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Genetic Control of Nitrate Assimilation in *Klebsiella oxytoca*

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Abstract: Some microorganisms can use nitrate as the sole source of nitrogen for biosynthesis. This project focuses on the bacterium *Klebsiella oxytoca*, an enterobacterium found in soil and water. Mutagenesis and molecular cloning identified the *nasFEDCBA* operon encoding enzymes for the uptake and reduction of nitrate to ammonium, and the adjacent *nasR* regulatory gene. Analysis of *nasF* operon expression revealed that transcription is activated by the Ntr (general nitrogen regulation) system in response to nitrogen limitation. Transcription antitermination control in response to nitrate and nitrite is mediated by the NasR protein. Additional work established that the NasR protein is an RNA-binding protein that interacts with *nasF* operon leader RNA to control transcription readthrough.

Report: Our major goals for the reporting period were threefold: (1) to identify and characterize the *nas* structural genes encoding enzymes for nitrate uptake and assimilation; (2) characterize the role of Ntr (general nitrogen regulation) control in regulating *nas* gene expression; and (3) identify and characterize the regulatory mechanism that controls nitrate- and nitrite-specific induction of *nas* gene expression. Virtually all of this work has been published in peer-reviewed journals. A list of these DOE-supported publications is presented below.

(1) *nas* Structural Genes:

When we began our studies, there was very little information on the genetics of nitrate assimilation in any bacterium. We chose to work with the enterobacterium *Klebsiella oxytoca* (*pneumoniae*), a close relative of *Escherichia coli* that has been extensively studied with respect to dinitrogen fixation (*nif* genes). We isolated several insertion mutants that failed to use nitrate as sole nitrogen source (*nas* genes), and found that they defined a single chromosomal *nas* locus. Molecular cloning, complementation analysis, identification of encoded polypeptides, insertion mutagenesis and DNA sequence analysis resulted in the discovery of the *nasFEDCBA* operon.

We further demonstrated that the *nasFED* genes encode the periplasmic binding protein, intrinsic membrane protein, and cytoplasmic ATPase protein, respectively, of the assimilatory nitrate and nitrite uptake system. We also demonstrated that the *nasC* and *nasA* genes encode the two subunits of assimilatory nitrate reductase, and that the *nasB* gene encodes assimilatory nitrite reductase. This work is described by Lin et al., 1993; Lin et al., 1994; and Wu & Stewart, 1998.

DISCLAIMER

(2) Ntr Control

In response to ammonium limitation, enterobacteria activate transcription of genes that encode permeases and enzymes for utilizing alternative nitrogen sources such as amino acids, purines, and, in the case of *K. oxytoca*, inorganic nitrogen (dinitrogen and nitrate). The well-studied NtrB-NtrC two-component regulatory system is responsible for Ntr activation. We demonstrated that the *nasF* operon is regulated by a conventional Ntr-dependent control region, with a σ^N- (σ^{54}-) dependent promoter and an upstream activating sequence (UAS) for phsopho-NtrC binding. As this control region is quite similar to others (*glnA*, *glnH*, *nifL*) that have been well-studied, we did not pursue these studies further. This work is described by Lin & Stewart, 1996.

We found that transcription of the *nasR* gene (see below) is also regulated by NtrC, and that *nasR* transcription originates from a σ^N-dependent promoter. However, the *nasR* upstream region does not contain a UAS for NtrC protein. Thus, the *nasR* promoter is an unconventional Ntr-controlled promoter. Our extensive documentation of this point is described by Wu et al., 1999.

(3) Nitrate Control

Expression of the *nas* operon is subject to dual control: global regulation in response to ammonium limitiation (Ntr control as described above) and pathway-specific induction by nitrate and nitrite. Both anions are equally effective inducers. We devoted most of our effort to the study of nitrate (and nitrite) induction, because the mechanism proved to have several novel features. Evidence indicates that the NasR protein (encoded by the nasR gene immediately upstream of the *nasF* operon) encodes a nitrate- and nitrite-responsive RNA-binding protein. In the presence of signal ligand, NasR protein binds to a stem-loop structure in the mRNA leader region immediately upstream of the *nasF* initiation codon and, through an as-yet unknown mechanism, allows RNA polymerase to read through a factor-independent transcription terminator in the leader. In the absence of signal ligand, RNA polymerase terminates transcription in the leader, resulting in low levels of *nasF* operon transcription.

We identified the *nasR* gene as encoding a pathway-specific activator of *nasF* operon transcription (Goldman et al., 1994). Our deletion analysis of the *nasF* operon control region revealed that cis-acting sequences for pathway-specific regulation lie downstream of the transcription initiation site, and sequence inspection of the transcribed leader region revealed two RNA stem-loop structures (Lin & Stewart, 1996). The structure formed by base pairing between stems 1 and 2 is a positive regulator of *nasF* operon induction, because deletions in 1:2 result in uninducible phenotypes. The structure formed by base pairing between stems 3 and 4 is the factor-independent transcription terminator; deletions in 3:4 result in constitutive phenotypes (Lin & Stewart, 1996). In vitro transcription with linear templates terminates at the 3:4 terminator; addition of NasR protein plus nitrate (or nitrite) results in efficient transcription readthrough. Furthermore, leader region deletions exert comparable effects on antitermination in vitro as they do on *nasF* operon expression in vivo (Chai & Stewart, 1998). Finally, NasR protein binds specifically to *nasF* leader mRNA in vitro, and binding is stimulated by the addition of nitrate or nitrite (Chai & Stewart, 1999). Further mutational analysis identified the hexanucleotide loop in structure 1:2 as critical for NasR binding and for regulated antitermination. Mutational analysis of stems 1 and 2 demonstrated that the base-paired stem, but not its primary sequence, is essential for antitermination. Finally, these results exclude antitermination models that invoke alternate stem-loop structures (Cahi & Stewart, 1999). The mechanism by which NasR protein effects transcription antitermination remains a challenge for future studies.

Summary

In eight years of research, we characterized and assigned specific functions to six *nas* structural genes and one *nas* regulatory gene. These studies have provided a foundation for identifying and characterizing nitrate assimilation genes in other bacterial species. We also uncovered an interesting variation of Ntr control (of the *nasR* promoter) that deserves further work. Finally, we discovered and extensively characterized a previously-unknown form of transcription antitermination control, the exact mechanism of which remains to be determined.

DOE-supported publications

Garzón, A., J. Li, A. Flores, J. Casadesus, and V. Stewart. 1992. Molybdenum cofactor (chlorate-resistant) mutants of *Klebsiella pneumoniae* M5al can use hypoxanthine as the sole nitrogen source. J. Bacteriol. 174:6298-6302.

Lin, J. T., B. S. Goldman, and V. Stewart. 1993. Structures of genes *nasA* and *nasB*, encoding assimilatory nitrate and nitrite reductases in *Klebsiella pneumoniae* M5al. J. Bacteriol. 175:2666-2672.

Lin, J. T., B. S. Goldman, and V. Stewart. 1994. The *nasFEDCBA* operon for nitrate and nitrite assimilation in *Klebsiella pneumoniae* M5al. J. Bacteriol. 176:2551-2559.

Goldman, B. S., J. T. Lin, and V. Stewart. 1994. Identification and structure of the *nasR* gene encoding the nitrate- and nitrite-responsive positive regulator of *nasFEDCBA* (nitrate assimilation) operon expression in *Klebsiella pneumoniae* M5al. J. Bacteriol. 176:5077-5085.

Lin, J. T., and V. Stewart. 1996. Nitrate- and nitrite-mediated transcription antitermination control of *nasF* (nitrate assimilation) operon expression in *Klebsiella pneumoniae* M5al. J. Mol. Biol. 256:423-435.

Wu, Q., and V. Stewart. 1998. NasFED proteins mediate assimilatory nitrate and nitrite transport in *Klebsiella oxytoca* (*pneumoniae*) M5al. J. Bacteriol. 180:1311-1322.

Chai, W., and V. Stewart. 1998. NasR, a novel RNA-binding protein, mediates nitrate-responsive transcription antitermination of the *Klebsiella oxytoca* M5al *nasF* operon leader *in vitro*. J. Mol. Biol. 283:339-351.

Lin, J. T., and V. Stewart. 1998. Nitrate assimilation by bacteria. Adv. Microbial Physiol. 38:1-30. (Invited Review)

Chai, W., and V. Stewart. 1999. RNA sequence requirements for NasR-mediated, nitrate-responsive transcription antitermination of the *Klebsiella oxytoca* M5al *nasF* operon leader. J. Mol. Biol. 292:203-216.

Wu, S. Q., W. Chai, J. T. Lin, and V. Stewart. 1999. General nitrogen regulation of nitrate assimilation regulatory gene *nasR* expression in *Klebsiella oxytoca* M5al. J. Bacteriol. 181:7274-7284.