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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.

ilitary 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 (NH₄)₂SO₄, (ii) 0.9 mM (NH₄)₂SO₄, (iii) 40 mg liter⁻¹ (180 μM) RDX, and (iv) 40 mg liter⁻¹ RDX plus 4 mM (NH₄)₂SO₄. 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 am-

monium-grown cultures of KTR9 (Fig. 1). At these times, 10 mg liter $^-$ (45 μ M) and 25 mg liter $^{-1}$ (112 μ M) RDX remained in the medium for cultures grown in the presence of RDX and RDX-(NH₄)₂SO₄, respectively. In comparison, all of the ammonium had been consumed in the low-nitrogen growth condition [0.9] mM (NH₄)₂SO₄], but a significant amount of ammonium remained in the nitrogen-rich growth condition [4 mM (NH₄)₂SO₄]. Using the 4 mM (NH₄)₂SO₄ 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 (NH₄)₂SO₄] and nitrogen-limiting conditions [0.9 mM (NH₄)₂SO₄]. 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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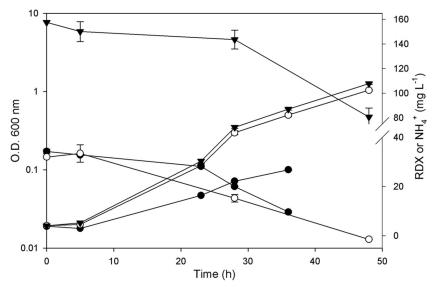


FIG 1 Growth and nitrogen utilization by *Gordonia* sp. KTR9 on 4 mM (NH₄)₂SO₄ (∇), 0.9 mM (NH₄)₂SO₄ (\bigcirc), and 180 μ M RDX (\blacksquare) as sole sources of nitrogen. Error bars represent the standard deviations from three replicates.

degradation.

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 (NH₄)₂SO₄. 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

biotic 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-

two strains, indicating that XplR does not play a role in RDX

limitation may represent a partial adaptation to scavenging xeno-

The coordination of *xplAB* with the cell's response to nitrogen

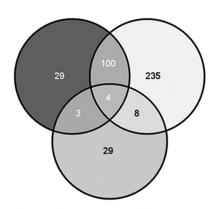


FIG 2 Analysis of gene induction patterns 2-fold or greater across the 0.9 mM (NH₄)₂SO₄ (dark gray), 180 μ M RDX (light gray), and 4 mM (NH₄)₂SO₄-180 μ M RDX (medium gray) transcriptomes using the 4 mM (NH₄)₂SO₄ growth condition as a baseline condition.

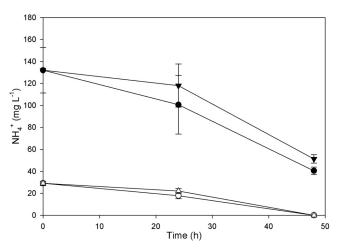


FIG 3 Nitrogen utilization by *Gordonia* sp. KTR9 (circles) and a *glnR* KTR9 mutant (triangles) grown under excess [4 mM (NH₄)₂SO₄; black] and nitrogen-limiting [0.9 mM (NH₄)₂SO₄; white] conditions. Error bars represent the standard deviations from three replicates.

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TABLE 1 Genes induced in the presence of either 40 mg liter $^{-1}$ (180 μ M) RDX or 0.9 mM (NH₄)₂SO₄ a

		Fold change				
		Cellular	0.9 mM ammonium		RDX + 4 mM ammonium	
Locus	Function	role	sulfate	RDX	sulfate	P value
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,	T	3.0	15	1.4	6.2E-05
VTD0 2425	periplasmic component	T	6.0	12	1.2	0 0E 0E
KTR9_3425	Formate/nitrite family of transporters		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	NT	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	GINA-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
	transport (T), nitrogen assimilation (N), urea degradation (U), amino acid, nucleon					

^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

		Microarray fold change		Real-time PCR fold change	
Locus	Function	KTR9 wild type	KTR9 ΔglnR strain	KTR9 wild type	KTR9 $\Delta glnR$ 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.

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.

of xplR (KTR9_4921) Gordonia sp. KTR9/AglnR KTR9 strain with glnR (KTR9_3806) deletion 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 BamH1 site of pK18mobsacB Real-time PCR Primers (5' to 3') KTR9_07977 CCTGCCACTCCGGGATT This study KTR9_1218f AACCGGCGGACCACCTT KTR9_1218f CGCAGCGACGACGGATCAG This study KTR9_1219f ACACCGATCGTGCGATGAG This study KTR9_1219r CCTTCGACTCCCGGATC This study KTR9_1306f CATCTCGAAGGACCAGAG KTR9_1306f CATCTCGAAGGCCAACTTCCT This study KTR9_1307f GGCCCCATGGAGGACCACTT This study KTR9_1309r CGAACCGCGATCTGGAG KTR9_1309r CGATCCGTGTATCGAGTTCG This study KTR9_1309r CGATCCGTGTATCGAGTTTCC This study KTR9_1309r CGATCCGTGTATCGAGGATCAG This study KTR9_1309r CATTGAATTCCGAGGATCAG This study KTR9_1685f CCCAAGGTCTGCCGTATTCC This study KTR9_1685f CCCAAGGTCTGCGGTGAGAA This study KTR9_1685f CCAAGGTCTGCGTGTTCC This study KTR9_2010r CTCGGTAACTCGGATAGAA This study KTR9_2010r CTCGGTGTGCCCATTCTC This study KTR9_3533r CCAGGCGAACTCCGTCTTCT This study KTR9_3533r CCAGGCGAACTCACTCCTT This study KTR9_3533r CCAGGCGAACTCACTCCTT This study KTR9_3533r CCAGGCGAACTCACTCCTT This study KTR9_3533r CCAGGCGAACTCACTCCTTCT This study KTR9_3533r CCAGGCCGATCTCTCT This study KTR9_3533r CCAGGCCGATCCGTTCTCT This study KTR9_3533r CCAGGCCGATCCGTTCTCT This study KTR9_3533r CCAGGCCAACTCCGTAACTC This study KTR9_3533r CGCGCCCTGGGTGAT This study KTR9_3533r CGCGCCCTGGGTGAT This study KTR9_3534r CGCGGCAGTCCGGAACACAC This study KTR9_3825r TGCGGGGAACACGCACTCCGTAACC This study KTR9_3827r TGGGTGAACAGTCCAAGACAC This study KTR9_3827r TGGGTGAACAGTCCAAGACC This study KTR9_3954f CCCGCAGCCGCATCCCAACCC This study KTR9_3954f TGGATGACCGCCATCCCAACCC This study KTR9_3954f	Bacterial strains, constructs, and primers	Catalog number, sequence, or description	Reference
Gordonia sp. KTR9/xplR:kan KTR9 strain with kan insertional inactivation of xplR (KTR9_4921) Gordonia sp. KTR9/AglnR KTR9 strain with glnR (KTR9_3806) (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 (xpl) near midpoint of gene cloned into BamH1 site of pK18mobsacB Real-time PCR Primers (5' to 3') KTR9_07971 ACCGCGATGATGGTCATGA This study KTR9_1218f AACCGGCGGACCACTCT This study KTR9_1218f CGCAGCGAACGGATCAG This study KTR9_1219f ACACCGGTGGGATGAG This study KTR9_1219f ACACCGATCACCAGGACAGAA This study KTR9_1306f CATCCGAGTACCAGGACAGAA This study KTR9_1306f CATCCGAGGACGACTCCT This study KTR9_1300f CGGAGCAACGCACCTCT This study KTR9_1307f GGCCCCATGGAGACGAACTGA This study KTR9_1307f CGGAACACCGCATCTCT This study KTR9_1307f CGGAACACCGCATCTCT This study KTR9_1309f CGATCCGTGTATCGAGTTTCC This study KTR9_1309f CGATCCGTGTATCGAGTTTCC This study KTR9_1309f CGATCCGTGTATCGAGTTTCC This study KTR9_1685f TCGCCAATTTCGGTTTTCC This study KTR9_1685f CCAAGGTCTGACAGAA This study KTR9_1685f CCAAGGTCGGTGAGAAA This study KTR9_1685f CCAAGGTCTGCGGTGAGAAA This study KTR9_2010f TGAAGCTGATCACTGCAATTTCC This study KTR9_2010f TGAAGCTGATCACTGCAATTTCC This study KTR9_3533f CCGCGCTGTCTCT This study KTR9_3533f TCGACGGCAACTCCTTCT This study KTR9_3533f TCGACGGCAACTCCGTTCT This study KTR9_3533f TCGACGGCCAACTCCGTAACTC This study KTR9_3534f ACGACACGCACTCCGTAACTC This study KTR9_3825f TGCCGGTGGAACACTCCGTAACTC This study KTR9_3825f TGCCGGTGGCCCTTCG This study KTR9_3826f TGCACGTCCGGAACAC This study KTR9_3827f TGGGTGAACAGTCCCGGAACCT This study KTR9_3827f TGGGTGAACAGTCCCGTAACTC This study KTR9_3827f TGGGTGAACAGTCCCGTAACTC This study KTR9_3827f TGGGTGAACAGTCCCGTAACTC This study KTR9_3827f TGGGTGAACAGTCCCGAACCT This study KTR9_3824f TGGCGCCCGCACACCT This study	<u>Strains</u>		
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KTR9_0797f	Real-time PCR Primers (5' to 3')		
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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