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Genetic Control of Nitrogen Assimilation in *Klebsiella oxytoca*

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Abstract *Klebsiella oxytoca*, an enterobacterium closely related to *Escherichia coli* and amenable to molecular genetic analysis, is a long-established model organism for studies of bacterial nitrogen assimilation. Our work concerned utilization of purines, nitrogen-rich compounds that are widespread in the biosphere. This project began with our observation that molybdenum cofactor (chlorate-resistant) mutants can use (hypo)xanthine as sole nitrogen source (Garzón et al., J. Bacteriol. **174**:6298, 1992). Since xanthine dehydrogenase is a molybdoenzyme, *Klebsiella* must use an alternate route for (hypo)xanthine catabolism.

We identified and characterized a cluster of 22 genes that encode the enzymes, permeases and regulators for utilizing hypoxanthine and xanthine as sole nitrogen source. (Hypoxanthine and xanthine arise from deamination of adenine and guanine, respectively.) Growth and complementation tests with insertion mutants, combined with protein sequence comparisons, allow us to assign probable functions for the products of these genes and to deduce the overall pathway.

We present genetic evidence that the first two enzymes for the *Klebsiella* purine utilization pathway have been recruited from pathways involved in catabolism of aromatic compounds. The first, HxaAB enzyme catalyzing (hypo)xanthine oxidation, is related to well-studied aromatic ring hydroxylating oxygenases such as phthalate dioxygenase. The second, HxbA enzyme catalyzing urate hydroxylation, is related to single-component monooxygenases. Thus, the *Klebsiella* purine utilization pathway has likely experienced non-orthologous gene displacement, substituting these oxygenases for the conventional enzymes, xanthine dehydrogenase and uricase.

We also present evidence that transcription of the *hxaAB* operon is subject to dual regulation: global general nitrogen regulation (Ntr) through an unknown mechanism, and (hypo)xanthine induction mediated by a LysR-type activator.

Report Our major goals for the reporting period were threefold: (1) identify and characterize the *nas* structural genes encoding enzymes for aerobic hypoxanthine utilization; (2) identify and characterize the regulatory mechanisms that control pathway-specific induction of *hxu* gene expression; and (3) characterize the role of Ntr (general nitrogen regulation) control in regulating *hxu* gene expression.

I am working hard to publish these studies this calendar year. I have a draft manuscript that describes all of our work in a single long paper. This is an information-rich, complex manuscript, and I have been struggling to find the optimal organization and level of detail. I am hesitant to break the work into two or more smaller papers, because the overall story is so strongly interconnected. However, from a practical standpoint, I may be forced to do so.

1. Structural genes (*hxu*) for aerobic hypoxanthine utilization

We identified and characterized a 23 kb gene cluster that encodes the enzymes for utilizing purines as sole nitrogen source. Growth and complementation tests with insertion mutants, combined with protein sequence comparisons, allowed us to assign probable functions for the products of these genes and also to deduce the five-step pathway for purine utilization.

Guanine to xanthine: the *guaD* gene. Guanine is deaminated to form xanthine. The *guaD* gene product is most similar to *E. coli* K-12 guanine deaminase. We constructed a *guaD* disruption strain to determine the role of this gene in purine utilization. The resulting mutant failed to grow with guanine as sole nitrogen source, but exhibited wild-type growth with adenine, hypoxanthine, xanthine and urate.

(Hypo)xanthine to urate: the *hxaAB-hxaR* module. Hypoxanthine is hydroxylated to form xanthine, which also is hydroxylated to form urate. Three MudJ insertion mutants exhibited the Hxa⁻ phenotype, failing to grow with hypoxanthine or xanthine as sole nitrogen source but displaying wild-type growth with urate and downstream purine catabolites. Two of these insertions disrupt the *hxaB* gene, whereas the third lies 20 nt upstream of the *hxaA* initiation codon. We also constructed a *hxaR* insertion to determine the role of this gene in regulation (see below). This *hxaR*::Ω-Cm mutant also exhibited the Hxa⁻ phenotype. Therefore, the *hxaA*, *hxaB* and *hxaR* genes encode functions required for conversion of (hypo)xanthine to urate.

Sequence comparisons indicate that the *hxaB* and *hxaA* gene products comprise the reductase and oxygenase, respectively, of a two-component aromatic ring-hydroxylating monooxygenase system. The HxaA sequence is that of a Rieske non-heme iron oxygenase, and the HxaB sequence is that of a corresponding reductase, both categorized in Class IA of Batie et al. Class IA reductases transfer electrons one at a time from the NADH-reduced FMN cofactor to the iron-sulfur cluster. Electrons then

pass to the oxygenase Reiske-type iron-sulfur cluster, and are used to reduce the non-heme iron. Dioxygen interacts with the reduced iron to hydroxylate the substrate.

Purines, which resemble two fused aromatic rings, do exhibit some aromatic properties. We suggest that the HxB and HxA enzymes comprise (hypo)xanthine monooxygenase reductase and bifunctional (hypo)xanthine monooxygenase, respectively. This enzyme complex likely functions by the same mechanisms as described for homologous aromatic ring-hydroxylating monooxygenases, hydroxylating first the C2 position of hypoxanthine to yield the enol form of xanthine, and second the C8 position to yield the enol form of urate. Purines undergo tautomeric interconversions to shift between the more unstable enol form and the more stable keto form.

Urate to S-(+)-allantoin: the *hxbA-hxbTMN* module. Urate is hydroxylated to form 5-hydroxyisourate (HIU), which is hydrolyzed to form 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU), which is decarboxylated to form S-(+)-allantoin. HIU also undergoes spontaneous conversion to racemic allantoin. Two insertion mutants exhibited the Hxb⁻ phenotype, failing to grow with hypoxanthine, xanthine or urate as sole nitrogen source but displaying wild-type growth with allantoin and downstream purine catabolites. Both insertions disrupt the *hxbA* gene. Therefore, the *hxbA* gene encodes a function required for catabolism of urate, namely hydroxylation of urate to form HIU.

Sequence comparisons indicate that the *hxbA* gene product is categorized in the ~45 kDa aromatic-ring flavoprotein monooxygenase family. 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. p-Hydroxybenzoate hydroxylase (PobA enzyme) is the archetype for the ~45 kDa aromatic-ring flavoprotein monooxygenase family. The reaction mechanism initiates with binding of the substrate (p-hydroxybenzoate). This leads to the reduction of the flavin isoalloxazine ring by NADPH, forming FADH₂. Dioxygen then binds to the complex and is reduced by two single electron transfers. The resulting C4a-flavin (hydro)peroxide hydroxylates the substrate. The HxB enzyme likely uses a similar mechanism to hydroxylate urate to form HIU. The HxB enzyme is distinct from urate oxidase (uricase), the conventional enzyme of the urate pathway.

Sequence comparisons indicate that the HxB and HxBN proteins are HIU hydrolase and OHCU decarboxylase, respectively, and that the HxBT protein is urate permease.

S-(+)-allantoin to allantate: the *hxcT-hxcRAB* module. Degradation of allantoin to allantate is the second of the two ring-opening steps in purine catabolism. Two insertion mutants exhibited the Hxc⁻ phenotype, failing to grow with allantoin or upstream purine catabolites as sole nitrogen source but displaying wild-type growth with allantate and ureidoglycolate. The insertions disrupt the *hxcA* and *hxcB* genes. Therefore, the *hxcAB* operon encodes functions required for opening the allantoin ring.

Sequence comparisons indicate that the HxcT protein is allantoin permease, and that the HxcR protein is a transcriptional regulator of the GntR family.

Allantoate via ureidoglycine to ureidoglycolate: the *hxdRJMQPBA* module.

Degradation of allantoate to ureidoglycolate, through the intermediate ureidoglycine, results in extraction of two of the four nitrogen atoms from the purine ring. One insertion mutant exhibits the Hxd⁻ phenotype, failing to grow with allantoate or upstream purine catabolites as sole nitrogen source but displaying wild-type growth with ureidoglycolate. This insertion disrupts the *hxdJ* gene, and likely prevents downstream gene expression through polarity.

Sequence analyses suggest that the HxdB and HxdA proteins are allantoate amidohydrolase and ureidoglycine aminohydrolase, respectively. Sequence comparisons indicate that the HxdJMQP proteins comprise a periplasmic binding protein-dependent allantoate transporter homologous to the HisJMQP histidine transporter, and that the HxdR protein is a transcriptional regulator of the RpiR family.

Ureidoglycolate degradation? the *hxeABC* module. We did not isolate any insertions in the *hxeABC* region. We hypothesize that these proteins are involved in the further catabolism of ureidoglycolate.

2. Pathway-specific regulation of *hxu* gene expression

As noted above, the HxcR and HxdR proteins are likely pathway-specific regulators for *hxcAB* and *hxdJMQPBA* transcription, respectively. Preliminary results indicate that the *hxcAB* operon is regulated by both pathway-specific and general nitrogen regulation. However, we have not studied these regulators further.

Pathway-specific regulation in the *hxaAB-hxaR* module. The two *hxaB::MudJ* insertion mutants expressed LacZ activity, indicating that they form $\Phi(hxaB-lacZ)$ operon fusions. Results indicate that *hxaAB* operon transcription is subject to dual control, first by global nitrogen regulation (Ntr) and second by pathway-specific induction.

The *hxaAB* operon shares a 125 nt intergenic region with the divergently-transcribed *hxaR* gene. The HxaR sequence is similar to those of LysR Family transcriptional regulators, many of which regulate degradation pathways for aromatic compounds.

We constructed a *hxaR::Ω-Cm* insertion strain to determine the role of this gene in purine utilization. We also constructed monocopy $\Phi(hxaA-lacZ)$ and $\Phi(hxaR-lacZ)$ operon fusions. We cultured both *hxaR*⁺ and *hxaR::Ω-Cm* derivatives of these strains in defined medium with nitrate as neutral nitrogen source, and measured LacZ specific activity. Expression from the $\Phi(hxaA-lacZ)$ *hxaR*⁺ strain was subject to both Ntr and

pathway-specific control, consistent with results from the *hxaB::MudJ* strains summarized above. By contrast, expression from the $\Phi(hxaA-lacZ)$ *hxaR:: Ω -Cm* strain was undetectable, demonstrating that the HxaR protein is essential for activating *hxaAB* operon transcription. This is consistent with the Hxa⁻ phenotype conferred by the *hxaR:: Ω -Cm* insertion (see above). Expression from the $\Phi(hxaR-lacZ)$ strain was essentially indifferent to added ammonium or xanthine, but was elevated about threefold in the *hxaR:: Ω -Cm* insertion strain relative to the *hxaR*⁺ strain. This suggests that *hxaR* transcription is subject to weak negative autoregulation.

3. General nitrogen regulation (Ntr) of *hxu* gene expression

Sequence inspection reveals Ntr-type promoters upstream of the *hxbA*, *hxbTBC*, *guaD*, *hxcT*, *hxeAB*, *hxeC* and *hxdJMQPBA* operons, indicating that general nitrogen regulation here is direct. We have not studied this further.

General nitrogen regulation in the *hxaAB-hxaR* module. We presume that the HxaR protein, a LysR Family transcriptional regulator, is activated by binding (hypo)xanthine to stimulate *hxaAB* operon transcription initiation. The source of Ntr regulation is less clear. No RpoN-dependent promoter is evident in the *hxaA-hxaR* intergenic region in either *K. oxytoca* M5al or *K. pneumoniae* MGH78578. Expression of the $\Phi(hxaR-lacZ)$ fusion was not regulated by Ntr, indicating that Ntr regulation does not act by controlling HxaR protein synthesis. One possibility is that both the HxaR and the Nac proteins bind upstream of the *hxaAB* operon to activate transcription. We do not have a *nac* mutant of *K. oxytoca* M5al, so we were not able to test this.

Summary

With three years of funding (and two extension years), we characterized and assigned specific or provisional functions to 22 *hxu* structural and regulatory genes. The most significant finding is that of a molybdenum-independent pathway for conversion of (hypo)xanthine to urate. Our work also provide a foundation for identifying and characterizing hypoxanthine utilization genes in other bacterial species.

DOE-supported publication

Pope, S. D., L.-L. Chen, and V. Stewart. Molybdenum-independent hypoxanthine utilization by *Klebsiella oxytoca* M5al. Manuscript in preparation.