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Analysis of the *xplAB*-Containing Gene Cluster Involved in the Bacterial Degradation of the Explosive Hexahydro-1,3,5-Trinitro-1,3,5-Triazine

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Repeated use of the explosive compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) on military land has resulted in significant soil and groundwater pollution. Rates of degradation of RDX in the environment are low, and accumulated RDX, which the U.S. Environmental Protection Agency has determined is a possible human carcinogen, is now threatening drinking water supplies. RDX-degrading microorganisms have been isolated from RDX-contaminated land; however, despite the presence of these species in contaminated soils, RDX pollution persists. To further understand this problem, we studied RDX-degrading species belonging to four different genera (*Rhodococcus*, *Microbacterium*, *Gordonia*, and *Williamsia*) isolated from geographically distinct locations and established that the *xplA* and *xplB* (*xplAB*) genes, which encode a cytochrome P450 and a flavodoxin redox partner, respectively, are nearly identical in all these species. Together, the *xplAB* system catalyzes the reductive denitration of RDX and subsequent ring cleavage under aerobic and anaerobic conditions. In addition to *xplAB*, the *Rhodococcus* species studied here share a 14-kb region flanking *xplAB*; thus, it appears likely that the RDX-metabolizing ability was transferred as a genomic island within a transposable element. The conservation and transfer of *xplAB*-flanking genes suggest a role in RDX metabolism. We therefore independently knocked out genes within this cluster in the RDX-degrading species *Rhodococcus rhodochrous* 11Y. Analysis of the resulting mutants revealed that XplA is essential for RDX degradation and that XplB is not the sole contributor of reducing equivalents to XplA. While XplA expression is induced under nitrogen-limiting conditions and further enhanced by the presence of RDX, MarR is not regulated by RDX.

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is widely used in both military and commercial applications. Following decades of use on military training ranges, along with manufacturing and decommissioning processes, RDX is now a significant environmental contaminant (1). The low rates of biodegradation of RDX in the environment, coupled with its poor soil-binding capacity, mean that RDX readily leaches through soil and groundwater, with the potential to contaminate drinking water supplies. This is of concern; the U.S. Environmental Protection Agency has determined RDX to be a possible human carcinogen, and the use of RDX on some ranges has been restricted as a result of groundwater contamination.

A number of *Corynebacterineae* spanning four different genera (*Rhodococcus*, *Microbacterium*, *Gordonia*, and *Williamsia*) isolated from RDX-contaminated soils are able to use RDX as a sole nitrogen source for growth (2–8). These species originate from geographically distinct locations, and all of those species tested have been shown to encode the cytochrome P450 XplA and the associated reductase XplB; indeed, no aerobic RDX-degrading isolates lacking *xplA* have yet been isolated by selective enrichments (using RDX as a sole nitrogen source). Together, the encoded P450 system catalyzes the reductive denitration of RDX, resulting in subsequent ring cleavage under aerobic and anaerobic conditions (9, 10). Structural analyses, including X-ray studies (11), have revealed features not previously seen in cytochromes P450, including the fusion of the heme domain to a flavodoxin redox partner (12).

The highly conserved nature of the *xplAB* gene sequences be-

tween two phylogenetically disparate species, *Rhodococcus rhodochrous* 11Y and *Microbacterium* sp. strain MA1, isolated from RDX-contaminated soil in the United Kingdom and the United States, respectively, indicates that these genes have been rapidly distributed across the world by horizontal gene transfer (1). In support of this, the *xplAB* sequence was found to be located on a plasmid of approximately 145.5 kb in *Microbacterium* sp. MA1. Sequencing revealed that an open reading frame (ORF) upstream of *xplAB*, pMA1.030, and the neighboring downstream ORFs pMA1.033 and pMA1.034 were nearly identical between *Microbacterium* sp. MA1 and *R. rhodochrous* 11Y (6). The *xplAB* genes are also present on an extrachromosomal element in *Gordonia* sp. strain KTR9, although in this species, glutamine synthetase is fused to the N terminus of *xplB* (13).

pMA1.028 in *Microbacterium* sp. MA1 encodes a putative permease transporter, termed AroP (6), which shares 72% identity with a putative amino acid permease from *Mycobacterium vanbaalenii* PYR-1 (NCBI accession number [YP_952672.1](https://www.ncbi.nlm.nih.gov/nuclot/YP_952672.1)). Two

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TABLE 1 Origins and accession numbers of bacteria used in this study

Bacterial strain	Country of isolation	Reference	NCBI accession no.
<i>Rhodococcus rhodochrous</i> 11Y	UK	3	AF439261.1
<i>Rhodococcus</i> sp. BL1	Belgium	This study	KF700363
<i>Rhodococcus</i> sp. DS1	Belgium	This study	KF933858
<i>Rhodococcus</i> sp. 5U	Ukraine	This study	KJ566616
<i>Rhodococcus</i> sp. 9UK	UK	This study	KJ566615
<i>Rhodococcus</i> sp. YH1	Israel	Unpublished data (Z. Ronen)	AF103733.1
<i>Rhodococcus</i> sp. DN22	Australia	7	X89240.1
<i>Rhodococcus</i> sp. EG2	US	8	KF571869.1
<i>Williamsia</i> sp. EG1	US	8	KF571870.1
<i>Microbacterium</i> sp. MA1	US	6	FJ357539
<i>Gordonia</i> sp. KTR9	US	4	DQ068383

studies have documented permeases located close to genes with related function (14, 15). The mode of RDX removal by *R. rhodochrous* 11Y is unknown, but given the proximity to *xplA*, it is possible that the *aroP*-encoded permease is involved in the transport of RDX or its breakdown products.

The ORF pMA1.027 in *Microbacterium* sp. MA1 encodes a transcriptional regulator that shares 62% amino acid identity with the regulatory protein MarR (multiple-antibiotic-resistance regulator) from *Gordonia bronchialis* DSM 43247 (NCBI accession number YP_003274556). The MarR family of proteins is involved in environmental stress responses and in the catabolic pathways of aromatic compounds (16) and is known to bind a number of anionic lipophilic, and often phenolic, effector molecules (17). MarR is also reported to negatively regulate a small cluster of naphthalene catabolism-related genes called *nagR2-ORF2''KL* in the naphthalene-degrading bacterium *Polaromonas naphthalenivorans* CJ2 (18) and the *marRAB* operon in *Escherichia coli* (19). Additionally, MarR is part of the *phn* island in polycyclic aromatic hydrocarbon (PAH)-degrading bacteria and is possibly involved in the regulation of *phn* genes (20).

Despite the presence of RDX-degrading microorganisms in RDX-contaminated soil, the rate of RDX degradation in soils is insufficient to remediate polluted environments resulting in groundwater contamination. It is known that the expression of *xplAB* is repressed by the presence of other nitrogenous compounds (13, 21), and previous studies suggest that XplA activity is regulated and induced by the presence of RDX (3).

We have isolated RDX-degrading bacteria from different geographical regions and sequenced them for *xplAB* and neighboring genes. The identification of a highly conserved, nearly identical gene cluster harboring *xplAB* in *Microbacterium* sp. MA1 and *R. rhodochrous* 11Y indicates the involvement, within this cluster, of these genes in RDX metabolism. To investigate the potential role of genes adjacent to *xplA*, we created and characterized *R. rhodochrous* 11Y knockouts in *aroP*, *marR*, *xplA*, and *xplB* and tested RDX metabolism following growth in a range of nitrogen sources.

MATERIALS AND METHODS

Enrichment and phylogenetic analysis. Soil samples were obtained from military sites in the United Kingdom, Belgium, and Ukraine. Enrichment cultures for soil from the United Kingdom, Belgium, and Ukraine were developed as described previously by Binks et al. (2).

Taxonomic characterization of isolates was performed by amplifying

TABLE 2 Primers used to amplify the *xplAB* gene cluster region in the RDX-degrading isolates

Primer	Sequence (5'–3')
GAF-F	CGATTCCGTATCCGACCTC
TranscRp-504R	GATGTTCTCCGGTTGATCG
RegulatoryPrt-89F	GGTTCACATGGCTTTTCACC
SensorK-292R	CTATCGCATCGCTCAGGAGT
SensorK-129F	CATTCCGTCTATTCCGGTTC
SensorK-1087R	CGTGGTCGTGACAGTCATCT
Sensork-1035F	GATTCCGGTCAACGGGTA
Carbesterase-167R	AGATCGGGACATGACAC
MarR-194R	GCACGTGTTGAGTCCGAGATG
HypoPrt-453F	GGTCTCGCAACACACTGAAC
Carbesterase-6F	ACGTCGGAATTCAAGTGTCCGGCGGACC
Permease-6R	GAGTCGAAGAGGGCACTCAG
Permease-7F	CTGTGTGGTGTGGTGATGGT
Permease-7R	CTACTCCCCGGTCTTCTTCGGAAT
Permease-8F	ACTACATCGTGTCTGTCTGCC
Dihydro-8R	GTCGAGCATATGCACGTCCGGGATACG
Dihydro-9F	GATGTCAACGAATGGACGAT
xplB-9R	GTCGATCCGAAGTGAACAC
xplB-10F	CGGTGATGATGCGTGAACT
xplA-10R	TCTCCGTAGGTGGAGGTGAC
xplB-11F	ACCCGACATATGACACCCGAGATGGAG
xplA-11R	TCAGATAGCCGAAAGCGACT
xplA-12F	CACGCGTCTACAACATACCC
SSD12R	AACAGCTCCTCACGGTAAGC
xplA13F	ATCGTCCTGTCTCTGAAAACC
AMP13R	GTTGTGGCAGGTGTTGAGTT
AmMP14F	ATCAGCGATGGGACGATAC
AMP14R	ATGCCAGGTGAACGTGT
AMP15F	GTGATGTACGAGGGCAAGC

and sequencing a nearly full-length (1,500-nucleotide) sequence of the 16S rRNA gene. PCR amplifications were performed with Phusion high-fidelity DNA polymerase (NEB), according to the manufacturer's recommendations, by using primers 7f (5'-AGAGTTTGATYMTGGCTCAG-3') and 1510r (5'-ACGGYTACCTTGTACGACTT-3') (22). The 16S rRNA gene sequences were submitted to the NCBI database (Table 1). Alignment and tree construction were performed as described previously by Andeer et al. (6), using MEGA software version 6 (23).

Analysis of the *xplAB* gene cluster. A cosmid library of *R. rhodochrous* 11Y DNA was constructed by using the pWEB cosmid vector kit (Epicentre) and phage T1-resistant *Escherichia coli* EPI100-T^R according to the instructions provided by the manufacturer. Primers used for sequencing are listed in Table 2.

Construction of knockout strains. Using the primers listed in Table 3, regions of *aroP*, *marR*, *xplB*, and *xplA* were amplified from *R. rhodochrous* 11Y to produce PCR products with EcoRI and NdeI restriction sites for the upstream regions and NdeI and HindIII restriction sites for the downstream regions. Each pair of upstream and downstream regions was cloned together into EcoRI- and HindIII-digested pK18mobsacB to produce the mutagenic plasmids p11YaroP, p11YmarR, p11YxplB, and p11YxplA. The mutagenic plasmids were transformed into *E. coli* S17-1 and knockouts in the *marR*, *aroP*, *xplA*, and *xplB* genes created following recombination with homologous regions on either side of each gene of interest in *R. rhodochrous* 11Y (24). Sucrose-sensitive transconjugants were grown in Luria-Bertani (LB) medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl) overnight (16 h) to induce the second crossover and then screened on agar containing 10% sucrose. Sucrose-sensitive *Rhodococcus* mutants were identified following screening on complex medium agar containing 10% (wt/vol) sucrose. Incomplete crossover events were removed following screening on sucrose with 50

TABLE 3 Primers used for construction of *Rhodococcus rhodochrous* 11Y knockout strains^a

Primer	Sequence (5'–3')
USF- <i>aroP</i>	ACGTCGGAATTCAGTGTCCGGGCGGACC
USR- <i>aroP</i>	GAATTCCATATGGGACGTATAACCGAG
DSF- <i>permease</i>	ATTCCGCATATGAAGAAGACCGGGGAG
DSR- <i>permease</i>	GCGCAGAAGCTTCTCGGGCAGATCGAC
USF- <i>marR</i>	GGGGAATTCGAGGATCGCCGTGA
USR- <i>marR</i>	TGGTCCCATATGGCCCCGACACTCGACG
DSF- <i>marR</i>	CTGGCGCATATGTCAAAAAACCTAT
DSR- <i>marR</i>	ACAGACAAGCTTCGATCAGTGCGGCGG
USF- <i>xplA</i>	CGATCTGAATTCGCACCCGGACACCT
USR- <i>xplA</i>	ATTCTCCATATGGGGTTCACTGCAGGC
DSF- <i>xplA</i>	GTCCTGACATATGATCCGATCTCACCTGCC
DSR- <i>xplA</i>	CCCAAGCTTGACGGTCCAGGGCGTTGT
USF- <i>xplB</i>	ACCATGGAATTCACGAACATCAGAGCT
USR- <i>xplB</i>	GTCGAGCATATGCACGTCGGGGATACG
DSF- <i>xplB</i>	ACCCGACATATGACACCCGAGATGGAG
DSR- <i>xplB</i>	CGTCCAAAGCTTCCAGTTTCGGCGTGA

^a USF, upstream forward; USR, upstream reverse; DSF, downstream forward; DSR, downstream reverse.

μg/ml kanamycin. Gene deletions in sucrose-resistant, kanamycin-sensitive mutants were confirmed by PCR using gene-specific primers listed in Table 3 and spanning each putatively deleted gene.

Growth and RDX uptake measurements. Bacteria were initially grown for 1 to 3 days at 30°C on RDX dispersion agarose plates containing 2 mM RDX, minimal medium (1.55 g/liter KH₂PO₄, 4.95 g/liter K₂HPO₄, 10 mM glycerol, 5 mM glucose, 5 mM succinate, trace elements [pH 7.2] [25]), and 1.5% (wt/vol) agarose. Colonies were then transferred onto agar plates of LB medium and grown at 30°C for 2 days. Cell biomass was harvested, and 5 mg in 50 μl of phosphate buffer (pH 7.2) was inoculated into each 250-ml conical flask containing 50 ml minimal medium with 100 μM RDX as the sole source of nitrogen. The flasks were incubated at 30°C with shaking at 180 rpm. Samples were taken at intervals, the absorbance at an optical density at 600 nm (OD₆₀₀) was recorded, and RDX levels were measured by using high-performance liquid chromatography (HPLC) (9).

Resting-cell assays. Bacteria were grown at 30°C to an OD₆₀₀ of 0.3 in minimal medium supplemented with 5 mM KNO₃, 750 μM KNO₂, 750 μM KNO₃, or 250 μM RDX as a nitrogen source, as described in Table 4, and the cells were collected by centrifugation. The cells were then resuspended in minimal medium containing 5 mM KNO₃, 450 μM KNO₂, 450 μM KNO₃, no nitrogen, or 150 μM RDX, as described in Table 4, and grown for a further 3 h; cells were then harvested by centrifugation. Cells were washed twice in 40 mM phosphate buffer (pH 7.2) and resuspended at a concentration of 0.1 g (wet weight)/ml. Resting-cell assays were performed at 30°C with shaking at 180 rpm, using 50 μl of cells in a total volume of 700 μl containing 40 mM phosphate buffer (pH 7.2), 100 μM RDX, and 100 μg/ml kanamycin. Samples of 100 μl were taken at each time point, each reaction was then stopped by the addition of 10 μl 1 M trichloroacetic acid, and RDX levels were measured by using HPLC (9).

Western analysis and activity assays. Cells were prepared as described above for resting-cell assays and then disrupted by sonication and centrifuged (10,000 × g) at 4°C for 30 min. For Western blot analysis, 25 μg of supernatant protein was loaded per lane. Anti-rabbit polyclonal antibodies to the XplA and XplB proteins were prepared by using purified XplA and XplB proteins, respectively, in rabbit.

Activity of XplA in cell extracts was determined by using 75 μl of cell extract, 300 μM NADPH, 0.1 U/ml spinach ferredoxin reductase (Sigma), and 100 μM RDX, in a final volume of 150 μl. Reactions were performed aerobically at 20°C, and NADPH oxidation was measured by the A₃₄₀.

Gene expression analysis. Total RNA was extracted by using an RNeasy kit (Qiagen). cDNA synthesis and quantitative PCR (qPCR) anal-

TABLE 4 Growth conditions used for resting-cell assays and *xplA* expression studies of *Rhodococcus rhodochrous* 11Y

Treatment	Growth condition overnight (16 h)	3-h growth condition
a	5 mM NH ₄ Cl	5 mM NH ₄ Cl
b	750 μM KNO ₃	5 mM KNO ₃
c	750 μM NH ₄ Cl	450 μM NH ₄ Cl
d	750 μM KNO ₃	450 μM KNO ₃
e	750 μM KNO ₂	450 μM KNO ₂
f	450 μM RDX	150 μM RDX
g	750 μM KNO ₃	No additional nitrogen source

ysis were conducted as described previously (9). Primer sequences for *xplA* were 5'-CCGAGTGGGCGCAACAGT-3' and 5'-TCCTCCTCGTC GAGTTCGAT-3', and those for DNA gyrase subunit B (*gyrB*) were 5'-G CCGAGGAGCAGGAACAG-3' and 5'-TAGTGGTAGACGCGGGTCTT G-3'. Relative expression values were calculated by using *gyrB* as an internal reference.

Testing of putative transcription initiation sites. Rapid amplification of cDNA ends (RACE) PCR was performed by using a SMARTer RACE kit (Clontech Laboratories, Inc.). Total RNA was isolated from *R. rhodochrous* 11Y by using an RNeasy kit (Qiagen), and first-strand cDNA synthesis was carried out according to the manufacturer's protocol. RACE PCR was performed by using Universal Primer Mix (Clontech) in combination with the *Rhodococcus*-specific primer 5'-GGCGACCATCACCA CACCACACAG-3'. Nested PCR was then performed by using Universal Primer A (Clontech) and the nested *Rhodococcus*-specific primer 5'-ATG GTGACGAGCAGCGCGATCAGATAC-3'. The PCR products were cloned into TOPO pCR2.1 (Invitrogen) and sequenced. Primer extension analysis was performed as described previously (16). Reverse transcription was carried out with a volume of 20 μl using 20 μg total RNA, 400 U Superscript III reverse transcriptase (Invitrogen), 5 nM primer (5'-FAM [6-carboxyfluorescein]-CATAGGCGAAGACCGTGTAGATCCCCGAC-3'), and 750 μM dATP, dCTP, dTTP, and dGTP at 55°C for 1.5 h. The reverse transcription reaction was repeated as described previously (26). Primer extension products were mixed with 0.3 μl the Genescan LIZ600 ladder and run on the AB3130XL instrument (Applied Biosystems), using default parameters. Results were analyzed by using Peak Scanner Software (Applied Biosystems).

Nucleotide sequence accession numbers. The GenBank accession numbers for *Rhodococcus* sp. BL1, DS1, 5U, and 9UK sequences are KF700363, KF933858, KJ566616, and KJ566615, respectively.

RESULTS

Analysis of the *xplAB* gene cluster in RDX degraders. Following selective enrichments on contaminated soil from the United Kingdom, Belgium, and Ukraine, four *Rhodococcus* isolates were obtained (Table 1). Phylogenetic analysis of the 16S rRNA gene sequences shown in Fig. 1 was used to compare the four *Rhodococcus* isolates with other members of the *Corynebacterineae*. The new isolates are all closely related to *R. rhodochrous* 11Y, whereas the presence of the *xplAB* genes is distributed more widely across the *Corynebacterineae* suborder. While not infallible, a commonly used guideline for simple taxonomy of bacteria using 16S rRNA gene sequences is that percent identity scores of ≥97% and ≥99% can be used to classify isolates to the genus and species levels, respectively. Alignment of the regions of 16S rRNA gene sequences available for all seven *Rhodococcus* isolates presented in this study (a 1,324-bp region for *R. rhodochrous* 11Y and *R. rhodochrous* YH1 and a 1,323-bp region for the remaining isolates) revealed that *R. rhodochrous* YH1 shares 98.87% identity with *R.*

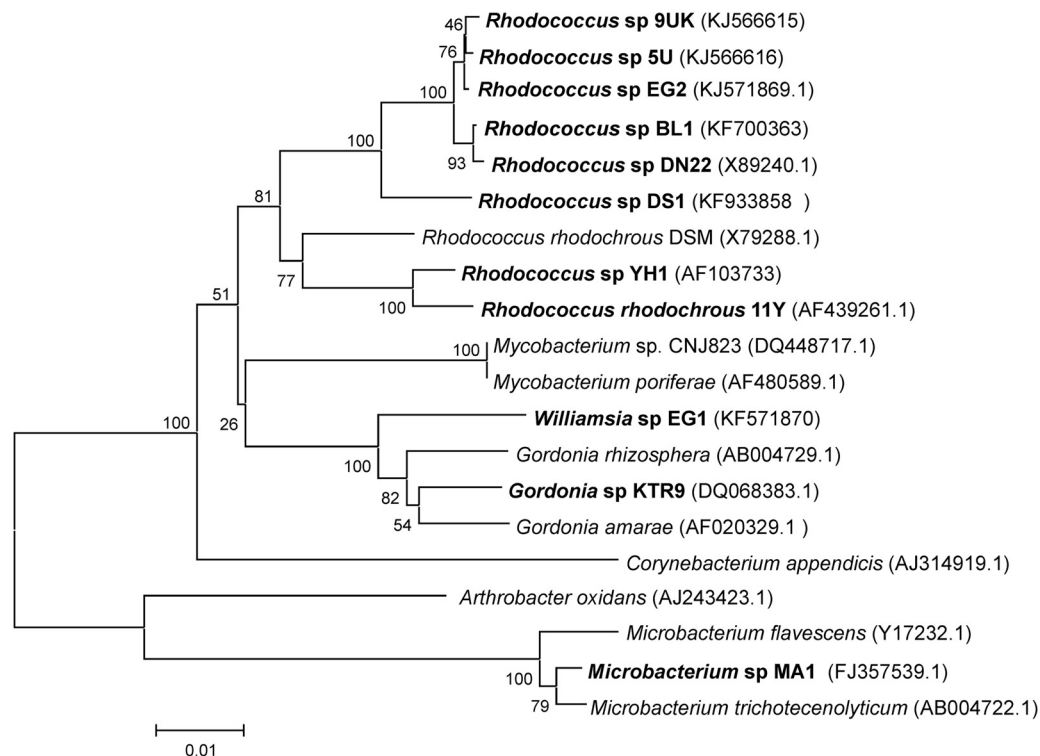


FIG 1 Phylogenetic analysis based on 16S rRNA gene sequences of 20 bacterial species. Sequences were analyzed by using the neighbor-joining method, bootstrapped with 1,000 replicate runs, using the MEGA 6 tree-building program. RDX-degrading species are in boldface type, and GenBank accession numbers are listed in parentheses after species names. The bar indicates substitutions per nucleotide.

rhodochrous 11Y, whereas the remaining 16S sequences share between 95.31 and 95.62% identity with *R. rhodochrous* 11Y. These alignments suggest that all seven *Rhodococcus* isolates are separate species.

The *xplAB* gene cluster sequence was determined for these species as well as for previously isolated RDX-degrading bacteria, including *Microbacterium* sp. MA1 (6); *Williamsia* sp. strain EG1 (8); *Rhodococcus* sp. strain EG2 (8); *Gordonia* sp. KTR9 (4); and three *Rhodococcus* species isolates from the United Kingdom (3), Israel (*Rhodococcus* sp. YH1), and Australia (7). Sequence analysis revealed that these 11 species all contained near-identical copies of the *xplAB* genes. Further sequencing of *R. rhodochrous* 11Y and

Microbacterium sp. MA1 revealed a 14-kb gene cluster encoding 12 genes (Fig. 2), including *xplAB*, with the ORFs pMA1.022 and pMA1.034 marking the borders. This region is almost identical between *R. rhodochrous* 11Y and *Microbacterium* sp. MA1. The exceptions are that a transposable element upstream of *xplAB* (pMA1.028) in *Microbacterium* sp. MA1 is absent from *R. rhodochrous* 11Y and a cyclic GMP, adenylyl cyclase, FhlA (GAF) domain in the transcription regulatory protein of *R. rhodochrous* 11Y is truncated in the pMA1.022 ORF in *Microbacterium* sp. MA1. Subsequent PCR analysis showed that the same ORFs were present in all eight *Rhodococcus* species. At the nucleotide level, there are only six nucleotide polymorphisms between *R. rhodochrous*

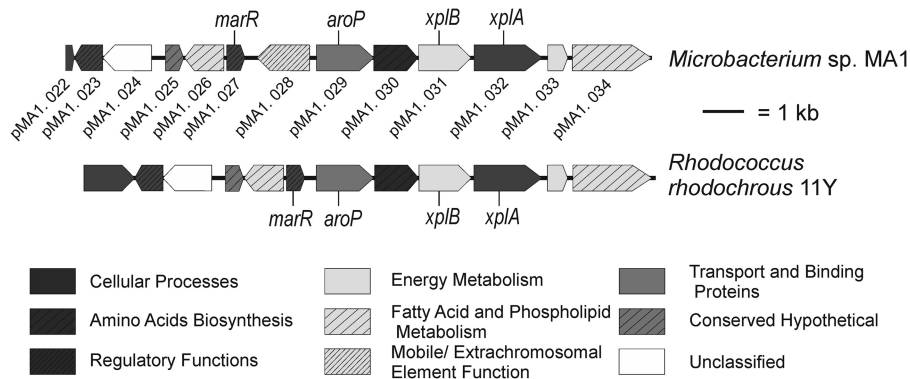


FIG 2 Alignment of the *xplAB*-containing gene cluster from *Rhodococcus rhodochrous* 11Y with *Microbacterium* sp. MA1 and classification of gene products therein.

TABLE 5 Amino acid residues in XplA and XplB that differ from *Rhodococcus rhodochrous* 11Y

Protein and species	Amino acid position from first Met	Encoded amino acid	Amino acid encoded in <i>Rhodococcus rhodochrous</i> 11Y
XplA			
<i>Rhodococcus</i> sp. EG2	13	Gly	Ala
<i>Gordonia</i> sp. KTR9	53	Asn	Asp
<i>Rhodococcus</i> sp. 5U			
<i>Microbacterium</i> sp. MA1			
<i>Rhodococcus</i> sp. EG2			
<i>Microbacterium</i> sp. MA1	393	Val	Ala
<i>Rhodococcus</i> sp. BL1	441	Ala	Val
<i>Microbacterium</i> sp. MA1			
<i>Rhodococcus</i> sp. DS1	551	Leu	Cys
<i>Rhodococcus</i> sp. DS1	551	Ser	Pro
XplB			
<i>Gordonia</i> sp. KTR9	1	Gly	Met
<i>Microbacterium</i> sp. MA1	172	Phe	Ile
<i>Gordonia</i> sp. KTR9	383	Trp	Ser

11Y and *Microbacterium* sp. MA1 across the 14-kb region, four of which are located in the *xplAB* region. Across all 11 species, the *xplAB* region is highly conserved (*Rhodococcus* sp. DS1 and YH1 are identical, and the remaining nine species share 99.87 to 99.99% identity with *R. rhodochrous* 11Y). The encoded amino acids that differ from the *xplAB* region in *R. rhodochrous* 11Y are presented in Table 5. The glutamine synthetase-*xplB* fusion found in *Gordonia* sp. KTR9 (4) was not found in any of the other RDX-degrading bacteria examined in this study.

Growth and RDX uptake by *Rhodococcus rhodochrous* 11Y and related isolates. The ability of the four newly isolated *Rhodococcus* species and seven previously reported species to grow in minimal medium with RDX as the sole source of nitrogen was tested. Figure 3A and C show that all the *Rhodococcus* species were able to grow in this medium. *Rhodococcus rhodochrous* 11Y had the highest growth rate, achieving an OD₆₀₀ of 0.348 in 16 h. At the same 16-h time point, *Rhodococcus* sp. strains EG2, BL1, 9UK, YH1, 5U, DN22, and DS1 achieved OD₆₀₀ densities that were 67.9, 60.8, 60.2, 55.4, 55.1, 52.3, and 32.4%, respectively, of the density of *R. rhodochrous* 11Y. These growth rates correlated with the rate of RDX removal from the medium, with the same *Rhodococcus* species removing 100, 100, 97, 93, 79, 65, 65, and 57%, respectively, of the RDX after 16 h (Fig. 3B and D).

The remaining species all exhibited lower rates of growth than did *R. rhodochrous* 11Y, with *Williamsia* sp. EG1 growing faster

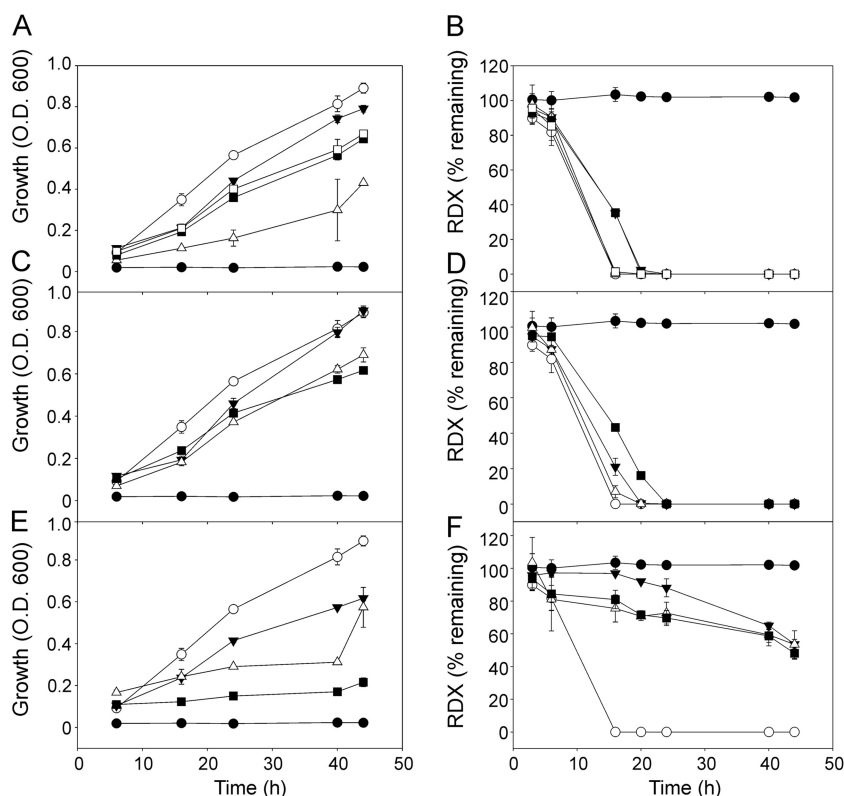


FIG 3 Growth of and RDX removal by *Rhodococcus rhodochrous* 11Y and related isolates. (A and B) Growth (A) and removal of 100 μ M RDX (B) for the no-bacterium control (●), *R. rhodochrous* 11Y (○), *Rhodococcus* sp. BL1 (▼), *Rhodococcus* sp. DS1 (△), *Rhodococcus* sp. 5U (■), and *Rhodococcus* sp. 9UK (□). (C and D) Growth (C) and removal of 100 μ M RDX (D) for the no-bacterium control (●), *R. rhodochrous* 11Y (○), *Rhodococcus* sp. YH1 (▼), *Rhodococcus* sp. DN22 (△), and *Rhodococcus* sp. EG2 (■). (E and F) Growth (E) and removal of 100 μ M RDX (F) for the no-bacterium control (●), *R. rhodochrous* 11Y (○), *Williamsia* sp. EG1 (▼), *Microbacterium* sp. MA1 (△), and *Gordonia* sp. KTR9 (■). Data shown are means of data from three biological replicates \pm standard deviations.

than *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9 having the lowest growth rate (Fig. 3E). Over the first 16 h of incubation, *Williamsia* sp. EG1, *Microbacterium* sp. MA1, and *Gordonia* sp. KTR9 reached OD₆₀₀ densities that were 69.4, 67.9, and 35.1%, respectively, of that reached by *R. rhodochrous* 11Y. As with the *Rhodococcus* species, these growth rates correlated approximately with the rate of RDX uptake (Fig. 3F): after 16 h, the level of RDX in the medium containing *Williamsia* sp. EG1 was not significantly different from the level of RDX in the no-bacterium control samples, and *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9 had removed only 25 and 19%, respectively, of the RDX.

RDX degradation by wild-type *R. rhodochrous* 11Y. In order to determine whether *xplA* expression and activity are induced by the presence of RDX or low nitrogen availability, *R. rhodochrous* 11Y was grown with the range of nitrogen treatments listed in Table 4. Resting-cell assays were then performed to monitor RDX removal. The growth of cells in 5 mM KNO₃ or NH₄Cl was measured as the OD₆₀₀ and was shown to be linear under the conditions used here, indicating that these nitrogen treatments did not limit growth (data not shown). As shown in Fig. 4A, cells grown under nitrogen-limiting conditions (treatments c, d, e, and f) or incubated with no nitrogen (treatment g) removed RDX faster than did cells grown under nonlimiting nitrogen conditions (treatments a and b). When cells were grown with RDX (treatment f) or in nitrogen-limiting NH₄Cl (treatment c) for 2 h, the subsequent rate of RDX removal was not significantly different between the two treatments. However, cells from both treatments f and c removed RDX faster than did cells grown in the alternative, nitrogen-limited regime of KNO₂ or KNO₃. To investigate the effect of the different nitrogen regimes on gene expression, *xplA* transcript abundance was measured by qPCR over a treatment time of 2 h. As shown in Fig. 4B, relative to treatment with no nitrogen (treatment g), there was little change in *xplA* expression levels with treatment with KNO₂ or KNO₃. In the cells supplied with RDX (treatment f), there was a 50% increase in *xplA* transcript levels relative to levels in cells cultured in medium without nitrogen.

Western blots of proteins from resting-cell assays 2 h after incubation were probed with XplA and XplB antibodies (Fig. 4C). In agreement with the *xplA* expression levels, the strongest signal was seen for cells supplied with RDX as the sole nitrogen source (treatment f). The XplA levels detected in the remaining treatments were approximately similar to each other, and this pattern was also seen on replicate blots (data not shown). To relate protein levels with XplA activity, crude extracts were assayed by monitoring the oxidation of NADPH at 340 nm. The activity of XplA in cells supplied with RDX as the sole nitrogen source (treatment f) was 6.9 ± 0.5 nmol NADPH · mg⁻¹ · min⁻¹. No XplA activity was detected for the treatments with the alternative nitrogen regimes tested (treatments b, d, and e).

Characterization of the knockout strains. To study the relative contributions of XplA, XplB, AroP, and MarR to RDX degradation, knockout strains (*xplA*, *xplB*, *aroP*, and *marR*) were successfully generated by using the unmarked gene deletion system (pK18mobsacB) (24, 27). Each gene deletion was confirmed by PCR using primers flanking the deleted region (data not shown). Sequencing confirmed the removal of target sequences shown in Fig. 5A and confirmed that coding sequences of the neighboring genes were unaltered, with the exception of the *xplB* knockout,

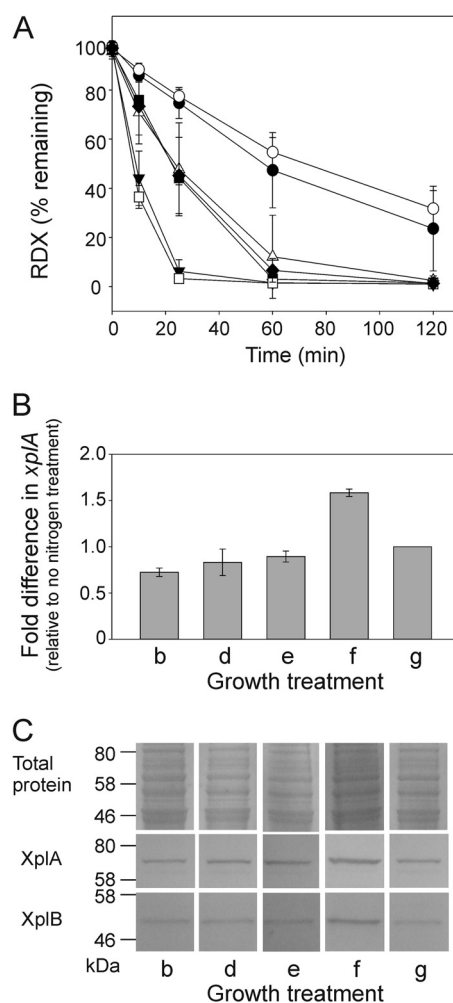


FIG 4 Effect of nitrogen sources on subsequent RDX removal and XplA expression in *Rhodococcus rhodochrous* 11Y. (A) Removal of 100 μ M RDX was measured in resting-cell assays. Growth conditions were as follows: overnight in 5 mM NH₄Cl and then 3 h in 5 mM NH₄Cl (treatment a) (●), 750 μ M KNO₃ and then 5 mM KNO₃ (treatment b) (○), 750 μ M NH₄Cl and then 450 μ M NH₄Cl (treatment c) (▼), 750 μ M KNO₃ and then 450 μ M KNO₃ (treatment d) (△), 750 μ M KNO₂ and then 450 μ M KNO₂ (treatment e) (■), 250 μ M RDX and then 150 μ M RDX (treatment f) (□), and the no-nitrogen control (treatment g) (◆). Data shown are means of data from three biological replicates \pm standard deviations. (B) Expression levels of *xplA* in resting cells after 2 h. Transcript levels were normalized to *gyrB* expression levels and expressed relative to values for treatment d. Results are standard errors of data from four biological replicates. (C) Total protein gel stained with Coomassie and Western blots of protein levels in resting cells after 2 h. Membranes were probed with XplA and XplB antibodies.

which removed an additional 131 bp from the 3' end of the homologous ORF pMA1.030 in *R. rhodochrous* 11Y.

Computational analysis of bacterial promoter elements using BPROM (Softberry, Inc.) revealed the presence of *E. coli*-characterized -10 and -35 hexamer sequences upstream of *aroP* and *xplB* (Fig. 5B). Primer extension analysis and RACE PCR confirmed a transcription start site upstream of *aroP*. No products were obtained for the putative promoter region upstream of *xplB*.

The wild-type and knockout strains were grown in 150 μ M RDX (treatment e) prior to resting-cell incubations. The results shown in Fig. 6A demonstrate that the rate of RDX removal by the

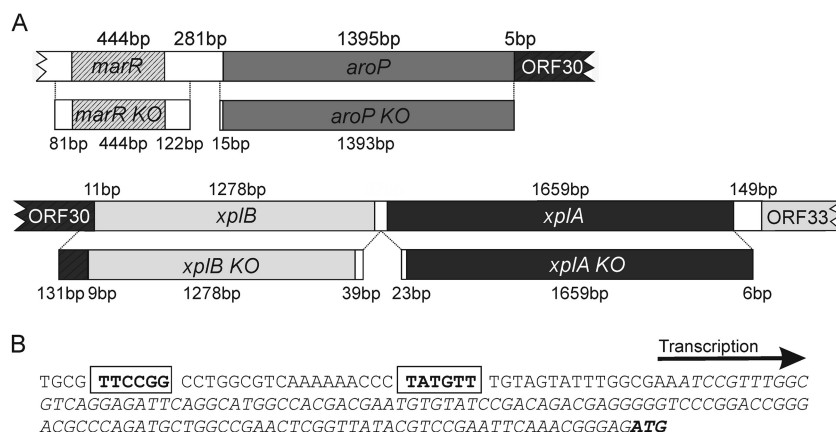


FIG 5 Creation of gene knockouts in *Rhodococcus rhodochrous* 11Y. (A) Scaled diagram showing DNA regions deleted in *xplA*, *xplB*, *marR*, and *aroP* knockout (KO) strains. Noncoding regions are represented by white boxes. (B) Transcription start site and open reading frame of *marR* predicted by RACE PCR and primer extension analyses. Boxes represent the -10 and -35 regions of the *marR* promoter, the transcribed region is in italic type, and the ATG start codon in boldface type.

marR and *aroP* knockout strains was not significantly different from that for wild-type *R. rhodochrous* 11Y cells, with all three strains removing all RDX within 1 h. In comparison to this, the *xplA* knockout was unable to remove RDX from the medium, and the *xplB* knockout was impaired in RDX removal, removing only 23% of the RDX in 1 h. To investigate whether the *aroP*-encoded permease had a higher affinity for RDX at lower RDX concentrations, the *aroP* knockout and wild-type strains were incubated in minimal medium containing 40 μ M RDX over 8 h of incubation. There were no differences in RDX degradation between these strains (data not shown). On Western blots of crude extracts probed with XplA and XplB antibodies (Fig. 6B), as expected, there was no XplA signal from the *xplA* knockout cells and no XplB signal from the *xplB* knockout cells. However, the reciprocal levels of XplA and XplB were also decreased in these strains, a result that was consistently observed across replicate blots (not shown). Signals for XplA and XplB in the *marR* and *aroP* knockout lines were not significantly different from wild-type levels.

Agarose containing RDX at concentrations above its aqueous solubility limit (180 μ M at 25°C) has an opaque appearance due to crystals of RDX. When wild-type *R. rhodochrous* 11Y was grown on these RDX dispersion plates, a zone of clearance appeared around the bacterial colonies as a result of RDX removal from this region. To determine whether XplA activity is involved in the removal of RDX from the agar, the *xplA* knockout strain was grown on RDX dispersion agar supplemented with 250 μ M the alternative nitrogen source NH_4Cl , KNO_3 , or NaNO_2 . While the expected zone of clearance was observed around colonies of wild-type *R. rhodochrous* 11Y growing on RDX dispersion plates, there was no zone of clearance around *xplA* knockout colonies grown on agar supplemented with 250 μ M NH_4Cl , KNO_3 , or NaNO_2 (Fig. 6C).

DISCUSSION

The *xplAB*-containing gene cluster. Here we demonstrated that 11 RDX-degrading bacterial species isolated from four different continents around the world, including 3 new isolates presented here, share near-identical *xplAB* sequences. The *xplAB* genes are part of a 14-kb gene cluster that is almost identical between *Rhodococcus* sp. and *Microbacterium* sp. MA1, isolated from RDX-

contaminated soils in the United Kingdom (3) and the United States (6), respectively. The highly conserved nature of this gene cluster between two different genera of actinomycetes indicates that genes have been moved by horizontal gene transfer. Conjugal transfer of RDX degradation among actinomycete bacteria *in vitro* has been reported (28). The environment has been exposed to RDX, a synthetic compound, for less than a century, and the evolution of its RDX-metabolizing activity is unclear. No alternative substrates have yet been identified for XplA. The closest match to XplA in the NCBI sequence database, an uncharacterized cytochrome P450 with a fused flavodoxin domain in *Gordonia terrae* strain NBRC 100016, shares only 46% identity with XplA and lacks detectable RDX-metabolizing ability.

Growth and RDX uptake by *Rhodococcus rhodochrous* 11Y and related isolates. It is interesting that, despite having high 16S percent identity scores and containing the *xplAB* gene cluster, there is still considerable range in the growth and RDX uptake rates among the *Rhodococcus* species presented here. For example, *Rhodococcus* sp. DS1 grew on medium containing RDX as the sole nitrogen source, at about half the rate of *R. rhodochrous* 11Y. Variation in growth rates was also observed between *R. rhodochrous* 11Y and species in the other four genera tested: *Williamsia* sp. EG1, *Microbacterium* sp. MA1, and *Gordonia* sp. KTR9. The growth and transformation rates for *Gordonia* sp. KTR9 are not consistent with previously reported rates for these species (4, 13); this may be due to the different growth conditions used in those studies. In this study, the bacteria were initially grown on LB medium, to increase biomass, prior to transfer into minimal medium. While it is possible that the growth conditions used in this study might have delayed growth of KTR9, these growth conditions were identical for all bacteria.

Regulation of the *xplAB* gene cluster. While the regulation of XplA has not yet been elucidated, resting-cell incubations of *R. rhodochrous* 11Y have shown that although activity toward RDX is present in the absence of RDX, activity significantly increased when cells were grown with RDX supplied as the sole nitrogen source (3). Previous reports have shown that the degradation of RDX by *Rhodococcus* sp. YH1 and *Gordonia* sp. KTR9 was inhibited when the cells were grown in the presence of nonlimiting

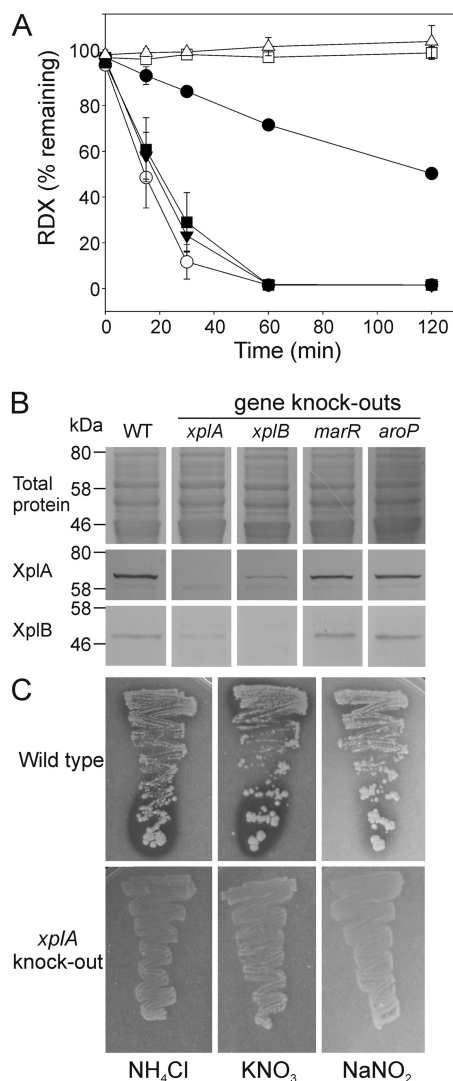


FIG 6 RDX removal, protein expression, and colony appearance of *Rhodococcus rhodochrous* 11Y wild-type and knockout strains. (A) Removal of 100 μ M RDX was measured in resting-cell assays. ■, wild type; □, *xplA* knockout; ●, *xplB* knockout; ○, *aroP* knockout; ▼, *marR* knockout; △, no-bacterium control. Data shown are means of data from three biological replicates \pm standard deviations. (B) Western blot analysis of crude cell extracts from wild-type (WT) and *xplA*, *xplB*, *marR*, and *aroP* knockout strains grown overnight in 750 μ M KNO₃ and then for 3 h in 150 μ M RDX. The total protein gel was stained with Coomassie. Membranes were probed with XplA and XplB antibodies. (C) Wild-type *R. rhodochrous* 11Y and the *xplA* knockout strain on dispersion plates containing 5 mM RDX after 14 days at 30°C.

concentrations of alternative nitrogen sources, suggesting that the expression of XplA is linked to central nitrogen metabolism (13, 21). Our results have shown that the level of RDX removal was higher when the cells were starved of nitrogen or grown under nitrogen-limiting conditions than when cells were grown with excess nitrogen, indicating that XplA expression in *R. rhodochrous* 11Y is regulated by nitrogen availability. In agreement with our findings, previous studies using *Gordonia* sp. KTR9 and a global nitrogen regulation (*glnR*) mutant of KTR9 indicated that nitrogen availability plays a role in the regulation of *xplA* (29). Beyond this conclusion, our studies also show that RDX removal was further increased in cells grown in RDX prior to resting-cell assays,

and under these conditions, XplA protein and activity levels increased correspondingly. These results indicate that XplA expression in *R. rhodochrous* 11Y could be further regulated by RDX availability. There are differences in the genetic components and arrangements of the *xplAB* gene cluster in *Gordonia* sp. KTR9 (13) compared with *R. rhodochrous* 11Y that may account for differences in the regulation of RDX degradation.

pMA1.027 is characterized as a regulator with 62% amino acid identity to MarR, a multiple-antibiotic-resistance regulator in *Gordonia bronchialis* DSM 43247. The family of MarR transcriptional regulators is involved in the regulation of a wide variety of cellular processes, including detoxification of aromatic aldehydes (30) and quinones (31) and biodegradation of aminophenol (32). The MarR family regulators have the ability to bind chemical compounds such as 2,4-dinitrophenol or salicylate (33). Under the conditions tested in this study, RDX uptake rates were not significantly different between the *marR* knockout and the wild type. This finding indicates that MarR is not involved in a signaling response to RDX. In parallel to *R. rhodochrous* 11Y, a gluconate R-type transcriptional regulator gene (*xplR*) is present in the *xplAB* gene cluster of *Gordonia* sp. KTR9. In agreement with our findings, studies of an *xplR* knockout demonstrated that XplR is not involved in RDX degradation (29). Studies of the *xplAB*-containing gene cluster in *Gordonia* sp. KTR9 identified -10 and -35 regions upstream of *xplR*. These regions were also identified upstream of a cytochrome P450 (*cyp151C*), along with a gluconate R binding site 5 bp upstream of the *cyp151C* -35 region (13). The *cyp151C*-*glnA*-*xplB*-*xplA* gene cluster in *Gordonia* sp. KTR9 has short intergenic spaces, with *xplR* being located approximately 1.5 kb upstream of the cluster. This organization suggests an operon that is regulated by XplR, with *cyp151C*, *glnA*-*xplB*, and *xplA* being transcribed as a single cistron (13). Such an arrangement has parallels with *R. rhodochrous* 11Y, where *marR* is located 281 bp upstream of *aroP*, while there are only 5, 9, and 62 bp separating *aroP* and pMA1.030 (a putative dihydrodipicolinate reductase gene), *xplB*, and *xplA*, respectively, with the latter four genes lacking Rho-independent terminator sequences. During our primer extension and RACE PCR analyses, a transcription start site upstream of *aroP* was detected.

Involvement of XplA and XplB in RDX catabolism. When *Gordonia* sp. KTR9 was cured of pGKT2, the plasmid carrying the *xplAB* gene cluster did not transform RDX, suggesting that *xplA* is essential for RDX degradation (13). Alongside this, expression of XplA in the non-RDX-catabolizing bacteria *E. coli*, *R. rhodochrous* CW25, and *Rhodococcus jostii* RHA1 conferred an RDX-catabolizing ability (3, 13). Our observation that the *xplA* knockout strain was unable to degrade RDX in resting-cell assays builds on these studies to demonstrate unequivocally that *xplA* and its gene product are necessary for RDX degradation and that there is no other RDX-degrading system present in *R. rhodochrous* 11Y. Furthermore, the absence of a zone of clearance around *xplA* knockout colonies grown on RDX dispersion agar establishes that the zone of clearance around colonies of wild-type *R. rhodochrous* 11Y is mediated by XplA activity: as RDX is degraded by XplA in cells of *R. rhodochrous*, a diffusion gradient of RDX in the surrounding agar occurs. This leads to solubilization of RDX crystals in the agar, and thus, a zone of clearance around the colonies develops.

In resting-cell assays, the *xplB* knockout strain showed a reduced rate of RDX removal compared to the wild type. Recombinant XplB has been shown to be the electron transfer partner for

XplA, transferring electrons from NADPH to XplA for RDX degradation (9) and accounting for the reduced level of RDX removal. However, significant XplA activity was observed for the *xplB* knockout, demonstrating that XplA is able to obtain reducing equivalents from another source in *R. rhodochrous* 11Y. Consistent with this, expression of XplA in non-RDX-catabolizing bacteria conferred an RDX-catabolizing ability (3, 13), demonstrating that one or more surrogate reductases present in the non-RDX degraders are able to partner with XplA. Moreover, the expression of *xplA* in *Arabidopsis* confers the ability to catabolize RDX (12), demonstrating that XplA can also utilize plant reductases. These observations also show that the transfer of *xplA* alone from donor to recipient naturally in the microbial environment would be sufficient to confer an RDX-degrading ability to the recipient.

Western blot analysis of crude extracts probed with XplA and XplB antibodies (Fig. 5C) demonstrated that levels of XplA and XplB were decreased in the *xplB* and *xplA* knockouts, respectively. When the *xplA* knockout was created, the open reading frame of *xplB* was not disrupted, and vice versa for the creation of the *xplB* knockout. It is possible that the mutations affected transcript stability or translational efficiency. Measurement of transcript and protein levels of the surrounding ORFs would reveal if this was the case. Alternatively, posttranslational coregulation of XplA and XplB could occur.

Route of RDX uptake. The *aroP* gene shares 76% sequence identity with an amino acid permease from *Mycobacterium vanbaalenii* PYR-1. Although studies of *Gordonia* sp. KTR9 show that bacteria do not require a dedicated transporter for RDX uptake (13), bacterial genes encoding related functions are frequently situated close to each other (14, 34, 35). Therefore, we speculated that the *aroP*-encoded permease might also be involved in the transport of RDX. However, resting cell assays performed on the *aroP* knockout strain showed that the rate of RDX removal was not significantly different from that of the wild-type (data not shown).

In summary, we have shown that *xplAB* genes are highly conserved among all four known genera of RDX-degrading bacteria isolated from different geographical locations. These genes are part of a larger gene cluster that is highly conserved between *R. rhodochrous* 11Y and *Microbacterium* MA1. Expression of XplA is induced under nitrogen-limiting conditions and can be further enhanced by the presence of RDX. We established that MarR must be controlled by nitrogen availability and is unlikely to sense RDX levels, as there was no difference in RDX removal between the *marR* knockout and wild-type cells when grown in RDX. Further experiments are required to fully understand the regulation of XplA by RDX and the involvement of RDX in the regulation of nitrogen metabolism.

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