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Biotechnology Letters

A cofactor consumption screen identifies promising NfsB family nitroreductases for dinitrotoluene remediation

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Funding Information:	<table border="1"> <tr> <td>Marsden Fund (VUW0704)</td> <td>Professor David Ackerley</td> </tr> <tr> <td>Marsden Fund (VUW1502)</td> <td>Professor David Ackerley</td> </tr> <tr> <td>Biotechnology and Biological Sciences Research Council (BB/P005713/1)</td> <td>Professor Neil C Bruce</td> </tr> <tr> <td>Strategic Environmental Research and Development Program (ER-2723)</td> <td>Professor Neil C Bruce</td> </tr> </table>	Marsden Fund (VUW0704)	Professor David Ackerley	Marsden Fund (VUW1502)	Professor David Ackerley	Biotechnology and Biological Sciences Research Council (BB/P005713/1)	Professor Neil C Bruce	Strategic Environmental Research and Development Program (ER-2723)	Professor Neil C Bruce
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Abstract:	<p>Objectives</p> <p>To survey a library of over-expressed nitroreductases to identify those most active with 2,4- and 2,6-dinitrotoluene substrates, as promising candidates for phytoremediation of soils and groundwater contaminated with poly-nitro toluene pollutants.</p> <p>Results</p> <p>To indirectly monitor dinitrotoluene reduction we implemented a nitroblue tetrazolium dye screen to compare relative rates of NADPH consumption for 58 nitroreductase candidates, over-expressed in a nitroreductase-deleted strain of Escherichia coli. Although the screen only provides activity data at a single substrate concentration, by</p>								

	<p>altering the substrate concentration and duration of incubation we showed we could first distinguish between more-active and less-active enzymes and then discriminate between the relative rates of reduction exhibited by the most active nitroreductases in the collection. We observed that members of the NfsA and NfsB nitroreductase families were the most active with 2,4-dinitrotoluene, but that only members of the NfsB family were proficient with 2,6-dinitrotoluene. Two NfsB family members, YfkO from <i>Bacillus subtilis</i> and NfsB from <i>Vibrio vulnificus</i>, appeared especially effective with these substrates. Purification of both enzymes as His6-tagged recombinant proteins enabled in vitro determination of Michaelis-Menten kinetic parameters with each dinitrotoluene substrate.</p> <p>Conclusions</p> <p><i>V. vulnificus</i> NfsB is a particularly promising candidate for bioremediation applications, being ca. 5-fold more catalytically efficient with 2,4-dinitrotoluene and over 26-fold more active with 2,6-dinitrotoluene than the benchmark <i>E. coli</i> nitroreductases NfsA and NfsB.</p>
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A cofactor consumption screen identifies promising NfsB family nitroreductases for dinitrotoluene remediation

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1 **Abstract**

2 *Objectives:* To survey a library of over-expressed nitroreductases to identify those most
3 active with 2,4- and 2,6-dinitrotoluene substrates, as promising candidates for
4 phytoremediation of soils and groundwater contaminated with poly-nitro toluene
5 pollutants.

6 *Results:* To indirectly monitor dinitrotoluene reduction we implemented a nitroblue
7 tetrazolium dye screen to compare relative rates of NADPH consumption for 58
8 nitroreductase candidates, over-expressed in a nitroreductase-deleted strain of *Escherichia*
9 *coli*. Although the screen only provides activity data at a single substrate concentration, by
10 altering the substrate concentration and duration of incubation we showed we could first
11 distinguish between more-active and less-active enzymes and then discriminate between
12 the relative rates of reduction exhibited by the most active nitroreductases in the collection.
13 We observed that members of the NfsA and NfsB nitroreductase families were the most
14 active with 2,4-dinitrotoluene, but that only members of the NfsB family were proficient
15 with 2,6-dinitrotoluene. Two NfsB family members, YfkO from *Bacillus subtilis* and NfsB from
16 *Vibrio vulnificus*, appeared especially effective with these substrates. Purification of both
17 enzymes as His₆-tagged recombinant proteins enabled *in vitro* determination of Michaelis-
18 Menten kinetic parameters with each dinitrotoluene substrate.

19 *Conclusions:* *V. vulnificus* NfsB is a particularly promising candidate for bioremediation
20 applications, being *ca.* 5-fold more catalytically efficient with 2,4-dinitrotoluene and over
21 26-fold more active with 2,6-dinitrotoluene than the benchmark *E. coli* nitroreductases NfsA
22 and NfsB.

23 *Keywords:* Bioremediation; Dinitrotoluene; NADPH depletion assay; NfsA; NfsB;
24 Nitroreductase; YfkO.

25 Introduction

26 2,4-dinitrotoluene and 2,6- dinitrotoluene, widely used as propellants or as precursors in the
27 manufacture of 2,4,6-trinitrotoluene (TNT), are toxic and persistent contaminants of soil and
28 groundwater (Dontsova et al, 2009). The strongly electron-withdrawing nitro groups on
29 poly-nitro toluenes delocalise the electronic charge distribution in the aromatic rings,
30 rendering them resistant to oxidative degradation by other enzymes (Roldan et al, 2008).
31 Bacterial type I nitroreductases, which reduce nitro substituents via concerted two-electron
32 transfer steps, yield derivatives that are far more amenable substrates for ring degrading
33 enzymes (Williams et al, 2015; Roldan et al, 2008).

34 Plants engineered for high-level expression of bacterial nitroreductases have shown
35 enhanced transformation and tolerance of poly-nitro toluenes, offering prospects for
36 phytoremediation of contaminated environments (Zhang et al, 2019; Van Dillewijn et al,
37 2008; Hannink et al, 2007). However, despite there being a great diversity of bacterial
38 nitroreductases available in nature (Akiva et al, 2017), very few candidates have been
39 surveyed to assess their relative potential for these applications. A dearth of characterised
40 nitroreductase candidates is particularly evident in the context of dinitrotoluene
41 remediation.

42 Effective surveying of novel enzyme candidates requires an efficient activity screen. We
43 previously described how *Escherichia coli* cells over-expressing nitroreductase enzymes that
44 are active with dinitrotoluene substrates became more sensitive to these toxins, and that
45 IC₅₀ data were somewhat predictive of relative levels of catalytic efficiency (k_{cat}/K_M) for the
46 purified enzymes (Rich et al, 2018). However, as IC₅₀ calculations require measurements to
47 be made across a broad range of substrate concentrations, this is not an approach

48 amenable to large-scale screening of candidate gene libraries. Here we describe our
49 implementation of an alternative assay, based on cofactor consumption, to rapidly identify
50 nitroreductase enzymes proficient in reduction of both 2,4- and 2,6-dinitrotoluene.

51

52 **Materials and Methods**

53 *Chemicals, plasmids, bacterial strains and nitroreductases*

54 All reagents were purchased from Sigma-Aldrich (Saint Louis, MO). For protein purification,
55 the genes encoding YfkO from *Bacillus subtilis* and NfsB from *Vibrio vulnificus* were
56 expressed from the His₆-tag expression vector pET28a+ in *E. coli* strain BL21(DE3) (Novagen,
57 Merck Millipore; Billerica, MA) as previously described (Prosser et al, 2013). For library
58 screening and IC₅₀ assays, nitroreductase candidates were over-expressed from plasmid
59 pUCX in *E. coli* 7NT, a gene-deleted derivative of strain W3110 that lacks seven endogenous
60 oxidoreductase genes (*nfsA*, *nfsB*, *azoR*, *nema*, *yieF*, *ycaK* and *mdaB*) to minimise
61 background nitroreductase activity and an efflux pump gene (*tolC*) to maximise substrate
62 retention (Copp et al, 2014). The 58-membered nitroreductase gene library was constructed
63 as previously described (Mowday et al, 2016) and the full list of candidate genes (with
64 UniProt default identifiers) is summarised in Table 1.

65 <Insert Table 1 here>

66

67 *Nitroblue tetrazolium assay*

68 Cells of *E. coli* 7NT pUCX:nitroreductase strains were used to inoculate wells of a sterile 96-
69 well microplate containing 200 μ L lysogeny broth (LB) supplemented with ampicillin (100
70 μ g.mL⁻¹) and glucose (0.2% w/v). Cultures were incubated at 30 °C, 200 rpm for 16 hours,

71 after which 10 μL of each overnight culture was used to inoculate wells of a sterile 96-well
72 microplate containing 190 μL induction medium (LB medium supplemented with ampicillin
73 ($100 \mu\text{g}\cdot\text{mL}^{-1}$), glucose (0.2% w/v) and IPTG (0.05 mM). Induced cultures were incubated at
74 30 °C, 200 rpm for 6 hours. Crude cell lysates were prepared by incubating 100 μL of each
75 culture with BugBuster® Protein Extraction Reagent at a 1:1 (v/v) ratio at room temperature
76 for 30 min. To monitor cell lysate NADPH consumption, duplicate 200 μL reactions were
77 prepared consisting of 100 mM KPO_4 buffer pH 8.0, crude cell lysate (40 μL for full library
78 screening assays or 5 μL for NfsB-family screening assays) and 150 μM substrate (2,4-
79 dinitrotoluene or 2,6-dinitrotoluene), and reactions were initiated by addition of 50 μL of
80 800 μM NADPH. Reactions were incubated at room temperature for 30 min for full library
81 screening assays, or 10 min for NfsB-family screening assays. Reactions were halted by the
82 addition of 50 μL nitroblue tetrazolium solution (nitroblue tetrazolium ($2 \text{ mg}\cdot\text{mL}^{-1}$),
83 phenazine methosulfate ($0.3 \text{ mg}\cdot\text{mL}^{-1}$, prepared fresh prior to use) and 100 mM KPO_4 buffer
84 (pH 8.0)). Levels of formazan production, corresponding to the amount of NADPH remaining
85 in each well, were quantified 3-5 min post addition of the nitroblue tetrazolium solution by
86 measuring absorbance at 590 nm using an EnSpire™ 2300 Multilabel Reader (PerkinElmer,
87 Waltham, MA). In pilot tests, four *E. coli* 7NT pUCX:nitroreductase strains (expressing the
88 genes encoding NfrA_Bli, NfsA_Vv, Ydgl_Bs and YcnD_Bs) exhibited a substantial decrease in
89 NADPH levels in the absence of substrate. Reasoning that this high background NADPH
90 oxidase activity would confound measurement of substrate consumption, these strains
91 were excluded from the full library analysis.

92

93 *IC₅₀ assays*

94 Cells of *E. coli* 7NT pUCX:*nitroreductase* strains were used to inoculate wells of sterile 96-
95 well microplates containing 200 μ L LB medium supplemented with ampicillin ($100 \mu\text{g}\cdot\text{mL}^{-1}$)
96 and glucose (0.2% w/v). Cultures were incubated at 30 °C, 200 rpm for 16 hours, after which
97 they were diluted 20-fold into 15 mL centrifuge tubes containing 2 mL induction medium (LB
98 medium supplemented with ampicillin ($100 \mu\text{g}\cdot\text{mL}^{-1}$), glucose (0.2% w/v) and IPTG (0.05
99 mM). Induced cultures were incubated at 30 °C, 200 rpm for 2.5 hours. Aliquots (30 μ L) of
100 each culture were added to wells of a sterile 384-well microplate containing 30 μ L induction
101 medium \pm two-fold the desired final dinitrotoluene concentration. In total, each strain was
102 challenged in duplicate across 7 to 15 conditions containing 1.5-fold increasing titrations of
103 2,4-dinitrotoluene or 2,6-dinitrotoluene together with a medium-only control. The
104 absorbance of each well at 600 nm was measured using an EnSpire™ 2300 Multilabel
105 Reader (PerkinElmer, Waltham, MA). Cultures were incubated at 30 °C, 200 rpm for a
106 further 4 hours, after which absorbance readings at 600 nm were recorded once more. The
107 increase in absorbances at 600 nm of corresponding challenged and unchallenged wells for
108 each strain were compared and used to calculate percentage growth following challenge.
109 IC_{50} values (the concentration of 2,4-dinitrotoluene or 2,6-dinitrotoluene estimated to
110 reduce growth by 50% relative to the unchallenged control) were calculated using a dose-
111 response inhibition four-parameter variable slope equation in GraphPad Prism 7.0
112 (GraphPad Software Inc., La Jolla, CA).

113

114 *His₆-tagged protein purification and Michaelis-Menten kinetics*

115 Recombinant His₆-tagged nitroreductases were purified by nickel-affinity chromatography
116 using Ni/NTA resin (Novagen, Merck, Darmstadt, Germany), followed by incubation with a
117 molar excess of FMN cofactor and buffer exchange as previously described (Prosser et al,

118 2010). Apparent steady state enzyme kinetics were determined via monitoring decrease in
119 absorbance at 340 nm (NADPH extinction co-efficient, $6,220 \text{ M}^{-1} \text{ cm}^{-1}$) at a fixed
120 concentration of NADPH and varying concentrations of 2,4-dinitrotoluene or 2,6-
121 dinitrotoluene. Reactions were carried out in 60 μL volumes in UVettes™ (Eppendorf,
122 Hamburg, Germany), using a 1 cm path length, and the wavelength was measured using a
123 Helios γ UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Reactions
124 consisted of 10 mM Tris-Cl pH 7.0 buffer, 250 μM NADPH, 2% DMSO and substrate
125 concentrations ranging from 0 - 400 μM 2,4-dinitrotoluene and 0 -1600 μM 2,6-
126 dinitrotoluene. Reactions were initiated by addition of an appropriate dilution of enzyme
127 and the linear decrease in absorbance monitored for the first 20-40 s post enzyme addition.
128 Non-linear regression analyses and Michaelis-Menten curve fitting was performed using
129 GraphPad Prism v 7.0 (GraphPad Software, San Diego, California, USA).

130

131 **Results and Discussion**

132 To identify enzymes capable of activating diverse nitroaromatic prodrugs we previously
133 generated a 58-membered library of nitroreductase candidates in *Escherichia coli* reporter
134 strains that express either a β -galactosidase or GFP gene under control of a DNA damage
135 responsive (SOS) promoter (Mowday et al, 2016; Copp et al, 2017). We tested whether
136 dinitrotoluene reduction could likewise be monitored via SOS assays, reasoning that
137 reduction of TNT is known to yield genotoxic metabolites (Bolt et al, 2006). However, we
138 found that both 2,4-dinitrotoluene and 2,6 dinitrotoluene evoked high levels of background
139 signal in nitroreductase-expressing reporter strains that precluded accurate screening (not
140 shown). We therefore sought to implement an alternative screening procedure based on

141 consumption of the NAD(P)H cofactor that acts as an electron source for bacterial Type I
142 nitroreductases. For this, we adapted a colorimetric nitroblue tetrazolium assay, previously
143 described by Mayer and Arnold (2002) for directed evolution of 6-phosphogluconate
144 dehydrogenase enzymes.

145 In the presence of NADPH yellow nitroblue tetrazolium is reduced to a purple formazan dye
146 that can be quantified at 590 nm. This allowed us to measure the relative levels of
147 dinitrotoluene reduction in lysates derived from *E. coli* strains over-expressing
148 nitroreductase candidates. To minimise background nitroreductase activity, screening of the
149 full 58-membered nitroreductase candidate library was performed in a strain of *E. coli*
150 lacking endogenous *nfsA*, *nfsB*, *azoR*, *nemaA*, *yieF*, *ycaK* and *mdaB* genes (strain 7NT; Copp et
151 al, 2014). Lysates were incubated with NADPH and either 2,4-dinitrotoluene or 2,6-
152 dinitrotoluene, then reactions were stopped with nitroblue tetrazolium chloride and the
153 amount of formazan in challenged lysates compared to unchallenged duplicates.

154 The full library screen confirmed that, of the enzymes tested, NfsA and NfsB family
155 nitroreductases consistently consumed the most NADPH in the presence of dinitrotoluene
156 substrates (Figure 1A). Notably, although members of both the NfsA and NfsB families were
157 represented among the top 25 most active enzymes with 2,4-dinitrotoluene as substrate,
158 the NfsB family enzymes markedly outperformed those from the NfsA family with 2,6-
159 dinitrotoluene as substrate (Figure 1A). To distinguish the relative activities of the NfsB
160 family enzymes it was necessary to reduce the amount of culture lysate from 40 μ L for the
161 full library screening assay to 5 μ L and the incubation time from 30 min to 10 min (Figure
162 1B).

163 <Insert Figure 1 here>

164 We next sought to test whether results from the nitroblue tetrazolium assay were generally
165 congruent with IC₅₀ data by performing detailed IC₅₀ assays for all strains expressing *nfsB*
166 family genes (Table 2) and correlating the measured IC₅₀ values (μM) with NADPH
167 consumption (percent ΔA₅₉₀) (Figure 2). The inverse correlation between IC₅₀ and NADPH
168 consumption for 2,4-dinitrotoluene was moderate rather than strong ($r^2 = 0.35$); this is likely
169 a consequence of the nitroblue tetrazolium assay providing a ‘snapshot’ of activity at a
170 single substrate concentration, whereas IC₅₀ data derive from a more comprehensive
171 midpoint of activity across a titration range. However, an additional advantage of the rapid
172 nitroblue tetrazolium assay over performing more extensive IC₅₀ measurements was evident
173 in its ability to generate data for 2,6-dinitrotoluene, whereas IC₅₀ values could not be
174 calculated for the majority of *nfsB*-expressing strains owing to insufficient toxicity at the
175 achievable solubility limit (Table 2).

176 <Insert Table 2 here> <Insert Figure 2 here>

177 Based on NADPH consumption data, the NfsB family enzyme *Bacillus subtilis* YfkO (YfkO_Bs)
178 appeared most active with 2,4-dinitrotoluene, whereas *Vibrio vulnificus* NfsB (NfsB_Vv)
179 appeared most active with 2,6-dinitrotoluene (Figure 1B). We purified each enzyme as a
180 His₆-tagged recombinant protein by nickel affinity chromatography and measured their
181 Michaelis-Menten kinetic parameters with either 2,4-dinitrotoluene or 2,6-dinitrotoluene as
182 the reductive substrate (Table 3). In terms of catalytic efficiency, the k_{cat}/K_M values for
183 NfsB_Vv and YfkO_Bs with 2,4-dinitrotoluene were 4- to 7-fold higher than those previously
184 reported for either of the canonical Type I nitroreductases NfsA_Ec and NfsB_Ec (Rich et al,
185 2018). Their superiority with 2,6-dinitrotoluene was even more profound, with the
186 measured k_{cat}/K_M values for NfsB_Vv and YfkO_Bs being 20- to 37-fold higher than those
187 previously reported for NfsA_Ec and NfsB_Ec (Rich et al, 2018). NfsB_Vv was particularly

188 effective with each substrate, and appears a promising candidate for expression in
189 transgenic dinitrotoluene remediation systems.

190 <Insert Table 3 here>

191

192 **Conclusions**

193 We have previously used activation of the *E. coli* SOS response to report on genotoxicity as a
194 measure of nitroreductase-mediated activation of prodrug substrates. Here, to monitor
195 activity with dinitrotoluene substrates that did not yield a clear SOS signal upon reduction,
196 we implemented a more generally applicable screen for nitroreductase activity based on
197 consumption of exogenously added NADPH that provided a dominant electron source for
198 the reaction. In addition to providing much higher throughput than IC₅₀ assays, we showed
199 that our screen could provide relative activity data with substrates such as 2,6-
200 dinitrotoluene that are insufficiently toxic at achievable concentrations for IC₅₀ values to be
201 calculated. The linear range of the assay was insufficient to discriminate between the more
202 active NfsB family nitroreductases while still detecting the less active NfsA and AzoR family
203 enzymes. However, by reducing both the substrate concentration and duration of
204 incubation we were able to discriminate between the different NfsB family nitroreductases
205 and identified *V. vulnificus* NfsB and *B. subtilis* YfkO as two of the most active dinitrotoluene
206 reducing enzymes in our collection. Purification of these nitroreductases as His₆-tagged
207 proteins enabled steady state kinetic assays to be performed, demonstrating *V. vulnificus*
208 NfsB to be over 5-fold more active with 2,4-dinitrotoluene and over 26-fold more active
209 with 2,6-dinitrotoluene than either of the canonical nitroreductases *E. coli* NfsA and NfsB.

210

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216

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Table 1. Nitroreductase candidate library

<i>Bacterial strain nitroreductase sourced from</i>	Nitroreductase gene	UniProt identifier
<i>Bacillus amyloliquefaciens</i> CAMR 0454	<i>ywrO</i>	Q8VSR5
<i>Bacillus coagulans</i> ATCC 7050	<i>nfsA</i>	G2TLJ0
<i>Bacillus licheniformis</i> ATCC14580	<i>nfrA</i>	Q65DM9
	<i>ycnD</i>	Q65ND8
	<i>yfkO</i>	Q65MG6
<i>Bacillus subtilis</i> NZ isolate	<i>nfrA</i>	P39605
	<i>ycnD</i>	P94424
	<i>ydgl</i>	P96707
	<i>yfkO</i>	O34475
	<i>ywrO</i>	P80871
<i>Bacillus thuringiensis</i> serovar konkukian str. 97-27	<i>nfsA</i>	Q6HKT9
<i>Citrobacter koseri</i> ATCC 27156	<i>nfsA</i>	A8AIR6
	<i>nfsB</i>	A8AJP2
<i>Cronobacter</i> (previously <i>Enterobacter</i>) <i>sakazakii</i> ATCC 29544	<i>nfsA</i>	A7MF39
	<i>nfsB</i>	A7MK61
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i> SCRI1043	<i>nfsA</i>	Q6D3R4
<i>Escherichia coli</i> W3110	<i>azoR</i>	P41407
	<i>mdaB</i>	P0AEY5
	<i>nemaA</i>	P77258
	<i>nfsA</i>	P17117
	<i>nfsB</i>	P38489
	<i>wrbA</i>	A1A9Q9
	<i>ycaK</i>	P43340
	<i>ycdI</i>	P75894
	<i>ydjA</i>	P0ACY1
	<i>yieF</i>	P0AGE6
<i>Klebsiella pneumoniae</i> ATCC 13883	<i>nemaA</i>	A6T9Z9
	<i>nfsA</i>	A6TT6U7
	<i>nfsB</i>	A6T5Y2
	<i>ycdI</i>	A6T798
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> 23K	<i>nfsA</i>	Q38UW5
<i>Listeria innocua</i> Clip11262	<i>nfsA</i>	Q92D83
	<i>ywrO</i>	Q92AM4
<i>Listeria welshimeri</i> ATCC 35897	<i>nfsA</i>	A0AH51
<i>Mycobacterium smegmatis</i> mc ² 155	<i>nfsA</i>	A0QWF2
<i>Neisseria meningitidis</i> strain H44/76	<i>nfsB</i>	E6N0E6
<i>Nostoc punctiforme</i> PCC73102	<i>nfsA</i>	B2J8C5
<i>Pseudomonas aeruginosa</i> PAO1	<i>ycaK</i>	Q9I4B3
	<i>ydgl</i>	Q9HTZ9
	<i>yieF</i>	O31038
<i>Pseudomonas putida</i> KT2440	<i>azoR</i>	Q88EC8
	<i>nfsA</i>	Q88K03

	<i>nfsB</i>	Q88K59
<i>Pseudomonas savastanoi</i> pv. <i>phaseolicola</i>	<i>mdaB</i>	Q48NL7
	<i>wrbA</i>	E7PSG4
<i>Salmonella typhi</i> ATCC 19430	<i>azoR</i>	P63462
	<i>nema</i>	Q8Z6P3
	<i>nfsA</i>	Q8Z848
	<i>nfsB</i>	Q8Z8M5
<i>Vibrio fischeri</i> ATCC 7744	<i>frasel</i>	P46072
	<i>nfsA</i>	B5EVB7
<i>Vibrio harveyi</i> ATCC 14126	<i>frp</i>	Q56691
	<i>nfsB</i>	A6AP86
<i>Vibrio harveyi</i> KCTC 2720	<i>frp</i> , herein referred to as <i>frp2</i>	AAM73721 (Genbank)
<i>Vibrio vulnificus</i> ATCC 27562	<i>azoR</i>	Q8DA68
	<i>nfsA</i>	Q8D4B5
	<i>nfsB</i>	Q7MCD2
	<i>nema</i>	Q8D549

Table 2. Dinitrotoluene IC₅₀ values for *E. coli* 7NT pUCX:nitroreductase strains

Nitroreductase	IC ₅₀ (μM)	
	2,6-dinitrotoluene	2,4-dinitrotoluene
YfkO_Bs	670 ± 60	330 ± 60
NfsB_Nme	680 ± 70	310 ± 60
NfsB_Vv	800 ± 70	510 ± 110
NfsB_Cs	940 ± 130	530 ± 60
YfkO_Bli	>1000	470 ± 40
NfsB_Pp	>1000	700 ± 140
NfsB_Kp	>1000	740 ± 20
Frasel_Vf	>1000	750 ± 50
NfsB_Vh	>1000	770 ± 60
NfsB_St	>1000	790 ± 20
NfsB_Ck	>1000	800 ± 60
NfsB_Ec	>1000	840 ± 60

Table 3. Michaelis-Menten kinetic parameters for reduction of 2,4-dinitrotoluene and 2,6-dinitrotoluene by purified His₆-tagged nitroreductases

Nitroreductase	Substrate	K_M^1 (μM)	k_{cat}^1 (s^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$)
<i>B. subtilis</i> YfkO	2,4-dinitrotoluene	62 ± 7	140 ± 42	440,000 ± 140,000
	2,6-dinitrotoluene	510 ± 80	22 ± 2	43,000 ± 7,000
<i>V. vulnificus</i> NfsB	2,4-dinitrotoluene	320 ± 50	200 ± 20	640,000 ± 110,000
	2,6-dinitrotoluene	1600 ± 110	90 ± 4	57,000 ± 5,000

¹Apparent kinetic parameters as measured at 250 μM NADPH

Figure Legends

Figure 1. NADPH consumption by *E. coli* 7NT pUCX:*nitroreductase* cell lysates in the

presence of 2,4- or 2,6-dinitrotoluene compounds. A. Crude cell lysates (40 μ l) of nitroreductase-expressing *E. coli* 7NT strains were incubated with 200 μ M NADPH and 150 μ M of either 2,4-dinitrotoluene (2,4-DNT) or 2,6-dinitrotoluene (2,6-DNT) for 30 min. Addition of nitroblue tetrazolium post-incubation yielded formazan dye in proportion to the remaining NADPH, which was quantified by measuring absorbance at 490 nm. Plotted values indicate the percentage of NADPH consumed by dinitrotoluene-challenged lysates during the reaction compared to unchallenged duplicate controls. Individual *E. coli* 7NT pUCX:*nitroreductase* strains are labelled according to the name of the nitroreductase (NTR) followed by a two- or three-letter abbreviation that defines the genus and species of the bacteria that the enzyme was derived from (a full list of all NTRs tested in this study and their accession codes is provided in the Materials and Methods). Data are the average of three independent assays \pm SD. **B.** To delineate the activities of NfsB-family NTRs, the assay was repeated as in panel **A**, only using a smaller volume of crude cell lysate (5 μ l) and shorter incubation time (10 min).

Figure 2. Scatter diagram and correlation analysis of NfsB-family nitroreductase activities

with 2,4-dinitrotoluene in nitroblue tetrazolium and IC₅₀ assays. Correlation analysis showed that 2,4-dinitrotoluene (2,4-DNT) IC₅₀ values for *E. coli* 7NT pUCX:*nitroreductase* strains were negatively correlated ($p < 0.05$, Pearson's correlation test) with the percentage of NADPH consumed by the corresponding strain lysates in nitroblue tetrazolium assays ($r^2=0.35$).

Figure 1

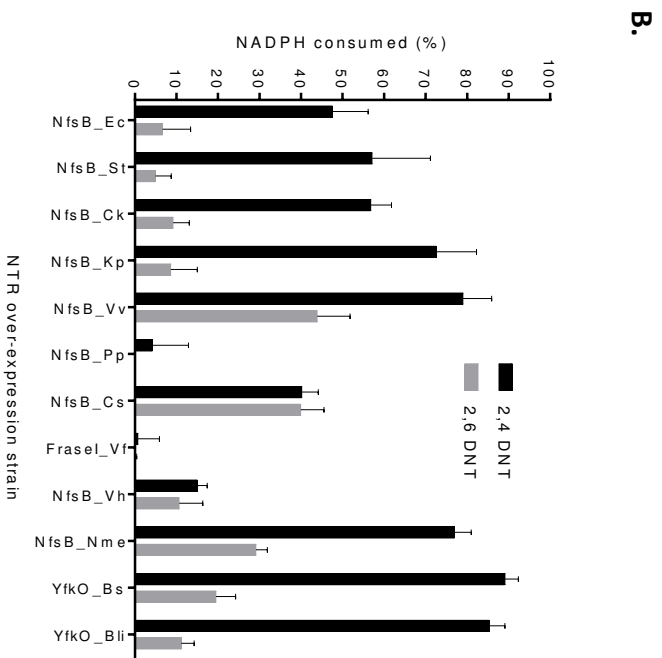
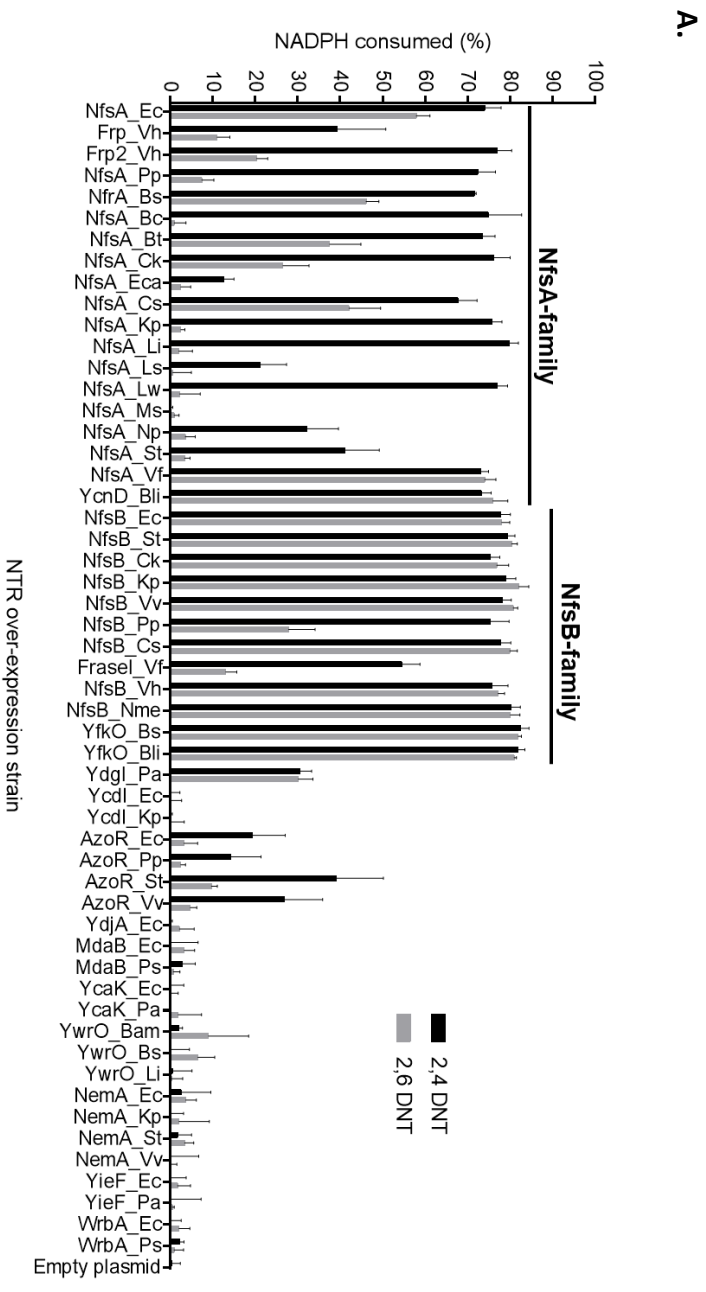
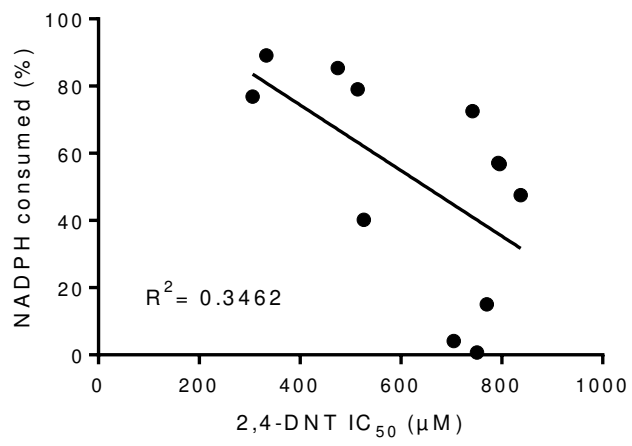


Figure 2



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David Akerley 25-MAY-2019