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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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# 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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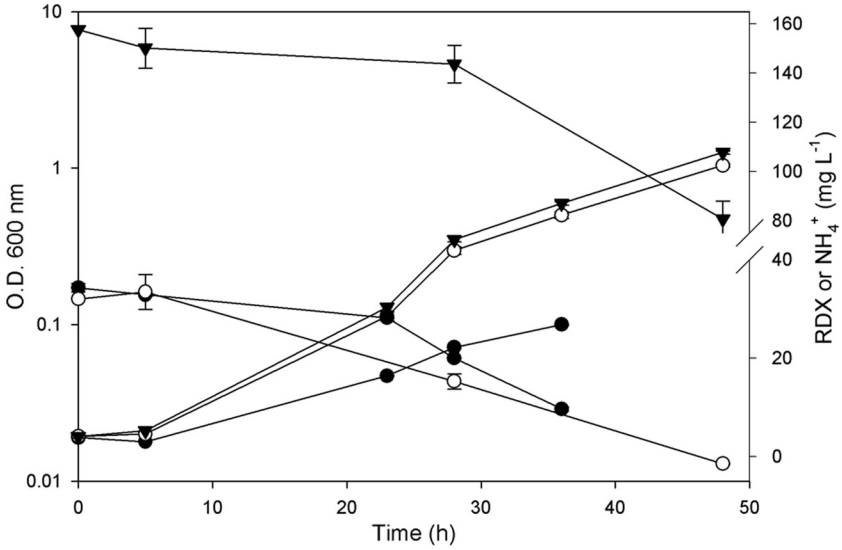


**FIG 1** Growth and nitrogen utilization by *Gordonia* sp. KTR9 on 4 mM $(NH_4)_2SO_4$ (▼), 0.9 mM $(NH_4)_2SO_4$ (○), and 180 μM RDX (●) as sole sources of nitrogen. Error bars represent the standard deviations from three replicates.

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-

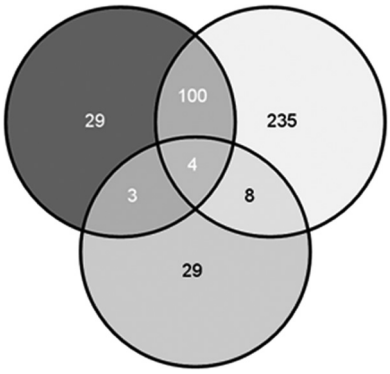


**FIG 2** Analysis of gene induction patterns 2-fold or greater across the 0.9 mM $(NH_4)_2SO_4$ (dark gray), 180 μM RDX (light gray), and 4 mM $(NH_4)_2SO_4$-180 μM RDX (medium gray) transcriptomes using the 4 mM $(NH_4)_2SO_4$ growth condition as a baseline condition.

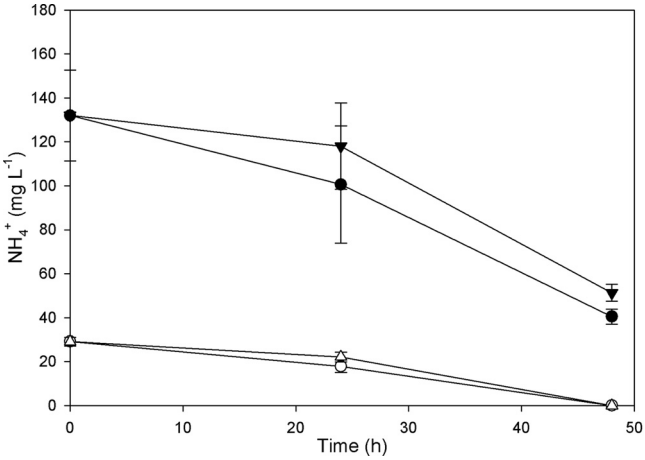


**FIG 3** Nitrogen utilization by *Gordonia* sp. KTR9 (circles) and a *glnR* KTR9 mutant (triangles) grown under excess [4 mM $(NH_4)_2SO_4$; black] and nitrogen-limiting [0.9 mM $(NH_4)_2SO_4$; white] conditions. Error bars represent the standard deviations from three replicates.

**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 |