Purine Utilization by *Klebsiella oxytoca* M5al: Genes for Ring-Oxidizing and -Opening Enzymes†

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The enterobacterium *Klebsiella oxytoca* uses a variety of inorganic and organic nitrogen sources, including purines, nitrogen-rich compounds that are widespread in the biosphere. We have identified a 23-gene cluster that encodes the enzymes for utilizing purines as the sole nitrogen source. Growth and complementation tests with insertion mutants, combined with sequence comparisons, reveal functions for the products of these genes. Here, we report our characterization of 12 genes, one encoding guanine deaminase and the others encoding enzymes for converting (hypo)xanthine to allantoate. Conventionally, xanthine dehydrogenase, a broadly distributed molybdoflavoenzyme, catalyzes sequential hydroxylation reactions to convert hypoxanthine via xanthine to urate. Our results show that these reactions in *K. oxytoca* are catalyzed by a two-component oxygenase (HpxE-HpxD enzyme) homologous to Rieske nonheme iron aromatic-ring-hydroxylating systems, such as phthalate dioxygenase. Our results also reveal previously undescribed enzymes involved in urate oxidation to allantoin, catalyzed by a flavoprotein monoxygenase (HpxO enzyme), and in allantoin conversion to allantoate, which involves allantoin racemase (HpxA enzyme). The pathway also includes the recently described PnuE allantoinase (HpxB enzyme). The HpxE-HpxD and HpxO enzymes were discovered independently by de la Riva et al. (L. de la Riva, J. Badía, J. Aguilar, R. A. Bender, and L. Baldoma, J. Bacteriol. 190:7892–7903, 2008). Thus, several enzymes in this *K. oxytoca* purine utilization pathway differ from those in other microorganisms. Isofunctional homologs of these enzymes apparently are encoded by other species, including *Acinetobacter*, *Burkholderia*, *Pseudomonas*, *Saccharomyces*, and *Xanthomonas*.

Purines and purine derivatives comprise a large portion of biomass and are involved in almost every step of life. Not only a major constituent of nucleic acids, they also are central to energy transfer and storage (ATP) as well as protein synthesis and signaling (GTP). Plants, animals, and many microorganisms use purines and purine derivatives to store and translocate nitrogen for assimilation or excretion (96).

Salvage pathways operate to recycle purines, including hypoxanthine and xanthine, back into nucleoside pools (107). Additionally, some organisms can utilize purines as the sole source of nitrogen and carbon. Adenine and guanine are deaminated to form hypoxanthine and xanthine, respectively, which then are oxidized to form uric acid (urate at physiological pH) (Fig. 1). These oxidation steps are catalyzed by xanthine dehydrogenase, a well-studied molybdoflavoenzyme that is conserved from bacteria to humans (51). Two sequential oxidation steps convert urate to allantoin via allantoin racemase (Fig. 1). The allantoin racemase (HpxA enzyme). The pathway also includes the recently described PnuE allantoinase (HpxB enzyme). The HpxE-HpxD and HpxO enzymes were discovered independently by de la Riva et al. (L. de la Riva, J. Badía, J. Aguilar, R. A. Bender, and L. Baldoma, J. Bacteriol. 190:7892–7903, 2008). Thus, several enzymes in this *K. oxytoca* purine utilization pathway differ from those in other microorganisms. Isofunctional homologs of these enzymes apparently are encoded by other species, including *Acinetobacter*, *Burkholderia*, *Pseudomonas*, *Saccharomyces*, and *Xanthomonas*.

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While this paper was in review, the paper by de la Riva et al. (24), describing the hpxDE, hpxR, hpxO, and hpxPQT genes from *Klebsiella pneumoniae* W70, was posted in the “JB Accepts” section of the *Journal of Bacteriology* online edition. Results and conclusions concerning these seven genes are congruent between the two studies.
(Some of the work presented here was submitted by Danielle Carl in 1994 as part of an undergraduate thesis to the Cornell University Division of Biological Sciences Honors Program.)

MATERIALS AND METHODS

Media. Defined, complex, and indicator media for routine genetic manipulations were used as described previously (57). Nitrogen-free medium contained 0.2% (w/vol) glucose, 1% (wt/vol) sodium citrate, 0.74% (wt/vol) sodium phosphate (pH 8), and 1 mM MgSO₄ (53). This medium was supplemented with additional nitrogen sources (10 mM NaNO₃ or NH₄Cl and 2.5 mM hypoxanthine, xanthine, urate, allantoin, or potassium allantoate) as indicated. Urate solutions were prepared as described previously (79). 5-Bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal; 40 µg/ml) was included at 40 µg/ml to score the Lac phenotypes of MudJ insertion mutants. All nitrogen sources were from Sigma-Aldrich (St. Louis, MO).

Selection for *K. oxytoca* transformants carrying bla-containing plasmids was accomplished with a combination of carbenicillin and ampicillin (Ap) at 800 and 60 µg ml⁻¹, respectively (54). Streptomycin (Sm) was used at 500 µg ml⁻¹ for selecting *K. oxytoca* Sm⁻ segregants (102). Other antibiotics used were chloramphenicol (Cm) at 50 µg ml⁻¹, kanamycin (Km) at 100 µg ml⁻¹, spectinomycin (Sp) at 50 µg ml⁻¹, and tetracycline (Tc) at 20 µg ml⁻¹.

Defined medium used to grow cultures for β-galactosidase assays and for growth yield tests was buffered with 3-[N-morpholino]propanesulfonic acid (MOPS) as previously described (69). Glucose (40 mM) was used as the sole carbon source, and nitrogen sources were added as indicated.

Culture conditions. Liquid cultures and plates were incubated at 30°C (41). Cultures for β-galactosidase assays were aerated at 240 rpm in 10 ml of medium in 125-ml sidearm flasks. Culture densities were measured with a Klett-Summerson photoelectric colorimeter equipped with a no. 66 (red) filter. Cultures in the mid-exponential phase (about 40 Klett units) were harvested, chilled on ice, and washed with saline. Cell pellets were stored overnight at −20°C prior to the assay.

Cultures for nitrogen assimilation tests were grown in 5 ml of MOPS medium in 16- by 175-mm culture tubes, aerated on a roller drum. Nitrogen sources were added over a range of growth-limiting concentrations: for nitrate, 0.4 to 1.8 mM; for histidine, 0.2 to 0.9 mM; for xanthine, 0.1 to 0.45 mM; and for guanine, 0.08 to 0.36 mM. Culture densities (optical densities at 420 nm) were measured after 24 and 48 h.

Phenotypes were determined by growth on plates made with nitrogen-free medium and microbiological agar (Marine BioProducts, Delta, British Columbia) supplemented with the test nitrogen source at a final concentration of 10
mN atoms. Strains were first streaked for single colonies on plates with added nitrate, which then were replica printed to plates with test compounds (49). Plates for anaerobic nitrogen assimilation tests were incubated in Brewer-Allgeier jars (11).

**β-Galactosidase assays.** Activity in CHCl₃-sodium dodecyl sulfate-permeabilized cells was measured at room temperature, approximately 21°C, by monitoring the hydrolysis of p-nitrophenyl-β-D-galactopyranoside. Specific activities are expressed in arbitrary (Miller) units (63). Cultures were assayed in duplicate, and reported values are averaged from two independent experiments.

**Strains.** Table 1 lists the strains used in this study. Previously, strain M5a1 was classified under *Aerobacter aerogenes* and subsequently under *K. pneumoniae*. However, phenotypic properties (such as positive reaction in the indole test) place this strain in the species *K. oxytoca* (see reference 102). Genetic cassettes were performed by P1 kc-mediated generalized transduction (90).

MudJ, originally termed Mu dl 1734, is a transposase-null bacteriophage *Mu*; *lacR lacY* *::lacZ* was made by inserting the *lacZ* determinant from plasmid pEG5005 with restriction endonuclease EcoRI and determining the hydrolysis of *β-D-galactopyranoside*. Specific activities are expressed in arbitrary (Miller) units (63). Cultures were assayed in duplicate, and reported values are averaged from two independent experiments.

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hypoxanthine. Hpx\textsuperscript{+} colonies were isolated, and plasmid DNA was extracted and retransformed to confirm the phenotype.

This in vivo cloning method generates essentially random inserts whose boundaries are delimited by the ends of bacteriophage Mu DNA (38). Large inserts from the pEG5005-based clones were subcloned into the pHG165 or pSU18 medium-copy-number vector to generate the plasmids pVJS1435, pVJS1436, pVJS1454, and pVJS3941 (Table 1). Subcloning utilized HindIII or Sall restriction endonuclease sites near the Mu DNA ends. These sites are indicated in parentheses in Table 1. All other sites are native to the K. oxytoca hpx DNA region. Sites are denoted according to the nucleotide position within the sequenced region (see below).

**DNA sequencing.** Automated DNA sequencing reactions (82) were performed by the DNA Sequencing Facility, University of California—Davis. Double-stranded templates were sequenced on a model 373A stretch DNA sequencer by using dye terminator chemistry and AmpliTaq-FS DNA polymerase (Perkin Elmer/Applied Biosystems Division, Foster City, CA). Templates were prepared by using QIAprep spin plasmid kits (Qiagen, Inc., Chatsworth, CA).

Several subclones were generated by deleting or by recloning internal restriction fragments. Sequences were primed from flanking polylinker sequence in subclones or from oligonucleotide primers synthesized to match the ends of sequence reads (primer walking). The DNA sequence was determined from multiple reads on both strands. Sequence information was compiled and analyzed through programs from DNASTAR, Inc. (Madison, WI).

**DNA sequence analysis.** Gene identifications were based primarily on the results from BLAST searches (1) conducted through servers operated by the National Center for Biotechnology Information at the National Library of Medicine. Proteins with similar sequences are denoted by their locus tags or GenInfo Identifier (GI) accession numbers, as appropriate. Domains (58) are indicated by their database accession numbers: conserved domain (cd), clusters of orthologous groups (COG), or protein family (pfam).

Analysis was facilitated as unpublished whole-genome sequence data for K. pneumoniae strain MGH78578 were released by the Washington University Genome Center. These data were particularly useful for assigning likely promoters and sites for translation initiation and termination, which most likely are conserved between the two species. The K. pneumoniae MGH78578 chromosome sequence is available in GenBank under accession number NC_009696.

**Allelic replacement.** Two insertion alleles were constructed directly. The hpxD::pAH144 allele was made by cloning a BamHI-released Ω-Cm interposon (30) into the unique BclI site within the hpx\textsuperscript{+} gene. This insertion was cloned into the conditionally replicating vector pKAS46 and transplanted to the chromosome by integration-excision allelic exchange as described previously (102). The guaD::pAH144 allele was made by cloning an internal fragment spanning guaD codons 125 to 334 into the conditionally replicating vector pAH144. The single-crossover integration was selected by resistance to Sp. The veracity of both replacements was confirmed by PCR analysis.

ΦhpxDlacZ and ΦhpxElacZ operon fusions. The divergent hpxD-hpxR transcription control region was cloned as two approximately 320-nucleotide (nt) PCR-generated fragments with introduced XbaI and BamHI restriction endonuclease sites at either end. The endpoints lie within the codons encoding Ala-30 and Gln-37 of the hpxD and hpxR coding regions, respectively. Fragments were subcloned into BamHI- and XbaI-digested vector pVJS2354 to create ΦhpxDlacZ and ΦhpxElacZ operon fusions. The resulting constructs were transferred into the mu locus by integration-excision allelic exchange as described previously (103).

**Nucleotide sequence accession number.** The DNA sequence reported in this paper is available from the GenBank nucleotide sequence database under accession number EU884423. The sequence spans 23,740 nt, including 287 nt comprising the 3’ portion of a gene homologous to the Serratia marcescens hpxC gene (2) (adjacent to the hpxE gene) and 581 nt comprising the 3’ portion of a gene homologous to Erwinia carotovora gene ECA2135 (adjacent to the hpxK gene).

### RESULTS AND DISCUSSION

**Purine utilization.** K. oxytoca M5al can use purines, pyrimidines, and most amino acids as sole sources of nitrogen (66). The purine rings contain four N atoms (Fig. 1), so we wished to determine if all four can be assimilated. We measured the growth yield of K. oxytoca cultured with limiting amounts of nitrogen, as described in Materials and Methods. Nitrate (one assimilable N atom) and histidine (two assimilable N atoms) (56) served as calibration controls. Results indicate that all four N atoms were assimilated from xanthine and that all five N atoms were assimilated from guanine (Table 3 and data not shown). We conclude that K. oxytoca quantitatively assimilates all N atoms from purines. An identical conclusion was drawn by de la Riva et al., who used adenine instead of guanine (24).

**Insertion mutants.** We conducted several screens for transposon insertion mutants with specific blocks in purine utilization, as described in Materials and Methods. Mutants were tested for growth with hypoxanthine, xanthine, urate, allantoin, and allantoate (Fig. 1). Urea was not tested, since urease is encoded at a distinct, well-characterized locus (64). Four classes of mutants were identified. Mutants with the Hpx\textsuperscript{−} phenotype utilized neither hypoxanthine nor xanthine but did utilize urate, allantoin, and allantoate. Thus, these mutants are specifically blocked in the conversion of hypoxanthine and xanthine to urate (Fig. 1). Similarly, mutants with the Urt\textsuperscript{−}, Aln\textsuperscript{−}, and Atg\textsuperscript{−} phenotypes were specifically blocked in the utilization of urate, allantoin, and allantoate, respectively.

Our analysis of genes for utilizing allantoate (hpxFGHIJK), downstream purine catabolites (hpxWXYZ), and an associated regulator (hpxU) will be reported separately. Provisional functions for these genes are included in the deposited GenBank file.

We employed bacteriophage P1-mediated generalized transduction to backcross each insertion to the wild type in order to demonstrate linkage between the transposon and the Hpx\textsuperscript{−} phenotype. Genetic crosses (105) with pairs of insertions encoding different antibiotic resistance phenotypes established the order hpxD-hpxO-hpxB-hpxF (Fig. 2A). Additional crosses not depicted in Fig. 2A yielded congruent results. Subsequent molecular genetic analysis confirmed this order (see below and Fig. 2B).

As we completed the DNA sequence of the hpx locus (see below), we employed whole-colony PCR amplification and DNA sequencing to determine the exact insertion site for each of the transposons. The results revealed 11 distinct insertions (Table 2 and Fig. 2B). Thus, the insertion mutagenesis fell far short of saturating the hpx locus. We used allelic replacement methods to construct two additional null alleles as described below. Overall, analysis of mutant phenotypes in conjunction with predictions from DNA sequence analysis allows functional assignments for each of the genes as described below.

**Gene product identification.** We cloned the hpx\textsuperscript{−} genes by complementation as described in Materials and Methods. Further subcloning and complementation analyses localized spe-
specific hpx functions to defined regions of the restriction map as described below. We determined the DNA sequence for the 22,872-nt hpx region, and concomitant computer-assisted analyses identified 23 genes (Fig. 2B).

Some functional assignments are based on homology to known proteins. In other instances, however, the proteins had not previously been characterized. In these cases, we initially attempted to identify orthologs present in genomes of other species (48). However, orthologous proteins do not necessarily perform identical functions (75); furthermore, it can be difficult to ascertain orthology (48). Therefore, we have identified what we presume to be “isofunctional homologs” in other species, based not only on overall sequence similarity and conservation of critical residues but also on genetic clustering of structural genes with those of other enzymes in the same or related pathways (i.e., synteny) (43). These assignments are described in detail below and are summarized in Table 4.

Transcriptional organization. Transcription of most K. oxytoca genes encoding nitrogen assimilation functions is regulated by general nitrogen control (Ntr) acting through the NtrC or Nac activator proteins (7, 55, 62). The NtrC protein activates promoters dependent upon the RpoN (σ54) form of RNA polymerase, which recognizes promoters whose consensus sequence is TGGYRYRRNNNYYGCW (where R represents purine, Y represents pyrimidine, and W represents A or T) (55). We identified six potential promoters by visual inspection.

FIG. 2. Klebsiella genes for purine utilization. (A) Genetic map as determined by generalized transduction. Map distances are calculated as 100 minus percent cotransduction. The arrow points to the selected marker. (B) Physical map as determined by DNA sequence analysis. Genes are denoted by arrows according to their encoded products: gray, enzymes; open, transporters; filled, regulators. Filled circles show sites of transposon insertion, and filled squares show directed allelic replacements. Line arrows indicate predicted RpoN-dependent transcripts. The five genetic modules are denoted by the substrate upon which their products act. (C) Gene organization in K. pneumoniae MGH78578. See also Table 4. Locus tags (KPN_) delimiting the boundary of each unit are shown. Arrowheads indicate relative orientation in the genome.

### Table 4. hpx genes, proposed functions, and isofunctional homologs

<table>
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<th>Gene</th>
<th>No. of codons</th>
<th>Proposed function</th>
<th>KPN_</th>
<th>XCC</th>
<th>BTH_I</th>
<th>ACIAD</th>
<th>W168</th>
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<tr>
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<td>(Hypo)Xanthine hydroxylase reductase</td>
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<td>0296</td>
<td></td>
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<tr>
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<td>0297</td>
<td></td>
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<tr>
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<td>hpxDE transcription activator (LysR type)</td>
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<td>0298</td>
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<td>0280</td>
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</table>

^a Locus tags: KPN_, K. pneumoniae MGH78578; XCC, Xanthomonas campestris ATCC 33913; BTH_I, Burkholderia thailandensis E264 (chromosome I); ACIAD, Acinetobacter sp. strain ADP1; W168, Bacillus subtilis W168 (genes).  
^b NA, not annotated. The orthologous gene is present at this location but not currently annotated.  
^c Nonorthologous “flavin-containing monoxygenase,” presumably catalyzing the same reaction.  
^d OHCU, 2-oxo-4-hydroxy-4-carboxy-5-ureidimidazoline.  
^e The region encoding the amino-terminal domain only.
tion, each of which is conserved in the *K. pneumoniae* MGH78578 sequence. These promoters are indicated in Fig. 2B as directing synthesis of the following hypothetical transcripts, shown by arrows: hpxO, hpxPQ1T, guaD, hpxC, hpxWXYZ, and hpxFGHIJK. Nucleotide sequence coordinates for these potential promoters are included in the deposited GenBank file. Thus, transcription of most hpx genes is likely activated directly by phospho-NtrC protein. We emphasize that we have not examined operon structure through direct experiments.

**Modularity of hpx gene organization.** Genes apparently involved in each step of the overall pathway are clustered together, so the overall hpx locus appears to comprise a supraoperonic cluster (100) containing five distinct modules of hpx genes plus the guaD and hpxU genes (Fig. 2B). The gene content and order within modules is essentially identical in the *K. pneumoniae* MGH78578 genome (Table 4). However, in *K. pneumoniae*, the five modules are separated into three distinct groups: hpxEDR–hpxOPQT – ~100 kb – hpxKJIHGF–hpxU–hpxWXYZ – ~16 kb – hpxBASC–guaD (Fig. 2C). Note that the relative orientations between modules also differ in the two species.

For *K. oxytoca* M5al, both the genetic and the physical maps indicate that these modules are immediately adjacent. For example, the cotransduction frequency between the hpxF111::Tn5d(Sp) and hpxD103::MudJ alleles corresponds to a physical distance of roughly 30 kb, as estimated from the formula of Sanderson and Roth (81), approximately twice the actual distance of about 16 kb. Similarly, the calculated distance between the hpxO112::TnI0d(Tc) and hpxB105::MudJ alleles, roughly 15 kb, is approximately twice the actual distance of about 8 kb. Thus, at least for the region encompassing the hpx genes, the genomes of these two *Klebsiella* species appear to be organized differently.

**Guanine to xanthine:** GuaD guanine deaminase. Guanine is deaminated to form xanthine (Fig. 1). The guaD gene product is most similar to *E. coli* K-12 guanine deaminase (60% sequence identity over 97% of its length), the product of the guaD (ygfP; cd01303) gene (61). The GuaD sequence includes a conserved nine-residue motif (P-G-X-V-I-D-X-H-T-V/I-H) also shared by members of the cyclic amidohydrolase family. This motif has been implicated in Mn$^{2+}$ or Zn$^{2+}$ binding in members of the family (46, 101). *E. coli* guanine deaminase contains Zn$^{2+}$ (61).

We constructed a guaD disruption strain to determine the role of this gene in purine utilization. The resulting mutant failed to grow on plates with guanine as the sole nitrogen source but exhibited wild-type growth with adenine, hypoxanthine, xanthine, and urate. The guaD coding sequence is preceded by an apparent RpoN-dependent promoter and thus likely constitutes a single-gene operon (Fig. 2B).

*K. oxytoca* M5al also uses adenine as the sole nitrogen source (66). However, the hpx locus does not include the gene for adenine deaminase.

**HypoXanthine to urate:** the hpxR-hpxDE module. Hypoxanthine is hydroxylated to form xanthine, which also is hydroxylated to form urate (Fig. 1). Xanthine dehydrogenase, a well-studied molybdoflavoenzyme, is the previously described enzyme catalyzing the sequential oxidation of hypoxanthine and xanthine to urate (51). Structural genes for this enzyme have been characterized for *Rhodobacter capsulatus* (52) and *Bacillus subtilis* (84). However, no such genes are present in the *K. oxytoca* hpx locus, and none were identified in the *K. pneumoniae* MGH78578 genome sequence.

Three MudJ insertion mutants exhibited the Hxn<sup>-</sup> phenotype, failing to grow with hypoxanthine or xanthine as the sole nitrogen source but displaying wild-type growth with urate and downstream purine catabolites. Two of these insertions disrupt the hpxE gene, whereas the third lies 20 nt upstream of the hpxD initiation codon (Table 2) and therefore blocks hpxDE operon transcription. We also constructed an hpxR insertion to determine the role of this gene in regulation (see below). This hpxR::Ω-Cm mutant also exhibited the Hxn<sup>-</sup> phenotype. All four insertion mutants were complemented to the Hxn<sup>-</sup> phenotype by plasmid pVJS3958, a subclone that contains only the hpxRAB region (Table 1). Therefore, the hpxD, hpxE, and hpxR genes encode functions required for conversion of (hypo)xanthine to urate, a conclusion reached independently by de la Riva et al. (24). The hpxD and hpxE genes are separated by only 13 nt and thus likely comprise an operon.

(i) HpxD and HpxE: (hypo)xanthine hydroxylase. Sequence comparisons indicate that the hpxE and hpxD gene products comprise the reductase and oxygenase, respectively, of a two-component Rieske nonheme iron aromatic-ring-hydroxylating oxygenase system. The HpxD sequence is that of a Rieske nonheme iron oxygenase, categorized in the phthalate family of Gibson and Parales (34), group I of Nam et al. (68), and class IA of Batie et al. (5). These oxygenases are homomultimeric aromatic-ring-activating mono- and dioxygenases with broad substrate specificity. They share relatively low sequence identity and range in length from 329 to 446 aminoacyl residues (the HpxD enzyme is 345 residues). The HpxD sequence includes a Rieske-type iron-sulfur cluster (consensus sequence, C-X-H-X<sub>15</sub>–26–C-X<sub>2</sub>–H; pfam00355; COG2146) near the amino terminus and a likely nonheme Fe(II)-binding 2-His-1-carboxylate facial triad (consensus sequence, H-X<sub>15</sub>–26–H-X<sub>1</sub>–H-X<sub>2</sub>–D) near the center (reviewed in references 31 and 73). The HpxD sequence shares 29% identity over a 160-residue span with that of the well-studied phthalate dioxygenase (OphA2 enzyme; GI 4128221; 443 residues) from *Burkholderia cepacia* DBO1 (6, 16).

The HpxE sequence (322 residues) is that of a corresponding reductase, categorized in class IA of Batie et al. (5), and includes the three defining cofactor domains: an amino-terminal FMN binding domain (consensus sequences, R-X-Y-S-L and G/S-R-G-G-S), a central NAD binding domain (consensus sequence, G-G-I-G-I-T-P; pfam00175) (in HpxE and KshB [95], the first Gly residue is replaced with Ala), and a carboxyl-terminal plant-type iron-sulfur cluster (consensus sequence, C-X<sub>2</sub>–X<sub>2</sub>–G–X–CG–X–C–X<sub>2</sub>–G–X; pfam00111; COG0633) (in HpxE, L–X<sub>2</sub> is Q–X<sub>2</sub>) (16, 18, 67, 95; reviewed in reference 60). The HpxE sequence shares 34% identity with the sequence of the phthalate dioxygenase reductase (OphA1 enzyme; GI 13432205; 322 residues) from *B. cepacia* DBO1 (6, 16) and about 40% identity with those of the vanillate O-demethylase reductases from a variety of species (e.g., the VanB enzyme from *Acinetobacter* sp. strain ADPI; GI 2271499; 318 residues) (86).

Purines, which resemble two fused aromatic rings, exhibit some aromatic properties (87). We suggest that the HpxE and
HpxD enzymes comprise (hypo)xanthine hydroxylase reductase and bifunctional (hypo)xanthine hydroxylase, respectively. This enzyme complex likely functions by the same mechanisms as those described for homologous aromatic-ring-hydroxylating oxygenases, hydroxylating first the C-2 position of xanthine to yield the enol form of xanthine and second the C-8 position to yield the enol form of urate (Fig. 1). Purines undergo tautomeric interconversions to shift between the relatively unstable enol form and the more stable keto form (8, 23, 87).

(ii) Transcriptional regulation in the hpxR-hpxDE module.

The two hpxE::MudJ insertion mutants expressed LacZ activity, indicating that they form Φ(hpxE-lacZ) operon fusions. We cultured these strains in defined medium, with glutamine as a neutral nitrogen source, and measured LacZ-specific activity. Both strains expressed approximately 4,000 Miller units after growth with added xanthine but less than 50 units after growth with added urate, allantoin, or allantoin and glucose, respectively. The genes encoding these proteins have elements homologous to those described for homologous aromatic-ring-hydroxylating oxygenases, with an amino-terminal helix-turn-helix motif (pfam00126) and a carboxyl-terminal LysR-type substrate-binding domain (pfam03466).

We constructed an hpxR::Ω-Cm insertion strain to determine the role of this gene in purine utilization. We also constructed monocopy Φ(hpxD-lacZ) and Φ(hpxR-lacZ) operon fusions at a separate locus in order to evaluate expression in hpxR− strains. We cultured both hpxR− and hpxR::Ω-Cm derivatives of these strains in defined medium, with nitrate as a neutral nitrogen source, and measured LacZ-specific activity. The results are shown in Table 5. Expression from the Φ(hpxD-lacZ) strain was essentially indifferent to added ammonium or hypoxanthine but was elevated about threefold in the hpxR::Ω-Cm insertion strain relative to the level for the hpxR+ strain. This suggests that hpxR transcription is subject to weak negative autoregulation.

These results indicate that the HpxR protein is activated by binding (hypo)xanthine to stimulate hpxDE operon transcription initiation. The source of Ntr regulation is less clear. No RpoN-dependent promoter is evident in the hpxD-hpxR intergenic region in either K. oxytoca M5al or K. pneumoniae MGH78578. Expression of the Φ(hpxR-lacZ) fusion was not regulated by Ntr, indicating that Ntr regulation does not act by controlling HpxR protein synthesis.

Very similar results were reported by de la Riva et al. (24), who further demonstrated specific binding of HpxR protein to the intergenic regulatory region. They additionally concluded that Ntr control is mediated by neither the NtrC nor the Nac proteins (7).

(iii) HpxD, HpxE, and HpxR isofunctional homologs in other species. The HpxD and HpxE sequences are most closely related to sequences from Xanthomonas spp. (63% and 47% identity, respectively), currently annotated as vanillate O-demethylase. Likewise, the HpxR sequence is most closely related to sequences from Xanthomonas spp. (46% identity). The genes encoding these proteins are adjacent to several other genes involved in purine catabolism (Table 4). Therefore, these genes comprise the hpxR-hpxDE module in Xanthomonas spp.

Urate to S-(+)-allantoin: the hpxO-hpxPQT module. Urate is hydroxylated to form 5-hydroxysurate (HIU), which is hydrolyzed to form 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU), which is decarboxylated to form S-(+)-allantoin (92) (Fig. 1). HIU also undergoes spontaneous conversion to racemic allantoin (45). These reactions comprise the first ring-opening step in purine utilization.

Uricase (urate oxidase) is the previously described enzyme catalyzing the initial oxidation of urate to HIU. Structural genes for this enzyme have been characterized for Bacillus subtilis (84) and for a range of eukaryotic species (71). However, no such genes are present in the K. oxytoca hpx locus, and none were identified in the K. pneumoniae MGH78578 genome sequence.

Two insertion mutants exhibited the Urt− phenotype, failing to grow with hypoxanthine, xanthine, or urate as the sole nitrogen source but displaying wild-type growth with allantoin and allantoin. Both insertions disrupt the hpxO gene (Table 2). Therefore, the hpxO gene encodes a function required for utilization of urate, a conclusion reached independently by de la Riva et al. (24).

The hpxP and hpxQ genes overlap (23 nt), as do the hpxO and hpxT genes (4 nt), and thus likely comprise the hpxPQT operon (Fig. 2B). Apparent RpoN-dependent promoters for both the hpxO gene and the divergently transcribed hpxPQT operon are present in the 346-nt intergenic region.

(i) HpxO: urate hydroxylase. Sequence comparisons classify the hpxO gene product (42 kDa) in the ~45-kDa aromatic-ring flavoprotein monooxygenase family (pfam01360), members of which share relatively low sequence similarity (40). For exam-

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* Strains were cultured to the mid-exponential phase in MOPS medium, with nitrate as the nitrogen source. Ammonium or hypoxanthine (Hyx) was added as indicated.

b Operon fusion construct integrated at the chromosomal rha locus.

c +, hpxR+; −, hpxR−Cm.

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TABLE 5. Effects of hypoxanthine and ammonium on expression from Φ(hpxD-lacZ) and Φ(hpxR-lacZ) constructs.

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ple, the HpxO sequence shares 28% sequence identity over 84% of its length with salicylate hydroxylase (NahG enzyme; GI 4104764; 437 residues) from Pseudomonas stutzeri AN10 (9).

Enzymes in this family cleave dioxygen without the use of a metal ion and incorporate one oxygen atom as a hydroxyl group ortho or para to an existing hydroxyl group while reducing the other oxygen atom to water (33; reviewed in reference 40). The HpxO sequence (384 residues) comprises three defining cofactor domains: an amino-terminal domain that binds the ADP moiety of flavin adenine dinucleotide (FAD) (consensus sequence, G-X-G-X2-G-X7-A/G-X6-G; pfam01494), a central domain that binds the pyrophosphate moiety of NAD(P)H and interacts indirectly with the pyrophosphate moiety of FAD (consensus sequence, D-X4-DG-X5-R; pfam01360), and a carboxyl-terminal domain that binds the ribose moiety of FAD. Sequences in the last domain vary considerably, save for a conserved G-D sequence (10, 28, 29, 59; reviewed in reference 40). The HpxO enzyme likely uses a similar mechanism to hydroxylate urate to form HIU (Fig. 1).

(ii) HpxT and HpxQ. Sequence comparisons indicate that the HpxT and HpxQ proteins are HIU hydroxylase and OHCU decarboxylase, respectively (15, 44, 77). The HpxT sequence (108 residues; COG2351) is 32% identical to that of HIU hydroxylase (PucM protein; 121 residues) from Bacillus subtilis 168 (44, 84). The HpxQ sequence (166 residues; COG3195) is 27% identical to that of the amino-terminal 165 residue OHCU decarboxylase domains of urate oxidase (uricase) from Bacillus sp. strain TB-90 (GI 21431959) (104) and B. subtilis 168 (PucL protein) (84). The Bacillus uricase is synthesized as a precursor of about 500 residues, from which the amino-terminal OHCU decarboxylase is proteolytically cleaved (70).

(iii) HpxP purine permease. The HpxP sequence (460 residues) shares 32% identity over 95% of its length with the high-affinity, high-capacity urate-xanthine permease UapA (GI 6136091; 615 residues) (36) and 30% identity over 90% of its length with the general purine permease UapC (GI 1351342; 586 residues), both from A. nidulans (26). Additionally, the HpxP sequence is 34% identical to those of the urate permeases PucJ (449 residues) and PucK (430 residues) from B. subtilis 168. The HpxP sequence meets the criteria for inclusion in the nucleobase ascorbate transporter family (COG2233) (37), including the nucleobase ascorbate transporter signature motif (Q/E/P-N-X-G-X7-T-R/K/G), the QH motif in the middle of transmembrane segment 1, and a conserved alanyl residue important for function but not specificity (Ala-404 in UapA and Ala-272 in HpxP).

The region spanning residues 378 to 446 in UapA and 336 to 404 in UapC (analogous to the region spanning residues 246 to 314 in HpxP) determines uptake specificity, with UapA residues Glu-412 and Arg-414 and UapC residues Gln-370 and Glu-372 possibly forming part of a purine binding site (27). The corresponding residues in the HpxP sequence are more similar to those of UapC than to those of UapA: Ser-280HpxP and Gln-370UapC are both uncharged, with polar side chains, whereas Glu-412UapA is acidic, and Asp-282HpxP and Gln-372UapC are both acidic, whereas Arg-414UapA is basic. This suggests that HpxP may be a general purine permease, an appealing hypothesis since this is the only hpx cluster-encoded permease for transport of guanine, hypoxanthine, xanthine, and urate.

(iv) HpxO isofunctional homologs in other species. The HpxO sequence is most similar (53% identical) to a protein sequence annotated as “putative flavoprotein monoxygenase acting on aromatic compound” from Acinetobacter sp. strain ADP1 (lucus tag ACIAD3540; GI 49532471; 385 residues). This gene is adjacent to those encoding HIU hydroxylase and OHCU decarboxylase (Table 4), supporting its assignment as encoding the isofunctional homolog of HpxO protein. Intriguingly, Xanthomonas genomes contain a gene for a presumptive flavin-containing monoxygenase (e.g., XCC0279), not homologous to HpxO, adjacent to those encoding HIU hydroxylase and OHCU decarboxylase (Table 4). Thus, it appears that at least two different monoxygenases have been recruited into the urate hydroxylase step of the purine utilization pathway.

R-(-)-allantoin to S-(-)-allantoin to allantoate: the hpxC-hpxSAB module. Conversion of allantoin to allantoate is the second of the two ring-opening steps in purine utilization (Fig. 1). Five insertion mutants exhibited the Aln- phenotype, failing to grow with allantoin or upstream purine catabolites as the sole nitrogen source but displaying wild-type growth with allantoate. The insertions disrupt the hpxA and hpxB genes (Table 2). Therefore, the hpxAB operon encodes functions involved in allantoin utilization. However, the hpxA::Tn10D(Tc) insertion was complemented by subclones carrying the hpxB but not the hpxA gene, indicating that the hpxA gene is not essential for growth on allantoate. Presumably, the hpxA::Tn10D(Tc) insertion is polar on hpxB gene expression.

Allantoinase is the previously described enzyme catalyzing the conversion of allantoin to allantoate (91). Structural genes for this enzyme have been characterized for Bacillus subtilis (84) and E. coli (21) and for a range of eukaryotic species (12). However, no such genes are present in the K. oxytoca hpx locus, and none were identified in the K. pneumoniae MH78578 genome sequence.

Most characterized allantoinases are specific for the S iso- mer. However, the nonenzymatic conversion of HIU to allantoate creates a racemic mixture of R- and S-allantoin (45), and allantoin is present as the racemic mixture in decaying organic matter (94). Allantoin racemase has been characterized for bacteria (94) and for Candida utilis (72). To our knowledge, the structural gene for an allantoin racemase has not been identified.

An apparent RpoN-dependent promoter for the hpxC gene (but not for the hpxSAB operon) is present in the 52-nt hpxC and hpxSAB intergenic region. The hpxS and hpxA genes overlap (1 nt; TAATG), and the hpxA and hpxB genes are separated by only 16 nt, so these likely comprise the hpxSAB operon (Fig. 2B).

(i) HpxB: allantoinase. Sequence comparisons suggested that the HpxB enzyme catalyzes conversion of allantoin to
allantoate. The HpxA sequence (310 residues; COG0726) shares 26% identity over 74% of its length with an imidase (291 residues; GI 14040045) from Ralstonia eutropha (97). The imidase catalyzes the hydrolysis of hydantoin and related compounds. Since allantoin is a 5-substituted hydantoin (ureidohy-}
dantoin), this suggests that the HpxB enzyme acts similarly to the imidase by hydrolyzing a cyclic C-N bond in the hydantoin ring to form allantoate (Fig. 1).

This conjecture was confirmed by Ramazzina et al., who recently identified the HpxB homolog from Pseudomonas fluorescence DSM 50090 as an (S)-allantoin-specific allantoinase (76). They denote this enzyme PuuE.

(ii) HpxA: allantoin racemase. Sequence comparisons indicate that the HpxA protein is allantoin racemase. The HpxA sequence (247 residues; COG 4126; pfam01177) shares 45% identity over 96% of its length with 5-substituted hydantoin racemase (HuyE enzyme; 229 residues; GI 266318) from Pseudomonas sp. strain NS671 (98). The HuyE enzyme catalyzes racemization of a variety of 5-substituted hydantoins (99).

Since HpxB allantoinase is specific for the enantiomer (76), allantoin racemase could be important for efficient allantoin utilization in natural environments. However, allantoin racemase likely is not required for colony formation on plates with abundant hydantoin racemase. In these genomes, the HpxA homolog from Pseudomonas aeruginosa is most similar (about 70% identity) to protein sequences typically annotated as “polysaccharide decacetylase” (76), and the HpxA sequence is most similar (up to 62% identity) to protein sequences typically annotated as “hydantoin racemase.” Representative examples are shown in Table 4. In the case of Burkholderia spp. and related species, the hpxA and hpxB homologs are present as an hpxSCAB module within a larger cluster of purine utilization genes, including those for HIU hydrolase and OHCU decarboxylase. In contrast, the Acinetobacter sp. hpxB homolog is clustered with the hpxO, hpxT, and hpxQ homologs, encoding enzymes of urate catabolism, whereas the hpxA and hpxC homologs are adjacent to each other at a separate location. This indicates that the HpxA allantoin racemase may function physiologically to scavenge exogenous, racemic allantoin. Consistent with this notion, the Xanthomonas spp. have hpxB homologs but none for hpxA or hpxC.

Van der Drift et al. identified allantoin racemase in P. fluorescens and Pseudomonas putida (among others) but not in Pseudomonas aeruginosa or P. stutzeri (94). Database searches reveal that hpxA homologs are present in genomes of P. fluorescens, P. putida, and Pseudomonas syringae but not in P. aeruginosa or P. stutzeri (locus tag Ppu_1557 in P. putida F1). This reinforces the conclusion that hpxA encodes allantoin racemase. In these genomes, the hpxA and hpxC genes are adjacent to each other but not to other purine utilization genes, as described above for the Acinetobacter sp. (The P. syringae clusters include an hpxS homolog as well.) In contrast, the hpxB gene, encoding allantoinase, is present in the genomes of all five Pseudomonas species examined (locus tag Ppu_1582 in P. putida F1), clustered with several other genes involved in purine utilization.

The HpxA sequence shares 25% identity with the Dcg1p proteins of S. cerevisiae (244 residues; locus tag YIR030C) and Candida albicans (232 residues; locus tag CaO19.244). The S. cerevisiae DCG1 gene was identified as a nitrogen-regulated gene within the DAL cluster, encoding allantoinase, allantoin perermase, and allantoicase (106), but the function of the Dcg1p protein has not been determined. Allantoin racemase activity has been characterized for Candida utilis (72). The analysis presented here indicates that Dcg1p may function as allantoin racemase.

Conclusions. Microbial genome sequencing projects reveal large numbers of genes with unknown functions. Here, Klebsiella purine utilization (Fig. 1) provides numerous examples of unpredicted enzymes catalyzing an established pathway (96). Several enzymes were evidently recruited from pathways for catabolism of aromatic compounds. Aromatic compounds and the pathways for their catabolism are diverse and widespread, and so, in retrospect, it is unsurprising that such enzymes are involved in oxidizing purines (Fig. 1).

Oxidation of (hypo)xanthine to form urate is catalyzed by a two-component Rieske nonheme iron aromatic-ring-hydroxy-
lating oxygenase system (HpxE-HpxD enzyme), and oxidation of urate to form 5-hydroxisourate is catalyzed by an ~45-kDa aromatic-ring flavoprotein monooxygenase (HpxO enzyme). These enzymes were identified independently by de la Riva et al. (24). Genome sequence comparisons suggest that a distinct flavin-containing monooxygenase catalyzes the latter step in Xanthomonas spp. (Table 4).

Yet another enzyme is involved in xanthine utilization by A. nidulans, for which purine utilization has long been studied (83). Early work established that mutants lacking xanthine dehydrogenase (hxA) or molybdenum cofactor (cpx) exhibit leaky growth with xanthine (but not hypoxanthine) as the sole nitrogen source (22). This indicated the presence of a molybdenum cofactor-independent xanthine alternative pathway, dependent on the xanA gene (85). Recently, it was established that XanA protein, a member of the taurine dioxygenase group of enzymes active on aromatic compounds, is an α-ketogluta-
rate-dependent dioxygenase that hydroxylates xanthine to form urate (20, 65).

Finally, our analysis reveals that Klebsiella spp. employ HpxA allantoin racemase, related to hydantoin racemases (72, 94), as well as the HpxB (PuuE) allantoinase, related to poly-

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