATP-independent periplasmic) di-
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, YxxK and GxxGxxG, related to conserved domains (Fig. 5a). The YxxK motif is located between Tyr145 and Lys149 of P. syringae Udh and is the primary motif of the 3-alpha/beta hydroxysteroid dehydrogenase domain (11, 37). The GxxGxxG motif located in the Gly18-to-Gly24 region of P. syringae Udh is similar to Rossman folds, GxxxG or Gx1-2GxxG, which have been discovered in NAD+/H1001 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 gammaphageo-bacteria, and their homologies are relatively close those to those in the Archaea Halobrum 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 glucurionate 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 biotechnological 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 anti-carcinogenic 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).

ACKNOWLEDGMENTS

This work was supported by the Office of Naval Research Young Investigator Program (grant no. N000140510656). S.-H.Y. was supported by a Merck Undergraduate Research Grant (Bioprocess Investigator Program (grant no. N000140510656). S.-H.Y. was supported by the US National Science Foundation, for support of KdgR regulon in P. syringae pv. tomato DC3000. Proc. Natl. Acad. Sci. USA 102:8515–8520.

REFERENCES