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

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Review

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3 1 *Title:* Investigating differences in the ability of XplA/B-containing bacteria to degrade the  
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5 2 explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)  
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21 **Graphical Abstract and One-Sentence Summary**

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.

24

For Peer Review

## 25 Abstract

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

## 42 Introduction

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

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3 49 isolated in the UK from *Rhodococcus rhodochrous* 11Y and identified as an unusual  
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5 50 cytochrome P450, XplA, and accompanying flavodoxin reductase partner, XplB (Seth-Smith  
6  
7 51 *et al.*, 2002). A number of aerobic RDX-degrading bacteria have been reported (Binks *et al.*,  
8  
9 52 1995, Coleman, 1998, Thompson *et al.*, 2005, Indest *et al.* 2007, Nejdat *et al.* 2008, Andeer  
10  
11 53 *et al.*, 2009, Bernstein *et al.*, 2011,) from different geographical locations but all belonging to  
12  
13 54 the order Actinomycetales.

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17 55 Within all the species tested, *xplA* has been detected, and found to have greater than 99 %  
18  
19 56 identity (Indest *et al.*, 2007, Seth-Smith *et al.*, 2008, Andeer *et al.*, 2009, Bernstein *et al.*,  
20  
21 57 2011, Rylott *et al.*, 2011, Chong *et al.*, 2014). The highly conserved nature of *xplA* suggests  
22  
23 58 its rapid distribution by horizontal gene transfer (Seth-Smith *et al.*, 2008, Andeer *et al.*, 2009,  
24  
25 59 Rylott *et al.*, 2011, Chong *et al.*, 2014). Genes involved in xenobiotic catabolism are often  
26  
27 60 located on a mobile genetic element, accompanied by insertion elements, and are integrated  
28  
29 61 into the bacterial chromosomal or plasmid (Nojiri *et al.*, 2004). In agreement with this, *xplA*  
30  
31 62 is plasmid-encoded in *Microbacterium* sp. MA1, *R. rhodochrous* 11Y (Andeer *et al.*, 2009)  
32  
33 63 and *Gordonia* sp. KTR9 (Indest *et al.*, 2010). Partial sequence analysis of the plasmid  
34  
35 64 carrying *xplA* in *Microbacterium* sp. MA1 revealed that the gene is also associated with  
36  
37 65 transposable elements in this bacterium (Andeer *et al.*, 2009). Furthermore, a 6.7 kbp region  
38  
39 66 flanking *xplA* has been found that is nearly identical (> 99 %) between *Microbacterium* sp.  
40  
41 67 MA1 and *R. rhodochrous* 11Y (Andeer *et al.*, 2009).

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46 68 The partnering reductase for XplA is XplB (Seth-Smith *et al.*, 2002), an NADPH-dependent  
47  
48 69 flavoprotein which contains one molecule of FAD as a cofactor and shares sequence  
49  
50 70 homology (and 27 % sequence identity) with the bovine adrenodoxin reductase FDXR (US  
51  
52 71 National Library of Medicine National Institutes of Health (NCBI) accession number  
53  
54 72 P08165.3). The reductase XplB is involved in the activation of the catalytic centre of XplA

1  
2  
3 73 via the transfer of electrons from NADPH to a flavodoxin domain fused to the N-terminal of  
4  
5 74 the P450 domain of XplA (Jackson *et al.*, 2007).  
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8 75 The role of XplB in RDX degradation has been demonstrated in both *R. rhodochrous* 11Y  
9  
10 76 (Chong *et al.*, 2014) and transgenic plant lines (Jackson *et al.*, 2007, Bui *et al.*, 2012). In  
11  
12 77 *Gordonia* sp. KTR9 the 5' end of *xplB* is fused to a glutamine synthetase (GS)-encoding  
13  
14 78 gene, *glnA* (Indest *et al.*, 2010), an arrangement that has not been found in any of the other  
15  
16 79 RDX-degrading bacteria examined so far. Glutamine synthetase (EC 6.3.1.2) is an essential  
17  
18 80 enzyme in nitrogen metabolism, catalysing the ATP-dependant production of glutamine from  
19  
20 81 glutamate and ammonia.  
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24 82 Previously, we reported that although *xplA* and *xplB* are highly conserved amongst RDX-  
25  
26 83 degrading bacteria, the ability of *Gordonia* sp. KTR9 and *Microbacterium* sp. MA1 to grow  
27  
28 84 in minimal medium with RDX as the sole source of nitrogen was significantly less than that  
29  
30 85 of *R. rhodochrous* 11Y. The ability of these bacteria to remove RDX from the medium  
31  
32 86 correlated with growth rates (Chong *et al.*, 2014). It is possible that the fusion of *xplB* with  
33  
34 87 *glnA* and/or additional changes in the genetic components and arrangements of the genes in  
35  
36 88 this region may account for differences in the regulation of RDX degradation in *Gordonia* sp.  
37  
38 89 KTR9 (Zhu *et al.*, 2014).  
39  
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42

43 90 Here we present analysis of the *xplA* and *xplB*-containing gene clusters from *R. rhodochrous*  
44  
45 91 11Y, *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9, which reveal differences and  
46  
47 92 commonalities in the arrangement of genes; along with emphasis on the sequence of *xplB*,  
48  
49 93 and characterisation of the GS-XplB fusion from *Gordonia* sp. KTR9.  
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52

## 53 **Materials and Methods**

### 55 **Genome sequencing**



1  
2  
3 96 Whole genome sequencing was used to obtain the complete *xplA/B* genomic island of *R.*  
4  
5 97 *rhodochrous* 11Y (NCBI acc. no. KY488543; Figure 1). To do this, total genomic DNA was  
6  
7 98 extracted by lysing the cells in TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0)  
8  
9 99 containing 10 mg/ml lysozyme, 10 % SDS and 20 mg/ml proteinase K. Cell debris was  
10  
11 100 centrifuged at 10000rpm and protein removed by phenol-chloroform extraction. Nucleic  
12  
13 101 acids were precipitated with 3M sodium acetate and isopropanol, washed in 70 % ethanol and  
14  
15 102 resuspended in nuclease-free water. Prior to sequencing, the 16S rRNA region was sequenced  
16  
17 103 to ensure that the DNA sample was from the correct species. Additionally, the *xplA* gene was  
18  
19 104 amplified from the DNA sample to make sure that the plasmid had not been cured from the  
20  
21 105 bacterial genome during the extraction process. The DNA sample was analysed using an  
22  
23 106 Agilent TapeStation 2200 and sequenced using a Next Generation Sequencing platform Ion  
24  
25 107 Torrent (Life Technologies). Raw sequence reads were assembled using Newbler, version 2.7  
26  
27 108 (Roche Diagnostics).

### 109 **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.*  
112 *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 *rhodochrous* 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

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

## 130 Activity assays

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

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2  
3 144 was used as a standard. Glutamine synthetase was measured spectrophotometrically, based on  
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5 145 the method of Kingdon *et al.*, 1968 and using a pyruvate kinase and lactic dehydrogenase-  
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7 146 linked assay that followed reduction of NADH at wavelength 340nm. Each 3 mL reaction  
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10 147 mix cuvette contained 34.1 mM imidazole buffer pH 7.1, 102 mM sodium glutamate, 8.5 mM  
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12 148 adenosine 5'-triphosphate, 1.1 mM phosphoenolpyruvate, 60 mM magnesium chloride, 18.9  
13  
14 149 mM potassium chloride, 45 mM ammonium chloride, 0.25 mM b-nicotinamide adenine  
15  
16 150 dinucleotide, 28 units pyruvate kinase, 40 units L-lactic dehydrogenase and 0.4 - 0.8 unit  
17  
18 151 glutamine synthetase. One enzyme unit will convert 1.0  $\mu$ mole of L-glutamate to L-glutamine  
19  
20 152 in 1 minute at pH 7.1 at 37°C with specific activity defined as number of units per mg  
21  
22  
23 153 protein.

#### 24 25 26 154 **Measurement of FAD**

27  
28 155 The amount of FAD cofactor bound to the XplB and GS-XplB proteins was measured  
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30 156 following the method described by Aliverti *et al.*, 1999. Protein was boiled at 100 °C, in the  
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33 157 dark, for 20 min and precipitated protein removed by centrifugation at 13,000 rpm for 10  
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35 158 minutes. The UV-visible spectrum of the supernatant was recorded (200 to 600 nm)  
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37 159 spectrophotometrically. FAD was also determined by HPLC analysis using a C18 column  
38  
39 160 with a mobile phase of 5 mM ammonium acetate buffer, pH 6.5 (solvent A) and methanol  
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41 161 (solvent B) and the following gradient: 5 minutes solvent A 85 %: solvent B 15 %; 20  
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43 162 minutes solvent A 25 %: solvent B 75 %; 5 minute solvent A 0 %: solvent B 100 %; 5  
44  
45 163 minutes solvent A 85 %: solvent B 15 %). The flow rate was 0.75 ml/min and column  
46  
47 164 temperature 30 °C. Commercially available FAD (Sigma) was used as a reference.

#### 48 49 50 51 165 **Results**

##### 52 53 54 166 **The *xplA/B* gene clusters**

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3 167 Analysis of the putative *xplA/B* genomic islands in *R. rhodochrous* 11Y, *Microbacterium* sp.  
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5 168 MA1 and *Gordonia* sp. KTR9 is shown in Figure 1. Within a 53 kb region in *R. rhodochrous*  
6  
7 169 11Y, there are 13.8 kb and 11.8kb gene clusters (termed the A and B regions respectively).  
8  
9 170 Further downstream, is a 570 bp sequence encoding a transposable element (termed the C  
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11 171 region). While the A region, which contains *xplA* and *xplB*, is highly conserved between *R.*  
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13 172 *rhodochrous* 11Y and *Microbacterium* sp. MA1, *Gordonia* sp. KTR9 shares homology only  
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15 173 with 3.1 kbp of the A region, although this includes *xplA* and *xplB*. Conversely, the B and C  
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17 174 regions are highly conserved between *R. rhodochrous* 11Y and *Gordonia* sp. KTR9, but  
18  
19 175 absent from *Microbacterium* sp. MA1. Two additional regions, (termed D1 and D2) are  
20  
21 176 nearly identical between *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9; yet absent from  
22  
23 177 *R. rhodochrous* 11Y. Scattered within these gene clusters are a number of transposable  
24  
25 178 elements, indicative of mobility within and between these regions.  
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### 30 179 ***Microbacterium* sp. MA1 XplB**

31  
32 180 The only difference between the *R. rhodochrous* 11Y and *Microbacterium* sp. MA1 XplB  
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34 181 sequences is that *R. rhodochrous* 11Y contains a Phe-172, which is conserved across a range  
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36 182 of RDX-degrading bacterial genera, whereas *Microbacterium* sp. MA1 contains an Ile-172  
37  
38 183 (Table 1). In *R. rhodochrous* 11Y XplB, a protein-bound flavin (FAD) has been reported to  
39  
40 184 be loosely bound (Jackson *et al.*, 2007). In agreement with this, purified *R. rhodochrous* 11Y  
41  
42 185 XplB contained 25 % of the predicted FAD and was yellow-colored. However, the  
43  
44 186 *Microbacterium* sp. MA1 XplB protein was colorless and lacked detectable FAD (Figure 2b  
45  
46 187 and c). Reductase activity in cell lysates was measured using the Griess assay to detect nitrite  
47  
48 188 released during the degradation of RDX by XplA. Figure 2d shows that in reaction mixtures  
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50 189 containing XplA and lysate from cells expressing *R. rhodochrous* 11Y XplB, nitrite release  
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52 190 was observed. However, although nitrite was detected in reaction mixtures containing lysate  
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3 191 from cells expressing the *Microbacterium* sp. MA1 XplB, the level was not significantly  
4  
5 192 different from that seen from lysate transformed with the empty vector control, indicating that  
6  
7 193 *Microbacterium* sp. MA1 XplB was inactive.  
8  
9

#### 10 194 **Characterisation of the XplB portion of the *Gordonia* KTR9 GS-XplB fusion**

11  
12 195 In *Gordonia* KTR9, the sequence of the XplB portion of the GS-XplB fusion was found to  
13  
14 196 differ from the *R. rhodochrous* 11Y XplB sequence by just two amino acids. Firstly, the Met-  
15  
16 197 1 in *Gordonia* KTR9 GS-XplB was missing, enabling the uninterrupted translation of XplB  
17  
18 198 following GS to produce the GS-XplB fusion. Secondly, *R. rhodochrous* 11Y XplB  
19  
20 199 contained a tryptophan (Trp-386) whereas the *Gordonia* KTR9 GS-XplB fusion contained  
21  
22 200 serine (Ser-385). This difference was the result of a single base change: TCG encoding  
23  
24 201 tryptophan and TGG encoding serine. A multiple sequence alignment, by % identity, of XplB  
25  
26 202 revealed that Trp-386 is highly conserved across a number of bacterial genera (Table 1).  
27  
28 203 Purified *Gordonia* KTR9 GS-XplB fusion protein was colorless and lacked detectable FAD  
29  
30 204 (Figure 2b and c). Subsequent Griess assays suggested that the GS-XplB fusion was inactive  
31  
32 205 (Figure 2d) and to confirm this, RDX removal rates by cell lysates were measured. When  
33  
34 206 lysate from cells expressing *R. rhodochrous* 11Y XplB was used as the source of reductase,  
35  
36 207 all the RDX was removed within 10 minutes. Although RDX degradation was detected in  
37  
38 208 reaction mixtures containing lysate from cells expressing the *Gordonia* KTR9 GS-XplB  
39  
40 209 fusion, this was lower than endogenous *E. coli* reductase activity measured in lysate from  
41  
42 210 cells transformed with just the empty vector control (Figure 2e). Together, these results  
43  
44 211 demonstrate that the XplB component of the *Gordonia* KTR9 GS-XplB was inactive.  
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50  
51 212 To investigate further, the role of Ser-385 and Trp-386 in XplB activity, reciprocal mutations  
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53 213 in *R. rhodochrous* 11Y XplB (W386S) and the *Gordonia* KTR9 GS-XplB-S385W fusion  
54  
55 214 were made. Lysate from cells expressing *R. rhodochrous* 11Y XplB-W386S lacked the  
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3 215 yellow coloration observed in lysate from unmutated *R. rhodochrous* 11Y XplB.  
4  
5 216 Furthermore, when used as the partnering reductase for XplA, activity towards RDX was  
6  
7 217 only observed when unmutated *R. rhodochrous* 11Y XplB was supplied as the reductase; no  
8  
9 218 activity was observed in assays using the mutated *R. rhodochrous* 11Y XplB-W386S (Figure  
10  
11 219 3a). Whereas lysate from cells expressing the *Gordonia* KTR9 GS-XplB fusion was not  
12  
13 220 yellow colored, lysate from cells expressing the mutated *Gordonia* KTR9 GS-XplB-S385W  
14  
15 221 fusion had the same yellow coloration observed in the cell lysate of *R. rhodochrous* 11Y  
16  
17 222 XplB. Figure 3b shows that when mutated *Gordonia* KTR9 GS-XplB-S385W fusion lysate  
18  
19 223 was supplied as a reductase in Griess assays, with purified *R. rhodochrous* 11Y XplA and  
20  
21 224 RDX as substrate, nitrite was detected. In assays measuring RDX using HPLC, lysate from  
22  
23 225 the mutated *Gordonia* KTR9 GS-XplB-S385W fusion removed significantly more RDX than  
24  
25 226 lysate from cells transformed with the empty vector (Figure 3c). However, upon purification,  
26  
27 227 the mutated *Gordonia* KTR9 GS-XplB-S385W fusion protein appeared colorless and FAD  
28  
29 228 was not detectable using HPLC. Assays monitoring the production of nitrite from RDX by *R.*  
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31 229 *rhodochrous* 11Y XplA, confirmed that the purified, mutated *Gordonia* KTR9 GS-XplB-  
32  
33 230 S385W fusion was inactive.

### 231 **Characterization of the GS portion of the *Gordonia* 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- $\alpha$  class, which lack the insertion  
235 signature sequence found in the GS type I- $\beta$  class (Brown *et al.*, 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  
238 truncated, missing 71 amino acids from the C-terminus. To investigate whether the missing

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3 239 residues form part of the active site, a model structure of the GS portion was created based on  
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5 240 the closest homolog (29.1 % identity) in the protein database bank for which structural data  
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7 241 was available: GS from *B. subtilis* (acc. no. P12425). Superimposing the model structure of  
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9 242 *Gordonia* KTR9 GS-XplB on the GS from *B. subtilis* (RMSD: 1.141 Å) revealed the  
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11 243 structure, and position, of the missing region (Figure 4a). Based on this homology modelling,  
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13 244 and knowledge from the structure of the GS type I- $\alpha$  of *B. subtilis* (Murray *et al.*, 2013), it  
14  
15 245 was found that the missing residues in the GS fusion included two residues involved in the  
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17 246 formation of a Tyr loop in the active site. Residues located on the other loops mediating the  
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19 247 catalytic activity of the enzyme present in GS from *B. subtilis* were also found in the  
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21 248 *Gordonia* KTR9 GS-XplB. This included an Asn loop which shares remarkable similarity  
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23 249 with GS from *B. subtilis*, while the Asp<sup>50</sup>, on a latch loop was found to be extended in the  
24  
25 250 *Gordonia* KTR9 GS-XplB when compared to the GS from *B. subtilis*. Remarkably, despite  
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27 251 fusion and truncation, the GS-XplB protein has glutamine synthetase activity (Figure 4b).

## 252 Discussion

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



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3 263 In addition to *xplA* and *xplB*, there are several neighboring genes which are nearly identical  
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5 264 between the bacteria studied here. In *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 (Ander  
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7 265 *et al.*, 2009) and *Gordonia* sp. KTR9 (Indest *et al.*, 2010), there are transposable elements in  
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10 266 neighboring regions, suggesting that *xplA* and *xplB* are part of a larger mobile element such  
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12 267 as an Integrative and Conjugative Element (ICE) or genomic island in a conjugative plasmid.  
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14 268 Similarly, the genes for the degradation of xenobiotics such as chlorobenzoate, by  
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16 269 *Pseudomonas* sp. strain B13 (Ravatn *et al.*, 1998, Gaillard *et al.*, 2006); and biphenyl  
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18 270 degradation by *Ralstonia eutropha* A5 (Springael *et al.*, 2001), are also found partly on larger  
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20 271 mobile elements. Genomic islands and ICEs are known to excise and integrate into  
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22 272 chromosomes or plasmids through conjugation (Burrus *et al.*, 2002, van der Meer &  
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24 273 Sentchilo, 2003). In support of this, *R. rhodochrous* 11Y.058 (Figure 1) encodes a phage  
25  
26 274 related integrase belonging to the tyrosine recombinase family, which shares complete  
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28 275 identity with a site-specific recombinase from *R. erythropolis* PR4. Moreover, the RDX-  
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30 276 degradation capacity of *Gordonia* sp. KTR9 was successfully transferred into the non-RDX  
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32 277 degrading species *Gordonia polyisoprenivorans*, *Rhodococcus jostii* RHA1 and *Nocardia* sp.  
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34 278 TW2 through conjugation (Jung *et al.*, 2011), demonstrating that this is a possible transfer  
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36 279 mechanism.  
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41 280 Sequence analysis of the genes surrounding *xplA* and *xplB* showed that there are regions of  
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43 281 identity between the three species, and although *xplA* alone is sufficient for the catabolism of  
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45 282 RDX (Rylott *et al.*, 2006, Indest *et al.*, 2010), it has been speculated that some of these  
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47 283 neighboring genes contribute towards RDX degradation (Indest *et al.*, 2010, Indest *et al.*,  
48  
49 284 2013, Chong *et al.*, 2014, Zhu *et al.*, 2014). For example, downstream of *xplB* in *R.*  
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51 285 *rhodochrous* 11Y and *Microbacterium* sp. MA1 are genes encoding a putative permease  
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53 286 (AroP; 11Y.026 and MA1.029 in Figure 1) and transcriptional regulator (MarR; 11Y.025 and  
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3 287 MA1.027 in Figure 1). Orthologous permeases have been found close to genes with related  
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5 288 function (Wehrmann *et al.*, 1995, Yu *et al.*, 2007), and members of the MarR family shown  
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7 289 to play a role in regulating catabolism of aromatic compounds (Bussmann *et al.*, 2010; Chong  
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10 290 *et al.*, 2014). However, gene deletion analysis has demonstrated that AroP and MarR from *R.*  
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12 291 *rhodochrous* 11Y do not affect RDX degradation in this species (Chong *et al.*, 2014). In  
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14 292 *Gordonia* sp. KTR9, sequence analysis of pGKT2, the 182 kb plasmid carrying *xplA* and  
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16 293 *xplB* indicated that they are integrated into an operon involved in the degradation of N-  
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18 294 heterocyclic compounds (Zhu *et al.*, 2014). On this operon, the genes upstream of *xplA* and  
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20 295 *xplB* (*xplR*, *cyp151C* and *glnA*) share high sequence similarity and arrangement to the *mor*  
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22 296 and *pip* gene clusters involved in the degradation of morpholine, piperidine and related  
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25 297 compounds in *Mycobacterium* and *Rhodococcus* (Indest *et al.*, 2010).

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28 298 An *xplB* knock-out in *R. rhodochrous* 11Y demonstrated that although XplB is not required  
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30 299 for XplA activity; the absence of XplB reduces the rate of RDX-degradation by 70 % (Chong  
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32 300 *et al.*, 2014). The fact that an *xplB* knock-out can still degrade RDX indicates that alternative  
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34 301 endogenous reductases can substitute in bacteria, and this has also been demonstrated in  
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36 302 XplA-transformed plants (Jackson *et al.*, 2007). A previous comparison between the RDX  
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38 303 removal rates of RDX-degrading isolates showed that all *Rhodococcus* spp. had faster RDX  
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40 304 removal rates than *Microbacterium* sp. MA1 or *Gordonia* sp. KTR9 (Chong *et al.*, 2014).  
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42 305 Considering that XplA alone is able to denitrate the RDX structure in the organism (Rylott *et*  
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44 306 *al.*, 2006, Indest *et al.*, 2010), and is identical in species across all three genera (Chong *et al.*,  
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46 307 2014), the differences in the RDX-removal rate are due to the impairment of XplB,  
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48 308 differences in bacterial physiology, or a combination of both.

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53 309 The studies presented here indicate that mutations present in XplB in *Gordonia* sp. KTR9 and  
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55 310 *Microbacterium* sp. MA1 explain, to a degree, why these species exhibit reduced rates of

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3 311 RDX degradation when compared with *R. rhodochrous* 11Y. Our studies comparing the *R.*  
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5 312 *rhodochrous* 11Y Trp-386 with the Ser-385 found in *Gordonia* sp. KTR9 showed that Trp-  
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7 313 386 clearly plays a critical role in retaining FAD in XplB. This residue also appears to be  
8  
9 314 important for the functionality of other FAD containing proteins as multiple sequence  
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11 315 alignments revealed the residue to be highly conserved amongst FAD-containing proteins  
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13 316 that share as low as 42 % sequence identity with XplB. Additionally, in *Gordonia* sp. KTR9,  
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15 317 the fusion of GS to XplB is likely to further inhibit the reductase activity of XplB, and thus  
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17 318 RDX-degrading activity of *Gordonia* sp. KTR9. Considering that *Gordonia* sp. KTR9 was  
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19 319 isolated from soil where RDX was not detected (Thompson *et al.*, 2005), it is possible that  
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21 320 the recombination and reduction of the genomic island, which resulted in the fusion of the  
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23 321 XplB to GS, arose from the absence of selective pressure from RDX.  
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28 322 Despite the fact that the GS component of the *Gordonia* sp. KTR9 GS-XplB fusion is  
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30 323 truncated, it was found to be active, demonstrating that the purified GS protein is correctly  
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32 324 folded and the missing residues are not essential for functionality. The GS portion of the  
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34 325 *Gordonia* sp. KTR9 GS-XplB fusion belongs to GS type I- $\alpha$ . It is already known that the GS  
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36 326 type I- $\alpha$  from *B. subtilis* plays an important role in regulating cellular nitrogen levels by  
37  
38 327 controlling the expression of GlnR and TnrA. In the presence of glutamine, GS binds TnrA  
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40 328 directly onto the DNA, forming a GS-TnrA-DNA complex with its formation regulated by  
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42 329 the intracellular levels of ATP, AMP, glutamine, and glutamate. (Fisher & Wray, 2008, Wray  
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44 330 & Fisher, 2010, Murray *et al.*, 2013, Hauf *et al.*, 2016). Mutation of GS in *B. subtilis* resulted  
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46 331 in the constitutive expression of both GlnR and TnrA proteins (Wray & Fisher, 2010). In  
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48 332 *Gordonia* sp. KTR9, GlnR is important in the assimilation of nitrite in the cell. Knock-out of  
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50 333 *glnR* in *Gordonia* sp. KTR9 resulted in the accumulation of nitrite from RDX (Zhu *et al.*,  
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52 334 2014) and down-regulation of the nitrite reductase gene loci KTR9\_1306 and KTR9\_1307  
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3 335 (Indest *et al.*, 2013). Additionally, the *glnR* mutant lacked the ability to utilise RDX, nitrite or  
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5 336 nitrate as the sole source of nitrogen, but not ammonium or glutamine.  
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3 345 **Figure Legends**  
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5 346 **Figure 1.** Schematic representation of the RDX-degrading gene clusters in three genera of  
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7 347 aerobic RDX-degrading bacteria.

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11 348 White colored regions have no sequence homology. Dotted lines indicate unsequenced  
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13 349 regions, genes encoding transposable elements are shown in yellow.

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16 350 **Figure 2.** Purification and analysis of XplB proteins from *R. rhodochrous* 11Y,  
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18 351 *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9.

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21 352 (a) SDS-PAGE analysis of lysates (L) and purified proteins from *E. coli* cells expressing  
22  
23 353 XplA or XplB homologues. (b) Overlaid chromatograms showing FAD absorbance peaks  
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25 354 from purified proteins. (c) Appearance of purified proteins. (d) Reductase activity in cell  
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27 355 lysates expressing XplB homologues, measured using the Griess assay with purified XplA  
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29 356 and RDX as substrate ( $n = 3 \pm SD$ . Letters refer to significant differences; ANOVA, Tukey  
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31 357 HSD). (e) RDX removal from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and  
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33 358 *Gordonia* sp. KTR9 GS-XplB supplemented with purified XplA ( $n = 3 \pm SD$ ).  
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38 359 **Figure 3** Activities of XplB proteins carrying reciprocal mutations from *R. rhodochrous* 11Y  
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40 360 and *Gordonia* sp. KTR9.

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43 361 (a) Nitrite release from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *R.*  
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45 362 *rhodochrous* 11Y XplB-(W386S) measured using the Griess assay, with purified XplA and  
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47 363 RDX as substrate ( $n = 3 \pm SD$ ). (b) Nitrite release, observed using the Griess assay, by *E. coli*  
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49 364 cell lysates expressing *R. rhodochrous* 11Y XplB and *Gordonia* sp. KTR9 GS-XplB-  
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51 365 (S385W), with purified XplA and RDX as substrate. (c) RDX removal by *E. coli* cell lysates  
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53 366 expressing the *Gordonia* sp. KTR9 GS-XplB-(S385W) or empty vector ( $n = 3 \pm SD$ ).  
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3 367 **Figure 4** Characterization of the glutamine synthetase (GS) and XplB portions of *Gordonia*  
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5 368 sp. KTR9 GS-XplB fusion protein.  
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8 369 (a) Model structure of GS from GS-XplB (blue) superimposed on the GS structure of *B.*  
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10 370 *subtilis* (yellow). Sequence missing from GS-XplB (red). Root mean square deviation 1.14  
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12 371 °A). (b) GS activity in *E. coli* cell lysates ( $n = 3 \pm \text{SD}$ ).  
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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 (I) in *Microbacterium* sp. MA1; and the tryptophan-386 (W) residue that in the *Gordonia* sp. KTR9 GS-XplB fusion, corresponds to serine (S). Multiple species for genera have been omitted. The alignment was performed using ClustalW2 (EMBL European Bioinformatics Institute).

Organism	Identity (%)	UniProt AC	Sequence (N- to C-terminal)
<i>Rhodococcus rhodochrous</i> 11Y	100	Q8GPH8	...DIIRLLSKQPDE <b>F</b> TGSD... <b>W</b> MRIDEAEVASASPGRIRQKVREVD...
<i>Gordonia</i> sp. KTR9	99.8	E1R0R9	...DIIRLLSKQPDE <b>F</b> TGSD... <b>S</b> MRIDEAEVASASPGRIRQKVREVD...
<i>Microbacterium</i> sp. MA1	99.8	C3UMY2	...DIIRLLSKQPDE <b>I</b> TGSD... <b>W</b> MRIDEAEVASASPGRIRQKVREVD...
<i>Pseudomonas</i> sp.	47.7	A0A0V8SZM6	...DVLRLAKSAED <b>F</b> AGSD... <b>W</b> QRIDHAETRAAAPGRVRRKITDRA...
<i>Nitratireductor pacificus</i>	47.2	K2MCG5	...DLLRLLAKTPDE <b>L</b> AGSD... <b>W</b> KRIDAAEIAAAPENRCRVKINSRD...
<i>Marinovum algicola</i>	46.7	A0A0H4L107	...DLLRLLAKSPEE <b>L</b> EGSD... <b>W</b> CRIDSAETANPPPGRCAKITTRE...
<i>Ventosimonas gracilis</i>	44.5	A0A139SRD6	...DVLRLLIKPAHD <b>W</b> QGS... <b>W</b> QRIDTEEKQCAPAGRVRRQKITERV...
<i>Roseomonas mucosa</i>	44.5	A0A0W0A9P2	...DLARMLSKDEAE <b>L</b> AGSD... <b>W</b> LRIRAAEAAAASAGRVRRKGATRD...
<i>Mameliella alba</i>	44.4	A0A0B3RZQ4	...DLLRLLAKAPEE <b>L</b> DGSD... <b>W</b> SRIDAAETGAAPEGRCRTKLATRE...
<i>Ruegeria</i> sp.	44.1	A0A1E3D8F1	...DLLRLLAKAPEE <b>L</b> DGSD... <b>W</b> SRIDAAETGAAPEGRCRTKLATRE...
<i>Agrococcus jejuensis</i>	43	A0A1G8CDN7	...DVARLVARDAAD <b>F</b> DGTD... <b>W</b> RRIDAAETLAAAPGRRRAKLRTLD...
<i>Agrococcus pavilionensis</i>	43	U1LRN2	...DVVRLLRDAEGL <b>G</b> GGTD... <b>W</b> RRIDVREQLGAAPGRSRSKLRSA...
<i>Arthrobacter globiformis</i>	43	H0QK39	...DVVRFLIKTPGE <b>F</b> AGSD... <b>W</b> LRVDAEERAAAPGNRSRKKLPDHA...
<i>Leucobacter</i> sp.	42.1	A0A061LTW8	...DVLRFVLKDRDA <b>Y</b> EGSD... <b>W</b> LRLDEHERSVAPSGRVRRHKLPDHD...

**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- $\beta$  type GS (Brown *et al.*, 1994). The alignment was performed using ClustalW2 (EMBL European Bioinformatics Institute).

Organism	Class	UniProt AC	Sequence (N- to C-terminal)	
<i>Mycobacterium tuberculosis</i>	GSI- $\beta$	P9WN39	STGIADTAYFGAEAEFYIFDSVSVFDSRANGSFYEVDAISGWWNTGAATEA	170
<i>Streptomyces coelicolor</i>	GSI- $\beta$	P15106	STGIADTAFFGPEAEFYVFDVSRFATRENESFYHIDSEAGAWNTGALED-	165
<i>Synechococcus</i> sp.	GSI- $\beta$	P28605	ASGIGDTAYFGPEAEFFVFDVRFQDQENKGFYYVDSVEGRWNSGRKEP-	168
<i>Anabaena</i> sp.	GSI- $\beta$	K7W630	STGLGDTAFFGPEAEFFIFDDVRYDQTTNSGYYYVDSVEGRWNTGREE--	166
<i>Salmonella typhimurium</i>	GSI- $\beta$	P0A1P6	ATGIADTVLFGPEPEFFLFDDIRFGASISGSHVAIDDIEGAWNSSTKYE-	166
<i>Pyrococcus furiosus</i>	GSI- $\alpha$	Q05907	KE--GYKAYIGPEPEFYLFK-----KNGTWELEIPDV-	141
<i>Haloferax volcanii</i>	GSI- $\alpha$	P43386	ELGY-DVNVA-PEPEFFLFEE-----DEDGRATTVTNDA-	164
<i>Bacillus subtilis</i>	GSI- $\alpha$	P12425	DLGFSDFNLG-PEPEFFLFKL-----DEKGEPTLELNDK-	153
<i>Methanococcus voltae</i>	GSI- $\alpha$	P21154	EEFKGEYFVG-PEPEFFILK-----NENK--WVPGDD-	156
<i>Gordonia</i> sp. KTR9	GSI- $\alpha$	E1R0R9	ERTGLEMRTG-TEPEMTWEG-----EGFETTFRPDS-	176

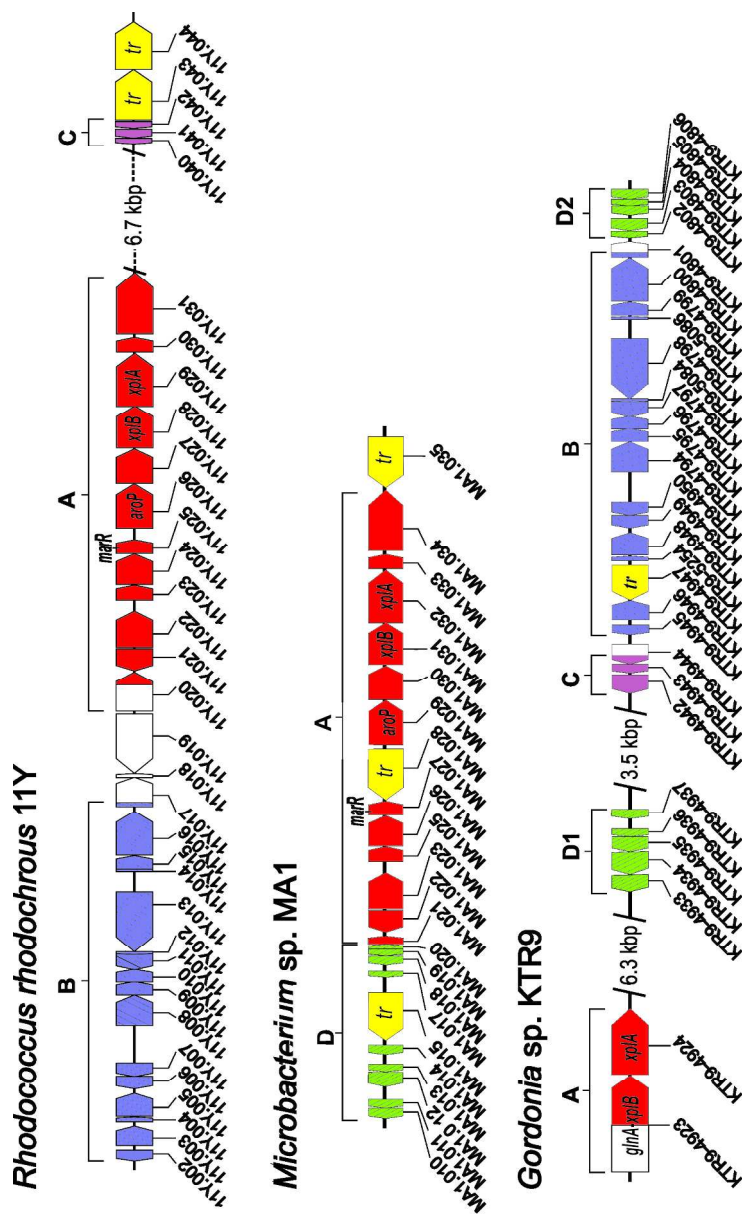


Figure 1. Schematic representation of the RDX-degrading gene clusters in three genera of aerobic RDX-degrading bacteria.

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)

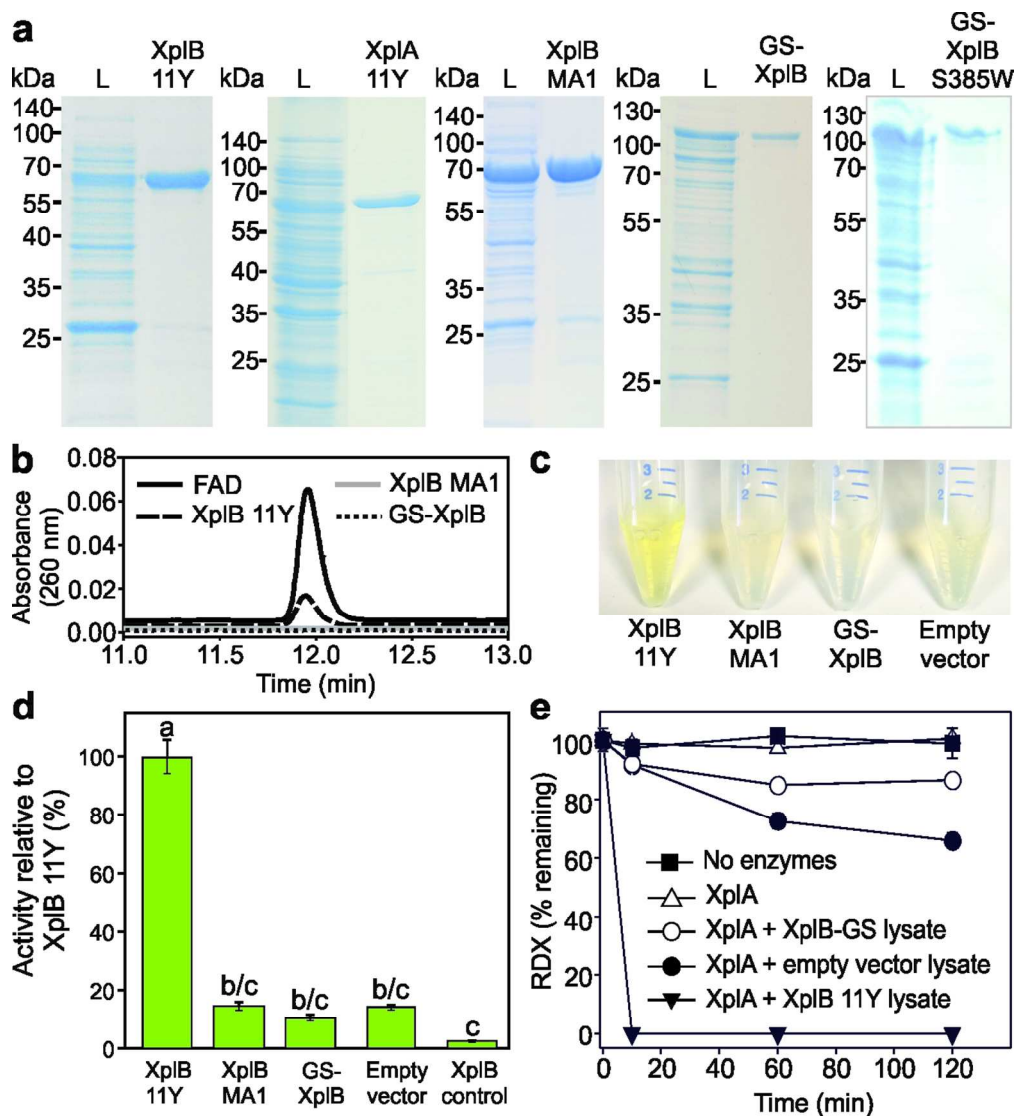


Figure 2. Purification and analysis of *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9 XplB!! + (a) SDS-PAGE analysis of lysates (L) and purified proteins from *E. coli* cells expressing XplA or XplB homologs. (b) Overlaid chromatograms showing FAD absorbance peaks from purified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

113x124mm (300 x 300 DPI)



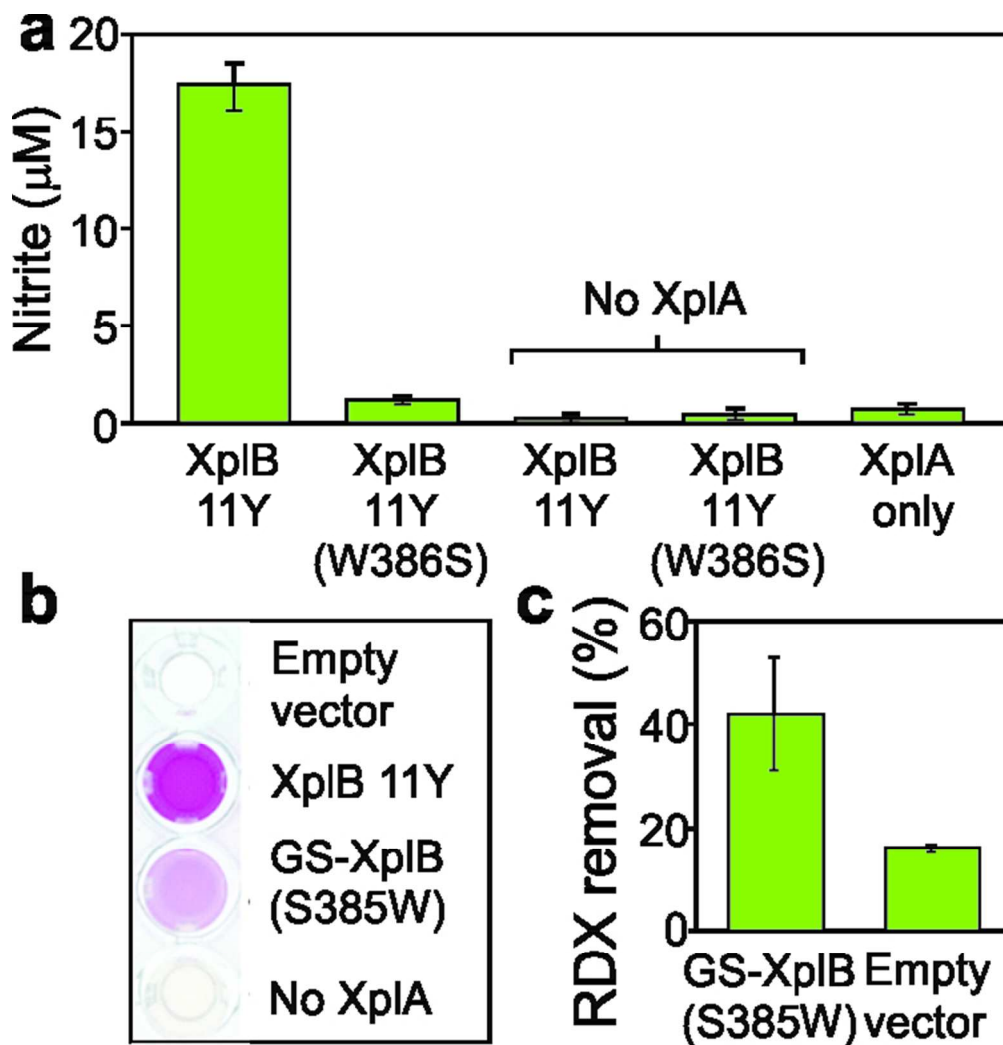
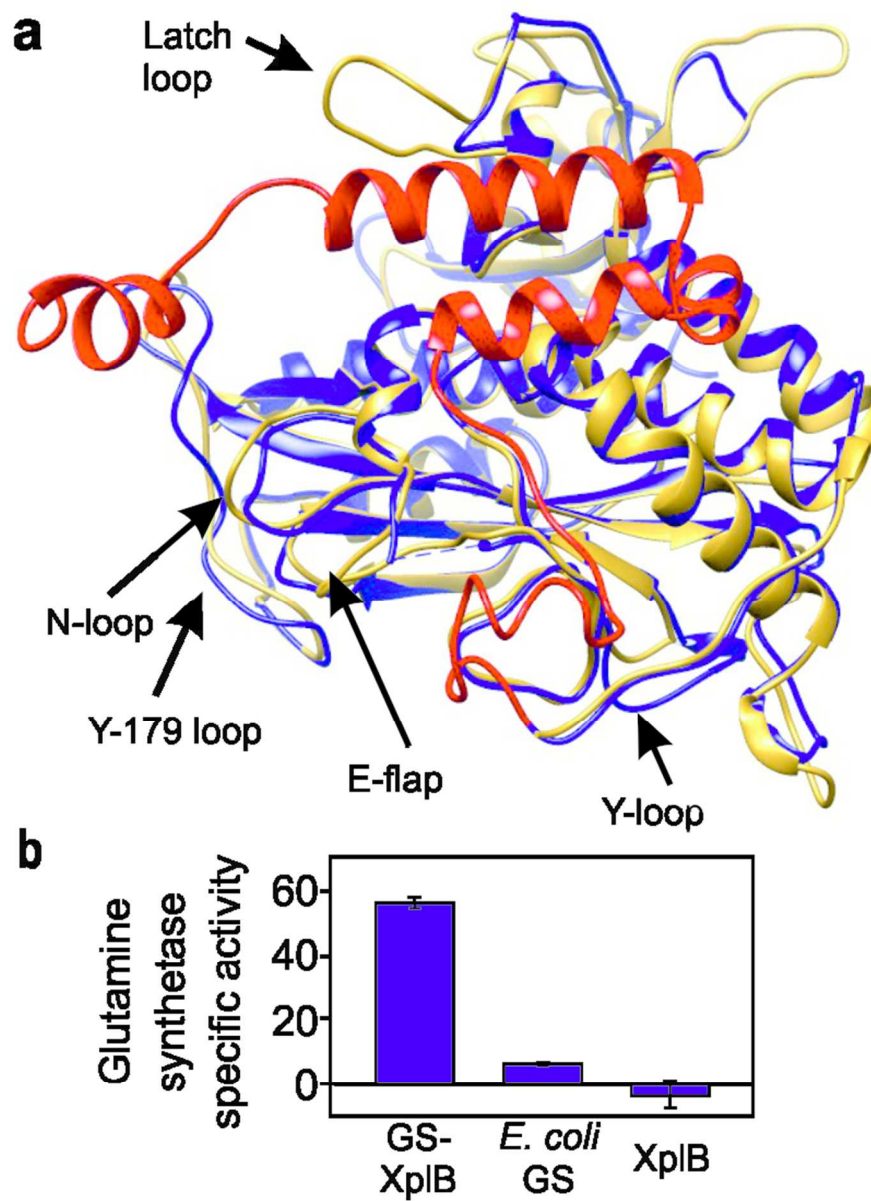


Figure 3 Activities of XplB proteins carrying reciprocal mutations from *R. rhodochrous* 11Y and *Gordonia* sp. KTR9.

(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 ( $n = 3 \pm SD$ ). (b) Nitrite release, observed using the Griess 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 ( $n = 3 \pm SD$ ).

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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 Å. (b) GS activity in *E. coli* cell lysates ( $n = 3 \pm \text{SD}$ ).

68x93mm (300 x 300 DPI)