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Role of Nitrogen Limitation in Transformation of RDX (Hexahydro-1,3,5-Trinitro-1,3,5-Triazine) by *Gordonia* sp. Strain KTR9

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The transcriptome of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)-degrading strain *Gordonia* sp. strain KTR9 and its *glnR* mutant were studied as a function of nitrogen availability to further investigate the observed ammonium-mediated inhibition of RDX degradation. The results indicate that nitrogen availability is a major determinant of RDX degradation and *xplA* gene expression in KTR9.

Military training activities and the production of explosives such as RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) have resulted in the contamination of soils with these compounds at military facilities around the world. As a result, there are numerous examples of RDX migrating into groundwater at military manufacturing and testing/training sites (1–4). *In situ* bioremediation offers an attractive, cost-effective cleanup option for contaminated sites with limited access. Microbially mediated RDX biodegradation has been reported under a number of conditions (5–14), with only a few genes implicated in RDX degradation (15–17). Of these, the cytochrome P450 system encoded by *xplAB* is the best characterized (18, 19).

Previously, we isolated and characterized environmental *Actinomycetes* able to use RDX as a sole nitrogen source (14). RDX degradation by a *Gordonia* sp. strain KTR9 isolate was inhibited in the presence of competing inorganic nitrogen sources, and reduction in RDX degradation correlated to expression of *xplA* (20). Recently, a role for the global nitrogen regulator, GlnR, in RDX degradation has been suggested for KTR9 since *glnR* mutants of KTR9 are unable to grow on RDX as a sole nitrogen source (21). To further investigate nitrogen limitation in the catabolism of RDX, gene expression analyses of KTR9 and a *glnR* KTR9 deletion mutant were conducted under excess and limiting nitrogen growth conditions.

KTR9 was grown as described previously (20), and triplicate cultures in the late exponential phase of growth were used to inoculate media containing the following nitrogen-containing compounds: (i) 4 mM $(NH_4)_2SO_4$, (ii) 0.9 mM $(NH_4)_2SO_4$, (iii) 40 mg liter^{-1} (180 μM) RDX, and (iv) 40 mg liter^{-1} RDX plus 4 mM $(NH_4)_2SO_4$. RDX concentrations were determined by high-performance liquid chromatography (HPLC) analysis (14), and ammonium concentrations were verified using the AQUANAL-plus test kit for ammonium (Sigma, St. Louis, MO). Total RNA extraction, cDNA synthesis/labeling, and microarray experimentation were performed as described previously (22). Microarray hybridizations were carried out using custom *Gordonia* sp. strain KTR9 arrays (Roche, Madison, WI) developed from the annotated genome (23). The microarray data set is available from NCBI (www.ncbi.nlm.nih.gov/geo) under accession number GSE42342.

Transcript levels were compared across the different growth conditions using mid-exponential stage (36-h) samples of RDX-grown cultures and late-exponential stage (48-h) samples of ammonium-grown cultures of KTR9 (Fig. 1). At these times, 10 mg liter^{-} (45 μM) and 25 mg liter^{-1} (112 μM) RDX remained in the medium for cultures grown in the presence of RDX and RDX-$(NH_4)_2SO_4$, respectively. In comparison, all of the ammonium had been consumed in the low-nitrogen growth condition [0.9 mM $(NH_4)_2SO_4$], but a significant amount of ammonium remained in the nitrogen-rich growth condition [4 mM $(NH_4)_2SO_4$]. Using the 4 mM $(NH_4)_2SO_4$ growth condition as a baseline for the transcriptome comparison, a 76% overlap was observed between the transcriptome of cells grown on 0.9 mM $(NH_4)_2SO_4$ and the transcriptome of cells grown on RDX (Fig. 2). A total of 100 genes involved in nitrogen transport, nitrogen assimilation, amino acid and nucleoside catabolism, transcription, and RDX degradation were common to both conditions (Table 1). A cluster of at least three hypothetical genes (KTR9_4925 to KTR9_4927) of unknown function, contiguous with the *xplAB* gene locus, were also upregulated. In contrast, the expression of these genes was significantly reduced in cells grown on RDX-4 mM $(NH_4)_2SO_4$ (Table 1).

Additional transcriptome studies using KTR9 and a global nitrogen regulation (*glnR*) mutant of KTR9 were conducted with cultures grown under nitrogen-rich [4 mM $(NH_4)_2SO_4$] and nitrogen-limiting conditions [0.9 mM $(NH_4)_2SO_4$]. Cells for transcriptome analysis were harvested at 48 h, when nitrogen became depleted in the low-nitrogen growth condition (Fig. 3). Effects of the *glnR* mutation on the expression of genes revealed significant reductions in transcript levels under nitrogen-limiting conditions in the mutant compared to those of the wild-type strain (Table 2). The *glnR* mutant was significantly impaired in its ability to upregulate key genes involved in nitrogen transport and assimilation, consistent with the regulatory role of GlnR in response to nitrogen starvation (24–27). In addition, the magnitude of upregulation of *xplAB* and additional genes surrounding *xplAB* was

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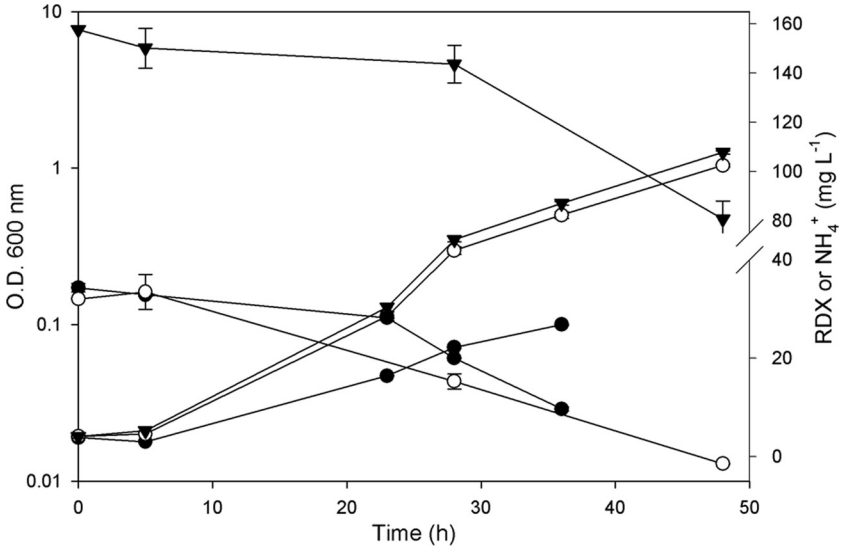

FIG 1 Growth and nitrogen utilization by *Gordonia* sp. KTR9 on 4 mM $(NH_4)_2SO_4$ (▼), 0.9 mM $(NH_4)_2SO_4$ (○), and 180 μM RDX (●) as sole sources of nitrogen. Error bars represent the standard deviations from three replicates.

also reduced under nitrogen limitation. Conversely, the lack of GlnR *cis*-acting elements upstream of *xplAB* may suggest that GlnR does not directly regulate *xplAB* expression. Real-time PCR analyses as described previously (20) confirmed the relative changes in transcript levels for genes listed in Table 2.

Atrazine catabolic genes are induced under nitrogen-limiting conditions by a combination of global nitrogen regulators and a specific regulator of the catabolic genes (28–30). We identified a regulator located upstream of the *xplAB* gene cluster, designated *xplR*, which is transcriptionally upregulated in the presence of RDX and subject to nitrogen repression (20). We hypothesized that XplR may repress *xplAB* in the presence of preferred nitrogen sources such as $(NH_4)_2SO_4$. To test this hypothesis, a kanamycin resistance marker (Km^r) was inserted into the *xplR* coding region (KTR9_4921) (31). Wild-type and *xplR* mutant cells were resuspended in cold 0.25 mM phosphate buffer containing 25 mg liter^{-1} RDX, and RDX levels were monitored over 24 h. No significant differences in degradation rates were observed between the two strains, indicating that XplR does not play a role in RDX degradation.

The coordination of *xplAB* with the cell's response to nitrogen limitation may represent a partial adaptation to scavenging xenobiotic sources of nitrogen under nitrogen-limiting conditions. In lieu of evolving specific regulators that respond directly to the presence of exogenous xenobiotics, the bacterium has instead evolved regulators that respond to the more common environmental state of nitrogen limitation. Perhaps the current regulation of *xplAB* in strain KTR9 represents a transition state toward specific regulation of RDX degradation, as the evolution of new catabolic activities can precede their respective regulators (32). The observation that nitrogen limitation induces RDX degradation in some bacteria has important practical implications for *in situ* RDX bioremediation projects that rely on natural attenuation, bioaug-

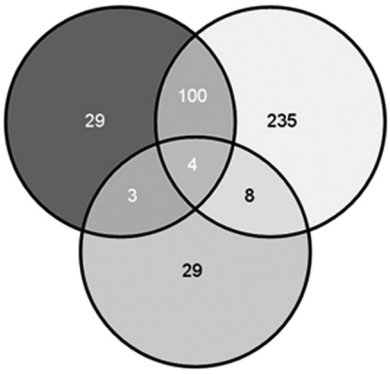

FIG 2 Analysis of gene induction patterns 2-fold or greater across the 0.9 mM $(NH_4)_2SO_4$ (dark gray), 180 μM RDX (light gray), and 4 mM $(NH_4)_2SO_4$-180 μM RDX (medium gray) transcriptomes using the 4 mM $(NH_4)_2SO_4$ growth condition as a baseline condition.

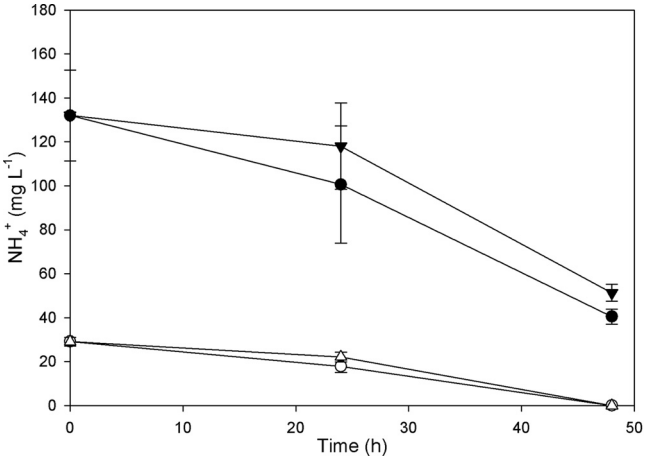

FIG 3 Nitrogen utilization by *Gordonia* sp. KTR9 (circles) and a *glnR* KTR9 mutant (triangles) grown under excess [4 mM $(NH_4)_2SO_4$; black] and nitrogen-limiting [0.9 mM $(NH_4)_2SO_4$; white] conditions. Error bars represent the standard deviations from three replicates.

TABLE 1 Genes induced in the presence of either 40 mg liter^{-1} (180 μM) RDX or 0.9 mM $(NH_4)_2SO_4$[a]

Locus	Function	Cellular role	Fold change			*P* value
			0.9 mM ammonium sulfate	RDX	RDX + 4 mM ammonium sulfate	
KTR9_1218	Proline dehydrogenase	A	24	12	1.6	4.0E−07
KTR9_1219	Putative delta-1-pyrroline-5-carboxylate dehydrogenase	A	25	12	1.4	1.4E−06
KTR9_1224	Glutamine synthetase, type III	N	4.3	9.0	1.3	1.4E−04
KTR9_1306	NAD(P)H-nitrite reductase	N	7.7	13	1.4	4.6E−05
KTR9_1307	Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	N	6.2	12	1.2	3.4E−05
KTR9_1309	Putative nitrate reductase/sulfite reductase	N	5.0	9.1	1.2	1.3E−04
KTR9_2009	Ammonia permease	T	5.2	7.0	0.9	1.5E−04
KTR9_2010	Nitrogen regulatory protein PII	R	3.0	4.3	0.9	7.0E−04
KTR9_2091	Alanine dehydrogenase	A	15	5.1	1.4	1.4E−03
KTR9_2634	Urea amidohydrolase (urease) gamma subunit	U	2.2	4.2	1.0	8.3E−05
KTR9_2769	Branched-chain amino acid ABC-type transport system, permease components	T	3.2	2.9	2.1	4.6E−02
KTR9_2770	ABC-type branched-chain amino acid transport system, permease component	T	3.4	4.2	1.7	1.9E−04
KTR9_2772	ABC-type branched-chain amino acid transport systems, ATPase component	T	2.8	4.4	1.6	1.5E−03
KTR9_2773	ABC-type branched-chain amino acid transport systems, periplasmic component	T	6.5	8.1	2.3	2.4E−03
KTR9_2930	Ethanolamine ammonia-lyase, large subunit	A	2.1	3.1	1.3	2.7E−05
KTR9_2938	Glutamate synthase, NADH/NADPH, small subunit	N	2.6	2.4	0.5	1.4E−03
KTR9_3418	Branched-chain amino acid ABC-type transport system, permease components	T	2.1	9.2	1.4	2.9E−03
KTR9_3419	ABC-type branched-chain amino acid transport systems, periplasmic component	T	3.0	15	1.4	6.2E−05
KTR9_3425	Formate/nitrite family of transporters	T	6.9	12	1.3	8.8E−05
KTR9_3426	Cyanate lyase	A	2.8	4.6	1.1	5.0E−04
KTR9_3533	Uroporphyrinogen III synthase		7.9	14	1.1	3.1E−05
KTR9_3534	Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	N	21	30	1.1	2.8E−07
KTR9_3535	NAD(P)H-nitrite reductase	N	15	24	1.0	4.1E−06
KTR9_3536	Nitrate/nitrite transporter	T	11	16	1.2	1.0E−06
KTR9_3594	Xanthine/uracil permeases	T	2.3	2.2	1.3	4.0E−03
KTR9_3723	Cytosine permease/uracil permease/thiamine permease/allantoin permease	T	4.5	2.9	1.4	2.9E−03
KTR9_3824	Allophanate hydrolase subunit 2	U	8.1	6.0	1.1	3.0E−04
KTR9_3825	Urea carboxylase-associated protein 1	U	25	14	1.1	1.2E−07
KTR9_3826	Urea carboxylase-associated protein 2	U	16	11	1.1	5.4E−06
KTR9_3827	Permease, urea carboxylase system	T	12	8.7	1.2	1.0E−05
KTR9_4060	ABC-type nitrate/sulfonate/bicarbonate transport system, permease component	T	3.2	5.4	1.4	3.2E−02
KTR9_4061	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	T	3.4	5.7	1.4	4.4E−04
KTR9_4063	Putative creatinine amidohydrolase	A	2.2	4.4	1.1	1.7E−04
KTR9_4065	Cytosine deaminase and related metal-dependent hydrolases	A	2.1	2.9	1.2	1.1E−03
KTR9_4672	Ferredoxin-dependent glutamate synthase	N	7.7	2.5	1.2	5.1E−05
KTR9_4673	Glutamate synthase domain 3	N	12	4.6	1.3	3.0E−06
KTR9_4674	Glutamate synthase domain 1	N	9.2	3.3	1.1	4.9E−06
KTR9_4676	Ammonia permease	T	2.4	2.4	1.3	6.2E−03
KTR9_4922	Cytochrome P450	X	10	11	0.8	2.0E−05
KTR9_4923	GlnA-XplB fusion protein,glutamine synthetase, GlnA, flavodoxin reductase, XplB	X	10	10	0.5	6.1E−06
KTR9_4924	Flavodoxin-cytochrome P450 XplA	X	10	8.5	0.6	2.5E−05
KTR9_4925	Hypothetical protein	X	20	17	0.7	1.7E−08
KTR9_4926	Hypothetical protein	X	25	18	1.0	1.0E−05
KTR9_4927	Hypothetical protein	X	6.4	6.9	1.1	6.8E−06

[a] Cellular roles: transport (T), nitrogen assimilation (N), urea degradation (U), amino acid, nucleoside, and other ammonia generating catabolic process (A), regulation (R), and RDX degradation (X). Fold change values were based on baseline comparisons, with transcriptome data from cells grown on 4 mM ammonium sulfate.

TABLE 2 Effects of a *glnR* mutation on the expression of genes induced by nitrogen limitation in wild-type KTR9[a]

Locus	Function	Microarray fold change		Real-time PCR fold change	
		KTR9 wild type	KTR9 Δ*glnR* strain	KTR9 wild type	KTR9 Δ*glnR* strain
KTR9_0797[b]	Permease, MFS superfamily	1.0	1.1	1.4	1.0
KTR9_1218	Proline dehydrogenase	31	120	4.6	171
KTR9_1219	Putative delta-1-pyrroline-5-carboxylate dehydrogenase	18	219	4.5	346
KTR9_1306	NAD(P)H-nitrite reductase	9.2	1.3	12	1.4
KTR9_1307	Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	4.8	1.2	12	1.3
KTR9_1309	Putative nitrate reductase/sulfite reductase	8.2	1.7	7.9	1.2
KTR9_1685[b]	DNA polymerase III, alpha subunit	1.1	1.2	2.4	1.1
KTR9_2010	Nitrogen regulatory protein PII	3.8	1.9	1.1	1.5
KTR9_3071	Glutamine synthetase, type I	2.3	0.2	0.2	0.1
KTR9_3533	Uroporphyrinogen III synthase	14	0.8	6.7	0.7
KTR9_3534	Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	38	0.9	23	0.7
KTR9_3825	Urea carboxylase-associated protein 1	18	1.4	4.3	0.8
KTR9_3826	Urea carboxylase-associated protein 2	10	1.1	4.2	0.9
KTR9_3827	Permease, urea carboxylase system	12	1.4	1	1.4
KTR9_3954[b]	Isochorismate synthase	0.9	0.9	1.8	0.5
KTR9_4922	Cytochrome P450	1	3.5	9.0	2.1
KTR9_4923	GlnA-XplB fusion protein	14	2.3	19	1.1
KTR9_4924	Flavodoxin-cytochrome P450 XplA	19	1.5	14	0.9
KTR9_4925	Hypothetical protein	36	1.0	7.8	0.6
KTR9_4926	Hypothetical protein	16	1.4	8.7	1.0

[a] Fold change values were based on baseline comparisons with microarray and/or real-time PCR data from corresponding cells grown on 4 mM ammonium sulfate. A correlation of 0.76 was observed for gene expression values generated by microarray and real-time PCR.
[b] Housekeeping genes shown not to be regulated in response to RDX or high- or low-nitrogen growth conditions.

mentation, or biostimulation. These approaches may be more suitable for sites with low inorganic background nitrogen levels, since aerobic RDX denitration may function more efficiently under these geochemical conditions.

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Table S1. Bacterial strains, constructs and primers used in study.

Bacterial strains, constructs, and primers	Catalog number, sequence, or description	Reference
Strains		
Gordonia sp. KTR9		(43)
Gordonia sp. KTR9/*xplR:kan*	KTR9 strain with *kan* insertional inactivation of *xplR* (KTR9_4921)	This study
Gordonia sp. KTR9/Δ*glnR*	KTR9 strain with *glnR* (KTR9_3806) deletion	(48)
Escherichia coli Top 10	Invitrogen #C4040	
Plasmid constructs		
pKTR9-4921:*kan*	1614 bp fragment containing insertion of 929 bp *kan* gene (gb AAB63351.1) into 685 bp KTR9_4921 (*xplR*) near midpoint of gene cloned into *Bam*H1 site of pK18*mobsacB*	This study
Real-time PCR Primers (5' to 3')		
KTR9_0797f	ACCGCGATGATGGTCATGA	This study
KTR9_0797r	CCTGCCACTCCGCGATT	This study
KTR9_1218f	AACCGGCGGACCACCTT	This study
KTR9_1218r	CGCAGCGAACGGATCAG	This study
KTR9_1219f	ACACCGATCGTGCGATGTAG	This study
KTR9_1219r	CCTTCGAGTACCAGGGACAGAA	This study
KTR9_1306f	CATCTCGAAGGCCAACTTCCT	This study
KTR9_1306r	CGGTGTCCCCATGTCGAA	This study
KTR9_1307f	GGCCCCATGGAGGATCTG	This study
KTR9_1307r	CGGAACACCGCGATCTGT	This study
KTR9_1309f	CGATCCGTGTATCGAGTTTGC	This study
KTR9_1309r	CATTGAATTCCGAGGATGCA	This study
KTR9_1685f	TCGCCAATTTCGGTTTCC	This study
KTR9_1685r	CCAAGGTCTGCGGTGAGTAGA	This study
KTR9_2010f	TGAAGCTGATCACTGCAATTGTC	This study
KTR9_2010r	CTCGGTGTGGCCCTTCTG	This study
KTR9_3071f	CCAGGCGGAGATCAACTACAA	This study
KTR9_3071r	TGACCGACTTGCCGTTCTG	This study
KTR9_3533f	TCGACGGCCAGTCGGTAT	This study
KTR9_3533r	GCCGCGCTGGGTGAT	This study
KTR9_3534f	ACGACACGCACTCCGTAACTC	This study
KTR9_3534r	CGCTGGGCAAGCAGATCT	This study
KTR9_3825f	TGCCGGTGGTCAGGAAGA	This study
KTR9_3825r	GACACGTCCAGACGGTATTCG	This study
KTR9_3826f	CGGACTCGCCGAATGC	This study
KTR9_3826r	TCGACTCTCGCCGGAAAC	This study
KTR9_3827f	TGGGTGAACAGTGCCAGGAT	This study
KTR9_3827r	TGGATGAGCCGCGTCAA	This study
KTR9_3954f	GCCGCAGTCTCCGAACCT	This study
KTR9_3954r	TTCAGGACCGTACGGAGTTTG	This study

KTR9_4922f	GGTATCGACGCGAAATGCA	This study
KTR9_4922r	TCACCTGGTCGGAAGTGATTG	This study
KTR9_4923f	CAACGTCGCCATGGACATC	This study
KTR9_4923r	GGTGTCCGGGTGCAGATC	This study
KTR9_4924f	CGACGAGGAGGACATGAGATG	This study
KTR9_4924r	GCAGTCGCCTATACCAGGGATA	This study
KTR9_4925f	AGGCATCTTCGTGCTGAACA	This study
KTR9_4925r	AGGTCAGCTGGCGAATCG	This study
KTR9_4926f	ACGACGAATGCATGTGAACAG	This study
KTR9_4926r	GGTGCGGGTATTCGACTATCC	This study
Miscellaneous Primers (5' to 3')		
gntR_knockout_F	ATGCAGATCGGAAGCATCTCC	This study
gntR_knockout_R	AACACTGCCAGCGCATCAAC	This study
gntR_568r	CGGTCACCGTGCTCATCAC	This study