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Investigating differences in the ability of XpIA/B-containing bacteria to degrade the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)

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4	Authors: Dana Khdr Sabir ^{1,2} Nicolas Grosiean ¹ Elizabeth L. Rylott ¹ Neil C. Bruce ^{1*}
т	Autoris. Dana Khar Saon , Alconas Grosjean , Enzadetn E. Ryfold , Hen C. Druce
5	¹ Centre for Novel Agricultural Products, Department of Biology, University of York,
6	Wentworth Way, York, YO10 5DD, UK
7	² Department of General Sciences, Charmo University, 46023 Chamchamal, Sulaimani,
8	Kurdistan Region- IRAQ
9	
10	*Correspondence to: Neil C. Bruce, Centre for Novel Agricultural Products, Department of
11	Biology, University of York, Wentworth Way, York, YO10 5DD, UK
12	Phone: +44 (0)1904 328777
13	Fax: +44 (0)1904 328801
14	E-mail: neil.bruce@vork.ac.uk
15	
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Page 3 of 31

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- 22 Differences in the ability of three bacterial strains to degrade RDX, an explosive and
- 23 environmental pollutant, were investigated using sequence and biochemical analyses.

25 Abstract

The xenobiotic hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a toxic explosive and environmental pollutant. This study examines three bacterial species that degrade RDX, using it as a sole source of nitrogen for growth. Although isolated from diverse geographical locations, the species contain near identical copies of genes encoding the RDX-metabolizing cytochrome P450, XplA, and accompanying reductase, XplB. Sequence analysis indicates a single evolutionary origin for *xplA* and *xplB* as part of a genomic island, which has been distributed around the world via horizontal gene transfer. Despite the fact that xplA and xplB are highly conserved between species, Gordonia sp. KTR9 and Microbacterium sp. MA1 degrade RDX more slowly than *Rhodococcus rhodochrous* 11Y. Both *Gordonia* sp. KTR9 and *Microbacterium* sp. MA1 were found to contain single base pair mutations in *xplB* which, following expression and purification, were found to encode inactive XplB protein. Additionally, the *Gordonia* sp. KTR9 XplB was fused to glutamine synthetase, which would be likely to sterically inhibit XplB activity. Although the glutamine synthetase is fused to XplB and truncated by 71 residues, it was found to be active. Glutamine synthetase has been implicated in the regulation of nitrogen levels; controlling nitrogen availability will be important for effective bioremediation of RDX.

42 Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a toxic explosive widely used in military
munitions. The use of RDX on military lands, coupled with its recalcitrance to
biodegradation, has resulted in the build-up of significant levels of pollution. This synthetic
nitramine, which has no known equivalent structure in nature, has been in the environment
for less than a century but nevertheless, microorganisms have evolved the ability to degrade it
(Rylott *et al.*, 2011). The enzymes involved in the aerobic biodegradation of RDX were first

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isolated in the UK from *Rhodococcus rhodochrous* 11Y and identified as an unusual
cytochrome P450, XplA, and accompanying flavodoxin reductase partner, XplB (Seth-Smith *et al.*, 2002). A number of aerobic RDX-degrading bacteria have been reported (Binks *et al.*,
1995, Coleman, 1998, Thompson *et al.*, 2005, Indest *et al.* 2007, Nejidat *et al.* 2008, Andeer *et al.*, 2009, Bernstein *et al.*, 2011,) from different geographical locations but all belonging to
the order Actinomycetales.

55 Within all the species tested, *xplA* has been detected, and found to have greater than 99 %

56 identity (Indest et al., 2007, Seth-Smith et al., 2008, Andeer et al., 2009, Bernstein et al.,

57 2011, Rylott *et al.*, 2011, Chong *et al.*, 2014). The highly conserved nature of *xplA* suggests

its rapid distribution by horizontal gene transfer (Seth-Smith *et al.*, 2008, Andeer *et al.*, 2009,

59 Rylott *et al.*, 2011, Chong *et al.*, 2014). Genes involved in xenobiotic catabolism are often

60 located on a mobile genetic element, accompanied by insertion elements, and are integrated

61 into the bacterial chromosomal or plasmid (Nojiri et al., 2004). In agreement with this, xplA

62 is plasmid-encoded in *Microbacterium* sp. MA1, *R. rhodochrous* 11Y (Andeer *et al.*, 2009)

and Gordonia sp. KTR9 (Indest et al., 2010). Partial sequence analysis of the plasmid

64 carrying *xplA* in *Microbacterium* sp. MA1 revealed that the gene is also associated with

65 transposable elements in this bacterium (Andeer *et al.*, 2009). Furthermore, a 6.7 kbp region

66 flanking *xplA* has been found that is nearly identical (> 99 %) between *Microbacterium* sp.

67 MA1 and *R. rhodochrous* 11Y (Andeer *et al.*, 2009).

68 The partnering reductase for XplA is XplB (Seth-Smith *et al.*, 2002), an NADPH-dependent

69 flavoprotein which contains one molecule of FAD as a cofactor and shares sequence

70 homology (and 27 % sequence identity) with the bovine adrenodoxin reductase FDXR (US

71 National Library of Medicine National Institutes of Health (NCBI) accession number

72 P08165.3). The reductase XplB is involved in the activation of the catalytic centre of XplA

via the transfer of electrons from NADPH to a flavodoxin domain fused to the N-terminal of
the P450 domain of XplA (Jackson *et al.*, 2007).

The role of XpIB in RDX degradation has been demonstrated in both *R. rhodochrous* 11Y (Chong *et al.*, 2014) and transgenic plant lines (Jackson *et al.*, 2007, Bui *et al.*, 2012). In *Gordonia* sp. KTR9 the 5' end of *xplB* is fused to a glutamine synthetase (GS)-encoding gene, *glnA* (Indest *et al.*, 2010), an arrangement that has not been found in any of the other RDX-degrading bacteria examined so far. Glutamine synthetase (EC 6.3.1.2) is an essential enzyme in nitrogen metabolism, catalysing the ATP-dependant production of glutamine from glutamate and ammonia.

Previously, we reported that although xplA and xplB are highly conserved amongst RDX-degrading bacteria, the ability of Gordonia sp. KTR9 and Microbacterium sp. MA1 to grow in minimal medium with RDX as the sole source of nitrogen was significantly less than that of R. rhodochrous 11Y. The ability of these bacteria to remove RDX from the medium correlated with growth rates (Chong *et al.*, 2014). It is possible that the fusion of *xplB* with glnA and/or additional changes in the genetic components and arrangements of the genes in this region may account for differences in the regulation of RDX degradation in *Gordonia* sp. KTR9 (Zhu et al., 2014).

90 Here we present analysis of the *xplA* and *xplB*-containing gene clusters from *R. rhodochrous*

91 11Y, Microbacterium sp. MA1 and Gordonia sp. KTR9, which reveal differences and

92 commonalities in the arrangement of genes; along with emphasis on the sequence of *xplB*,

93 and characterisation of the GS-XplB fusion from *Gordonia* sp. KTR9.

94 Materials and Methods

95 Genome sequencing

Page 7 of 31

FEMS Microbiology Letters

Whole genome sequencing was used to obtain the complete xplA/B genomic island of R. rhodochrous 11Y (NCBI acc. no. KY488543; Figure 1). To do this, total genomic DNA was extracted by lysing the cells in TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) containing 10 mg/ml lysozyme, 10 % SDS and 20 mg/ml proteinase K. Cell debris was centrifuged at 10000rpm and protein removed by phenol-chloroform extraction. Nucleic acids were precipitated with 3M sodium acetate and isopropanol, washed in 70% ethanol and resuspended in nuclease-free water. Prior to sequencing, the 16S rRNA region was sequenced to ensure that the DNA sample was from the correct species. Additionally, the *xplA* gene was amplified from the DNA sample to make sure that the plasmid had not been cured from the bacterial genome during the extraction process. The DNA sample was analysed using an Agilent TapeStation 2200 and sequenced using a Next Generation Sequencing platform Ion Torrent (Life Technologies). Raw sequence reads were assembled using Newbler, version 2.7 (Roche Diagnostics).

Construction of mutant strains

110 Point mutations in *xplB* and *glnA-xplB* were obtained using the QuikChangeII Site-Directed

111 Mutagenesis protocol (Agilent Technologies Inc.). To mutate Trp-386 to Ser in *R*.

rhodochrous 11Y XplB, primers *xplB* (W386S)-F 5' GTC GAT TTC GAC GGC TCG ATG

113 CGG ATC G-3' and *xplB* (W386S)-R 5'- CGT CGA TCC GCA TCG AGC CGT CGA AA-

114 3' were used. To mutate the Ser-385 to Trp in GS-XplB, primers *xplB* (S385W)-GS-F 5'-

115 GTC GAT TTC GAC GGC TGG ATG CGG ATC G-3' and *xplB* (S385W)-GS-R 5'-CGT

- 116 CGA TCC GCA TCC AGC CGT CGA AA-3' were used. To mutate Phe-172 to Ile in *R*.
- 117 rhododochrous 11Y XplB, primers xplB (F172I)-F 5' AAG CAG CCC GAC GAA ATC
- 118 ACC GGT TC-3' and *xplB* (F172I)-R 5' ATC GGA ACC GGT GAT TTC GTC GGG C-3'

119 were used. Mutations were confirmed by sequencing.

120 Cloning and expression

The glnA-xplB gene was amplified from Gordonia sp. KTR9 by PCR using primers pGEX-xplB-glnA-F 5'- GGTTCCGCGTGGATCCATGAGTACATCCGCGCTCG-3' and pGEX-xplB-glnA-R 5'- GTCGACCCGGGAATTCTCAGCAGACCGATTCGGCCG-3' and cloned at the *BamH*I and *EcoRI* restriction sites, using an In-Fusion® HD cloning system, into pGEX2T. The GS-XplB fusion protein was expressed in *Escherichia coli* BL-21 (DE3). The cells were grown at 37 °C to OD600 ~0.6, then induced with 0.5 mM IPTG supplemented with 50 µg/ml riboflavin and grown for 14 hours at 20 °C. All proteins were expressed and purified as described by Jackson et al. (2007), protein identities were confirmed by MALDI-MS sequence analysis.

130 Activity assays

Reductase activity of XplB homologs was determined using 75 µl of cell free extract or 100 μg purified protein, 50 mM potassium phosphate buffer (pH 6.8), 300 μM NADPH, 0.08 mg purified XpIA protein (Jackson et al., 2007) (0.65 mg for GS-XpIB assays) and 100 µM RDX, in a final volume of 1 mL. For this assay, R. rhodochrous 11Y XplA was first purified (Figure 2a), and its activity towards RDX verified using spinach ferredoxin reductase (Sigma-Aldrich), as reported previously (Rylott *et al.*, 2006). The reactions were initiated by the addition of 100 μ M of RDX at room temperature and time points samples stopped by the addition of 10 % (v/v) 1M trichloroacetic acid. Levels of RDX were measured using HPLC (Jackson *et al.*, 2007), and nitrite production using Griess reagent (Griess, 1879) as follows: To 180 μ l of sample, 50 μ L of 10 mg of sulfanilamide/ml in 0.68 M HCl was added and incubated for 5 minutes, and then 20 μ L of 10 mg of N-(1-naphthyl)-ethylenediamine dihydrochloride in water were added. Following mixing, and a further 10 min incubation at room temperature, absorbance at 540 nm was measured. Sodium nitrite (0 to 100 μ M nitrite)

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was used as a standard. Glutamine synthetase was measured spectrophotometrically, based on the method of Kingdon *et al.*, 1968 and using a pyruvate kinase and lactic dehydrogenase-linked assay that followed reduction of NADH at wavelength 340nm. Each 3 mL reaction mix cuvette contained 34.1 mM imidazole buffer pH 7.1, 102 mM sodium glutamate, 8.5 mM adenosine 5'-triphosphate, 1.1 mM phosphoenolpyruvate, 60 mM magnesium chloride, 18.9 mM potassium chloride, 45 mM ammonium chloride, 0.25 mM b-nicotinamide adenine dinucleotide, 28 units pyruvate kinase, 40 units L-lactic dehydrogenase and 0.4 - 0.8 unit glutamine synthetase. One enzyme unit will convert 1.0 µmole of L-glutamate to L-glutamine in 1 minute at pH 7.1 at 37°C with specific activity defined as number of units per mg protein.

154 Measurement of FAD

The amount of FAD cofactor bound to the XplB and GS-XplB proteins was measured following the method described by Aliverti et al., 1999. Protein was boiled at 100 °C, in the dark, for 20 min and precipitated protein removed by centrifugation at 13,000 rpm for 10 minutes. The UV-visible spectrum of the supernatant was recorded (200 to 600 nm) spectrophotometrically. FAD was also determined by HPLC analysis using a C18 column with a mobile phase of 5 mM ammonium acetate buffer, pH 6.5 (solvent A) and methanol (solvent B) and the following gradient: 5 minutes solvent A 85 %: solvent B 15 %; 20 minutes solvent A 25 %: solvent B 75 %; 5 minute solvent A 0 %: solvent B 100 %; 5 minutes solvent A 85 %: solvent B 15 %.). The flow rate was 0.75 ml/min and column temperature 30 °C. Commercially available FAD (Sigma) was used as a reference.

Results

166 The *xplA*/*B* gene clusters

Analysis of the putative *xplA/B* genomic islands in *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 and Gordonia sp. KTR9 is shown in Figure 1. Within a 53 kb region in R. rhodochrous 11Y, there are 13.8 kb and 11.8kb gene clusters (termed the A and B regions respectively). Further downstream, is a 570 bp sequence encoding a transposable element (termed the C region). While the A region, which contains *xplA* and *xplB*, is highly conserved between *R*. rhodochrous 11Y and Microbacterium sp. MA1, Gordonia sp. KTR9 shares homology only with 3.1 kbp of the A region, although this includes *xplA* and *xplB*. Conversely, the B and C regions are highly conserved between R. rhodochrous 11Y and Gordonia sp. KTR9, but absent from *Microbacterium* sp. MA1. Two additional regions, (termed D1 and D2) are nearly identical between *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9; yet absent from *R. rhodochrous* 11Y. Scattered within these gene clusters are a number of transposable elements, indicative of mobility within and between these regions. Microbacterium sp. MA1 XplB The only difference between the *R. rhodochrous* 11Y and *Microbacterium* sp. MA1 XplB sequences is that *R. rhodochrous* 11Y contains a Phe-172, which is conserved across a range of RDX-degrading bacterial genera, whereas *Microbacterium* sp. MA1 contains an Ile-172 (Table 1). In R. rhodochrous 11Y XplB, a protein-bound flavin (FAD) has been reported to be loosely bound (Jackson et al., 2007). In agreement with this, purified R. rhodochrous 11Y XplB contained 25 % of the predicted FAD and was yellow-colored. However, the Microbacterium sp. MA1 XplB protein was colorless and lacked detectable FAD (Figure 2b and c). Reductase activity in cell lysates was measured using the Griess assay to detect nitrite released during the degradation of RDX by XplA. Figure 2d shows that in reaction mixtures containing XplA and lysate from cells expressing R. rhodochrous 11Y XplB, nitrite release

was observed. However, although nitrite was detected in reaction mixtures containing lysate

FEMS Microbiology Letters

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191	from cells expressing the Microbacterium sp. MA1 XplB, the level was not significantly
192	different from that seen from lysate transformed with the empty vector control, indicating that
193	Microbacterium sp. MA1 XplB was inactive.
194	Characterisation of the XplB portion of the Gordonia KTR9 GS-XplB fusion
195	In Gordonia KTR9, the sequence of the XplB portion of the GS-XplB fusion was found to
196	differ from the <i>R. rhodochrous</i> 11Y XplB sequence by just two amino acids. Firstly, the Met-
197	1 in Gordonia KTR9 GS-XplB was missing, enabling the uninterrupted translation of XplB
198	following GS to produce the GS-XplB fusion. Secondly, R. rhodochrous 11Y XplB
199	contained a tryptophan (Trp-386) whereas the Gordonia KTR9 GS-XplB fusion contained
200	serine (Ser-385). This difference was the result of a single base change: TCG encoding
201	tryptophan and TGG encoding serine. A multiple sequence alignment, by % identity, of XplB
202	revealed that Trp-386 is highly conserved across a number of bacterial genera (Table 1).
203	Purified Gordonia KTR9 GS-XplB fusion protein was colorless and lacked detectable FAD
204	(Figure 2b and c). Subsequent Griess assays suggested that the GS-XplB fusion was inactive
205	(Figure 2d) and to confirm this, RDX removal rates by cell lysates were measured. When
206	lysate from cells expressing R. rhodochrous 11Y XplB was used as the source of reductase,
207	all the RDX was removed within 10 minutes. Although RDX degradation was detected in
208	reaction mixtures containing lysate from cells expressing the Gordonia KTR9 GS-XplB
209	fusion, this was lower than endogenous E. coli reductase activity measured in lysate from
210	cells transformed with just the empty vector control (Figure 2e). Together, these results
211	demonstrate that the XplB component of the Gordonia KTR9 GS-XplB was inactive.
212	To investigate further, the role of Ser-385 and Trp-386 in XplB activity, reciprocal mutations
213	in R. rhodochrous 11Y XplB (W386S) and the Gordonia KTR9 GS-XplB-S385W fusion
214	were made. Lysate from cells expressing R. rhodochrous 11Y XplB-W386S lacked the

215	yellow coloration observed in lysate from unmutated R. rhodochrous 11Y XplB.
216	Furthermore, when used as the partnering reductase for XplA, activity towards RDX was
217	only observed when unmutated R. rhodochrous 11Y XplB was supplied as the reductase; no
218	activity was observed in assays using the mutated R. rhodochrous 11Y XplB-W386S (Figure
219	3a). Whereas lysate from cells expressing the Gordonia KTR9 GS-XplB fusion was not
220	yellow colored, lysate from cells expressing the mutated Gordonia KTR9 GS-XplB-S385W
221	fusion had the same yellow coloration observed in the cell lysate of <i>R. rhodochrous</i> 11Y
222	XplB. Figure 3b shows that when mutated Gordonia KTR9 GS-XplB-S385W fusion lysate
223	was supplied as a reductase in Griess assays, with purified <i>R. rhodochrous</i> 11Y XplA and
224	RDX as substrate, nitrite was detected. In assays measuring RDX using HPLC, lysate from
225	the mutated Gordonia KTR9 GS-XplB-S385W fusion removed significantly more RDX than
226	lysate from cells transformed with the empty vector (Figure 3c). However, upon purification,
227	the mutated Gordonia KTR9 GS-XplB-S385W fusion protein appeared colorless and FAD
228	was not detectable using HPLC. Assays monitoring the production of nitrite from RDX by <i>R</i> .
229	rhodochrous 11Y XplA, confirmed that the purified, mutated Gordonia KTR9 GS-XplB-
230	S385W fusion was inactive.
231	Characterization of the GS portion of the <i>Gordonia</i> KTR9 GS-XplB fusion
232	Multiple sequence alignments with characterized GS type I protein sequences (Brown et al.,
233	1994, Hayward et al., 2009, Murray et al., 2013) revealed that the GS component of the
234	Gordonia KTR9 GS-XplB fusion belongs to the GS type I- α class, which lack the insertion
235	signature sequence found in the GS type I- β class (Brown <i>et al.</i> , 1994; Table 2). The
236	Gordonia KTR9 GS-XplB contains 390 amino acids and alignment with the three closest

- 237 homologs (> 78 % identity) available in public databases revealed that the fusion protein is
- truncated, missing 71 amino acids from the C-terminus. To investigate whether the missing

Page 13 of 31

FEMS Microbiology Letters

239	residues form part of the active site, a model structure of the GS portion was created based on
240	the closest homolog (29.1 % identity) in the protein database bank for which structural data
241	was available: GS from <i>B. subtilis</i> (acc. no. P12425). Superimposing the model structure of
242	Gordonia KTR9 GS-XplB on the GS from B. subtilis (RMSD: 1.141 Å) revealed the
243	structure, and position, of the missing region (Figure 4a). Based on this homology modelling,
244	and knowledge from the structure of the GS type I-a of B. subtilis (Murray et al., 2013), it
245	was found that the missing residues in the GS fusion included two residues involved in the
246	formation of a Tyr loop in the active site. Residues located on the other loops mediating the
247	catalytic activity of the enzyme present in GS from <i>B. subtilis</i> were also found in the
248	Gordonia KTR9 GS-XplB. This included an Asn loop which shares remarkable similarity
249	with GS from <i>B. subtilis</i> , while the Asp^{50} , on a latch loop was found to be extended in the
250	Gordonia KTR9 GS-XplB when compared to the GS from B. subtilis. Remarkably, despite
251	fusion and truncation, the GS-XplB protein has glutamine synthetase activity (Figure 4b).

Discussion

Both *xplA* and *xplB* are highly conserved amongst different genera of aerobic RDX-degrading bacteria isolated from distinct geographical locations. This conservation endorses the theory of the recent evolution of these genes, and dissemination around the world through horizontal gene transfer (Seth-Smith et al., 2008, Andeer et al., 2009, Jung et al., 2011). Such evolution and distribution has been reported for other xenobiotic-degrading genes, for example naphthalene degrading genes (nahAc) (Herrick et al., 1997), N-heterocycle morpholine degrading genes (morABC) (Sielaff & Andreesen, 2005) and atrazine degrading genes (atzABC) (de Souza et al., 1998) were also found to be highly conserved to the level of identical copies of the same gene found amongst different bacteria isolated from diverse geographical locations.

1

2 3	263	In addition to <i>xplA</i> and <i>xplB</i> , there are several neighboring genes which are nearly identical
4 5 6	264	between the bacteria studied here. In R. rhodochrous 11Y, Microbacterium sp. MA1 (Andeer
7 8	265	et al., 2009) and Gordonia sp. KTR9 (Indest et al., 2010), there are transposable elements in
9 10	266	neighboring regions, suggesting that <i>xplA</i> and <i>xplB</i> are part of a larger mobile element such
11 12	267	as an Integrative and Conjugative Element (ICE) or genomic island in a conjugative plasmid.
13 14 15	268	Similarly, the genes for the degradation of xenobiotics such as chlorobenzoate, by
16 17	269	Pseudomonas sp. strain B13 (Ravatn et al., 1998, Gaillard et al., 2006); and biphenyl
18 19	270	degradation by Ralstonia eutropha A5 (Springael et al., 2001), are also found partly on larger
20 21	271	mobile elements. Genomic islands and ICEs are known to excise and integrate into
22 23	272	chromosomes or plasmids through conjugation (Burrus et al., 2002, van der Meer &
24 25 26	273	Sentchilo, 2003). In support of this, R. rhodochrous 11Y.058 (Figure 1) encodes a phage
27 28	274	related integrase belonging to the tyrosine recombinase family, which shares complete
29 30	275	identity with a site-specific recombinase from <i>R. erythropolis</i> PR4. Moreover, the RDX-
31 32	276	degradation capacity of Gordonia sp. KTR9 was successfully transferred into the non-RDX
33 34	277	degrading species Gordonia polyisoprenivorans, Rhodococcus jostii RHA1 and Nocardia sp.
35 36 37	278	TW2 through conjugation (Jung et al., 2011), demonstrating that this is a possible transfer
38 39	279	mechanism.
40 41		
41	280	Sequence analysis of the genes surrounding <i>xplA</i> and <i>xplB</i> showed that there are regions of
43 44	281	identity between the three species, and although <i>xplA</i> alone is sufficient for the catabolism of
45 46 47	282	RDX (Rylott et al., 2006, Indest et al., 2010), it has been speculated that some of these
48 49	283	neighboring genes contribute towards RDX degradation (Indest et al., 2010, Indest et al.,
50 51	284	2013, Chong et al., 2014, Zhu et al., 2014). For example, downstream of xplB in R.
52 53	285	rhodochrous 11Y and Microbacterium sp. MA1 are genes encoding a putative permease
54 55	286	(AroP; 11Y.026 and MA1.029 in Figure 1) and transcriptional regulator (MarR; 11Y.025 and
56 57 58 59 60		13

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2 3	287	MA1.027 in Figure 1). Orthologous permeases have been found close to genes with related
4 5 6	288	function (Wehrmann et al., 1995, Yu et al., 2007), and members of the MarR family shown
7 8	289	to play a role in regulating catabolism of aromatic compounds (Bussmann et al., 2010; Chong
9 10	290	et al., 2014). However, gene deletion analysis has demonstrated that AroP and MarR from R.
11 12	291	rhodochrous 11Y do not affect RDX degradation in this species (Chong et al., 2014). In
13 14 15	292	Gordonia sp. KTR9, sequence analysis of pGKT2, the 182 kb plasmid carrying xplA and
16 17	293	<i>xplB</i> indicated that they are integrated into an operon involved in the degradation of N-
18 19	294	heterocyclic compounds (Zhu et al., 2014). On this operon, the genes upstream of xplA and
20 21	295	xplB (xplR, cyp151C and glnA) share high sequence similarity and arrangement to the mor
22 23 24	296	and <i>pip</i> gene clusters involved in the degradation of morpholine, piperidine and related
24 25 26 27	297	compounds in Mycobacterium and Rhodococcus (Indest et al., 2010).
28 29	298	An <i>xplB</i> knock-out in <i>R. rhodochrous</i> 11Y demonstrated that although XplB is not required
30 31	299	for XplA activity; the absence of XplB reduces the rate of RDX-degradation by 70 % (Chong
32 33	300	et al., 2014). The fact that an xplB knock-out can still degrade RDX indicates that alternative
34 35	301	endogenous reductases can substitute in bacteria, and this has also been demonstrated in
30 37 38	302	XplA-transformed plants (Jackson et al., 2007). A previous comparison between the RDX
39 40	303	removal rates of RDX-degrading isolates showed that all Rhodococcus spp. had faster RDX
41 42	304	removal rates than Microbacterium sp. MA1 or Gordonia sp. KTR9 (Chong et al., 2014).
43 44	305	Considering that XplA alone is able to denitrate the RDX structure in the organism (Rylott et
45 46 47	306	al., 2006, Indest et al., 2010), and is identical in species across all three genera (Chong et al.,
47 48 49	307	2014), the differences in the RDX-removal rate are due to the impairment of XplB,
50 51 52	308	differences in bacterial physiology, or a combination of both.
53 54	309	The studies presented here indicate that mutations present in XplB in Gordonia sp. KTR9 and
55 56	310	Microbacterium sp. MA1 explain, to a degree, why these species exhibit reduced rates of
57 58		14
59 60		

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311	RDX degradation when compared with R. rhodochrous 11Y. Our studies comparing the R.
312	rhodochrous 11Y Trp-386 with the Ser-385 found in Gordonia sp. KTR9 showed that Trp-
313	386 clearly plays a critical role in retaining FAD in XplB. This residue also appears to be
314	important for the functionality of other FAD containing proteins as multiple sequence
315	alignments revealed the residue to be highly conserved amongst FAD-containing proteins
316	that share as low as 42 % sequence identity with XplB. Additionally, in Gordonia sp. KTR9,
317	the fusion of GS to XplB is likely to further inhibit the reductase activity of XplB, and thus
318	RDX-degrading activity of Gordonia sp. KTR9. Considering that Gordonia sp. KTR9 was
319	isolated from soil where RDX was not detected (Thompson et al., 2005), it is possible that
320	the recombination and reduction of the genomic island, which resulted in the fusion of the
321	XplB to GS, arose from the absence of selective pressure from RDX.
322	Despite the fact that the GS component of the Gordonia sp. KTR9 GS-XplB fusion is
323	truncated, it was found to be active, demonstrating that the purified GS protein is correctly
324	folded and the missing residues are not essential for functionality. The GS portion of the
325	Gordonia sp. KTR9 GS-XplB fusion belongs to GS type I- α . It is already known that the GS
326	type I- α from <i>B. subtilis</i> plays an important role in regulating cellular nitrogen levels by
327	controlling the expression of GlnR and TnrA. In the presence of glutamine, GS binds TnrA
328	directly onto the DNA, forming a GS-TnrA-DNA complex with its formation regulated by
329	the intracellular levels of ATP, AMP, glutamine, and glutamate. (Fisher & Wray, 2008, Wray
330	& Fisher, 2010, Murray et al., 2013, Hauf et al., 2016). Mutation of GS in B. subtilis resulted
331	in the constitutive expression of both GlnR and TnrA proteins (Wray & Fisher, 2010). In
332	Gordonia sp. KTR9, GlnR is important in the assimilation of nitrite in the cell. Knock-out of
333	glnR in Gordonia sp. KTR9 resulted in the accumulation of nitrite from RDX (Zhu et al.,
334	2014) and down-regulation of the nitrite reductase gene loci KTR9_1306 and KTR9_1307

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- 335 (Indest et al., 2013). Additionally, the glnR mutant lacked the ability to utilise RDX, nitrite or
- 336 nitrate as the sole source of nitrogen, but not ammonium or glutamine.

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345 **Figure Legends**

Figure 1. Schematic representation of the RDX-degrading gene clusters in three genera of
aerobic RDX-degrading bacteria.

348 White colored regions have no sequence homology. Dotted lines indicate unsequenced

349 regions, genes encoding transposable elements are shown in yellow.

350 Figure 2. Purification and analysis of XplB proteins from *R. rhodochrous* 11Y,

351 *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9.

352 (a) SDS-PAGE analysis of lysates (L) and purified proteins from *E. coli* cells expressing

353 XplA or XplB homologues. (b) Overlaid chromatograms showing FAD absorbance peaks

354 from purified proteins. (c) Appearance of purified proteins. (d) Reductase activity in cell

355 lysates expressing XplB homologues, measured using the Griess assay with purified XplA

and RDX as substrate ($n = 3 \pm$ SD. Letters refer to significant differences; ANOVA, Tukey

357 HSD). (e) RDX removal from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and

358 *Gordonia* sp. KTR9 GS-XplB supplemented with purified XplA ($n = 3 \pm SD$).

Figure 3 Activities of XplB proteins carrying reciprocal mutations from *R. rhodochrous* 11Y
and *Gordonia* sp. KTR9.

361 (a) Nitrite release from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *R.*

362 *rhodochrous* 11Y XplB-(W386S) measured using the Griess assay, with purified XplA and

363 RDX as substrate ($n = 3 \pm SD$). (b) Nitrite release, observed using the Griess assay, by *E. coli*

- 364 cell lysates expressing *R. rhodochrous* 11Y XplB and *Gordonia* sp. KTR9 GS-XplB-
- 365 (S385W), with purified XplA and RDX as substrate. (c) RDX removal by *E. coli* cell lysates
- 366 expressing the *Gordonia* sp. KTR9 GS-XplB-(S385W) or empty vector ($n = 3 \pm SD$).

 367 Figure 4 Characterization of the glutamine synthetase (GS) and XplB portions of Gordonia

368 sp. KTR9 GS-XplB fusion protein.

- 369 (a) Model structure of GS from GS-XplB (blue) superimposed on the GS structure of *B*.
- *subtilis* (yellow). Sequence missing from GS-XplB (red). Root mean square deviation 1.14

371 °A). (**b**) GS activity in *E. coli* cell lysates ($n = 3 \pm SD$).

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Table 1

Multiple sequence alignment of the *Rhodococcus rhodochrous* 11Y XplB showing closest matches by % identity. The alignment shows the regions containing the phenylalanine-172 (F) residue that is replaced with isoleucine (<u>I</u>) in *Microbacterium* sp. MA1; and the tryptophan-386 (W) residue that in the *Gordonia* sp. KTR9 GS-XplB fusion, corresponds to serine (<u>S</u>). Multiple species for genera have been omitted. The alignment was performed using ClustalW2 (EMBL European Bioinformatics Institute).

0 :	Identity	UniProt	Sequence
Organism	(%)	AC	(N- to C-terminal)
Rhodococcus rhodochrous 11Y	100	Q8GPH8	DIIRLLSKQPDE F TGSD W MRIDEAEVASASPGRIRQKVREVD
Gordonia sp. KTR9	99.8	E1R0R9	DIIRLLSKQPDE F TGSD S MRIDEAEVASASPGRIRQKVREVD
Microbacterium sp. MA1	99.8	C3UMY2	DIIRLLSKQPDE I TGSD W MRIDEAEVASASPGRIRQKVREVD
Pseudomonas sp.	47.7	A0A0V8SZM6	DVLRLLAKSAED F AGSD W QRIDHAETRAAAPGRVRRKITDRA
Nitratireductor pacificus	47.2	K2MCG5	DLLRLLAKTPDELAGSDWKRIDAAEIAAAPENRCRVKINSRD
Marinovum algicola	46.7	A0A0H4L107	DLLRLLAKSPEELEGSDWCRIDSAETANPPPGRCRAKITTRE
Ventosimonas gracilis	44.5	A0A139SRD6	DVLRLLIKPAHDWQGSDWQRIDTEEKQCAPAGRVRQKITERV
Roseomonas mucosa	44.5	A0A0W0A9P2	DLARMLSKDEAE L AGSD W LRIRAAEEAAASAGRVRRKGATRD
Mameliella alba	44.4	A0A0B3RZQ4	DLLRLLAKAPEELDGSDWSRIDAAETGAAPEGRCRTKLATRE
<i>Ruegeria</i> sp.	44.1	A0A1E3D8F1	DLLRLLAKAPEE L DGSD W SRIDAAETGAAPEGRCRTKLATRE
Agrococcus jejuensis	43	A0A1G8CDN7	DVARLVARDAADFDGTDWRRIDAAETLAAAPGRRRAKLRTLD
Agrococcus pavilionensis	43	U1LRN2	DVVRLLARDAEGLGGTDWRRIDVREQLGAAPGRSRSKLRSRA
Arthrobacter globiformis	43	H0QK39	DVVRFLIKTPGE F AGSD W LRVDAAERAAAPGNRSRKKLPDHA
Leucobacter sp.	42.1	A0A061LTW8	DVLRFLVKDRDA y egsd w lrldehersvapsgrvrhklpdhd

Table 2.

Multiple sequence alignment of a region of the glutamine synthetase component of the GS-XplB fusion from *Gordonia* sp. KTR9. The shaded region is unique to class GS type I- β type GS (Brown *et al.*, 1994). The alignment was performed using ClustalW2 (EMBL European Bioinformatics Institute).

Organism	Class	UniProt	Sequence
		AC	(N- to C-terminal)
Mycobacterium tuberculosis	GSI-β	P9WN39	STGIADTAYFGAEAEFYIFDSVSFDSRANGSFYEVDAISGWWNTGAATEA 170
Streptomyces coelicolor	GSI-β	P15106	STGIADTAFFGPEAEFYVFDSVRFATRENESFYHIDS <mark>EAGAWNTGALED- 165</mark>
Synechococcus sp.	GSI-β	P28605	ASGIGDTAYFGPEAEFFVFDDVRFDQTENKGFYYVDSVEGRWNSGRKEP- 168
Anabaena sp.	GSI-β	K7W630	STGLGD <mark>TAFF</mark> GPEAEFFIFDDVRYDQTTNSGYYYVDS <mark>VEGRWNTGREE 166</mark>
Salmonella typhimurium	GSI-β	P0A1P6	ATGIADTVLFGPEPEFFLFDDIRFGASISGSHVAIDDIEGAWNSSTKYE- 166
Pyrococcus furiosus	GSI-α	Q05907	KEGYKAYIGPEPEFYLFKKNGTWELEIPDV- 141
Haloferax volcanii	GSI-α	P43386	ELGY-DVNVA-PEPEFFLFEEDEDGRATTVTNDA- 164
Bacillus subtilis	GSI-α	P12425	DLGFSDFNLG-PEPEFFL <mark>F</mark> KLDEKGEPTLELNDK- 153
Methanococcus voltae	GSI-α	P21154	EEFKGEYFVG-PEPEFFILKNENGKWVPGDD- 156
Gordonia sp. KTR9	GSI-α	E1R0R9	ERTGLEMRTG-TEPEMTWEGEGFETTFRPDS- 176



Figure 1. Schematic representation of the RDX-degrading gene clusters in three genera of aerobic RDXdegrading bacteria.

White colored regions have no sequence homology. Dotted lines indicate unsequenced regions, genes encoding transposable elements are shown in yellow.

Figure 2. Purification and analysis of XplB proteins from R. rhodochrous 11Y, Microbacterium sp. MA1 and Gordonia sp. KTR9.

162x267mm (300 x 300 DPI)



Figure 2. Purification and analysis of *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9
XpIB! + (a) SDS-PAGE analysis of lysates (L) and purified proteins from *E. coli* cells expressing XpIA or XpIB homologs. (b) Overlaid chromatograms showing FAD absorbance peaks from purified proteins. (c)
Appearance of purified proteins. (d) Reductase activity in cell lysates expressing XpIB homologs, measured using the Griess assay with purified XpIA and RDX as substrate (n = 3 ± SD. Letters refer to significant differences; ANOVA, Tukey HSD). (e) RDX removal from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XpIB and *Gordonia* sp. KTR9 XpIB-GS supplemented with purified XpIA (n= 3 ± SD).

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Figure 3 Activities of XpIB proteins carrying reciprocal mutations from *R. rhodochrous* 11Y and *Gordonia* sp. KTR9.

(a) Nitrite release from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *R. rhodochrous* 11Y XplB-(W386S) measured using the Griess assay, with purified XplA and RDX as substrate (n = 3 ± SD). (b) Nitrite release, observed using the Greiss assay, by *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *Gordonia* sp. KTR9 GS-XplB-(S385W), with purified XplA and RDX as substrate. (c) RDX removal by *E. coli* cell lysates expressing the *Gordonia* sp. KTR9 GS-XplB-(S385W) or empty vector (n = 3 ± SD).

63x66mm (300 x 300 DPI)



Figure 4 Characterization of the glutamine synthetase (GS) and XplB portions of Gordonia sp. KTR9 GS-XplB fusion protein.

(a) Model structure of GS from GS-XplB (blue) superimposed on the GS structure of B. subtilis (yellow). Sequence missing from GS-XplB (red). Root mean square deviation 1.14 °A). (b) GS activity in E. coli cell lysates (n = $3 \pm$ SD).

68x93mm (300 x 300 DPI)