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

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Abstract:

Objectives

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.

Results

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

	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 Bacillus subtilis and NfsB from Vibrio vulnificus, 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. Conclusions V. vulnificus 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 E. coli nitroreductases NfsA and NfsB.
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A cofactor consumption screen identifies promising NfsB family nitroreductases for dinitrotoluene remediation

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Abstract

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Conclusions: V. vulnificus 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 *E. coli* nitroreductases NfsA and NfsB.

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

Introduction

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

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

Effective surveying of novel enzyme candidates requires an efficient activity screen. We previously described how *Escherichia coli* cells over-expressing nitroreductase enzymes that are active with dinitrotoluene substrates became more sensitive to these toxins, and that IC_{50} data were somewhat predictive of relative levels of catalytic efficiency (k_{cat}/K_M) for the purified enzymes (Rich et al, 2018). However, as IC_{50} calculations require measurements to be made across a broad range of substrate concentrations, this is not an approach

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

Materials and Methods

Chemicals, plasmids, bacterial strains and nitroreductases

All reagents were purchased from Sigma-Aldrich (Saint Louis, MO). For protein purification, the genes encoding YfkO from *Bacillus subtilis* and NfsB from *Vibrio vulnificus* were expressed from the His_6-tag expression vector pET28a+ in *E. coli* strain BL21(DE3) (Novagen, Merck Millipore; Billerica, MA) as previously described (Prosser et al, 2013). For library screening and IC_{50} assays, nitroreductase candidates were over-expressed from plasmid pUCX in *E. coli* 7NT, a gene-deleted derivative of strain W3110 that lacks seven endogenous oxidoreductase genes (*nfsA, nfsB*, *azoR*, *nemA*, *yieF*, *ycaK* and *mdaB*) to minimise background nitroreductase activity and an efflux pump gene (*tolC*) to maximise substrate retention (Copp et al, 2014). The 58-membered nitroreductase gene library was constructed as previously described (Mowday et al, 2016) and the full list of candidate genes (with UniProt default identifiers) is summarised in Table 1.

<Insert Table 1 here>

Nitroblue tetrazolium assay

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

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

IC_{50} assays

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

His_6-tagged protein purification and Michaelis-Menten kinetics

Recombinant His_6-tagged nitroreductases were purified by nickel-affinity chromatography using Ni/NTA resin (Novagen, Merck, Darmstadt, Germany), followed by incubation with a molar excess of FMN cofactor and buffer exchange as previously described (Prosser et al,

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

Results and Discussion

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

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

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

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

<Insert Figure 1 here>

We next sought to test whether results from the nitroblue tetrazolium assay were generally congruent with IC_{50} data by performing detailed IC_{50} assays for all strains expressing *nfsB* family genes (Table 2) and correlating the measured IC_{50} values (µM) with NADPH consumption (percent ΔA_{590}) (Figure 2). The inverse correlation between IC_{50} and NADPH consumption for 2,4-dinitrotoluene was moderate rather than strong ($r^2 = 0.35$); this is likely a consequence of the nitroblue tetrazolium assay providing a 'snapshot' of activity at a single substrate concentration, whereas IC_{50} data derive from a more comprehensive midpoint of activity across a titration range. However, an additional advantage of the rapid nitroblue tetrazolium assay over performing more extensive IC_{50} measurements was evident in its ability to generate data for 2,6-dinitrotoluene, whereas IC_{50} values could not be calculated for the majority of *nfsB*-expressing strains owing to insufficient toxicity at the achievable solubility limit (Table 2).

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

Based on NADPH consumption data, the NfsB family enzyme *Bacillus subtilis* YfkO (YfkO_Bs) appeared most active with 2,4-dinitrotoluene, whereas *Vibrio vulnificus* NfsB (NfsB_Vv) appeared most active with 2,6-dinitrotoluene (Figure 1B). We purified each enzyme as a His_6-tagged recombinant protein by nickel affinity chromatography and measured their Michaelis-Menten kinetic parameters with either 2,4-dinitrotoluene or 2,6-dinitrotoluene as the reductive substrate (Table 3). In terms of catalytic efficiency, the k_{cat}/K_M values for NfsB_Vv and YfkO_Bs with 2,4-dinitrotoluene were 4- to 7-fold higher than those previously reported for either of the canonical Type I nitroreductases NfsA_Ec and NfsB_Ec (Rich et al, 2018). Their superiority with 2,6-dinitrotoluene was even more profound, with the measured k_{cat}/K_M values for NfsB_Vv and YfkO_Bs being 20- to 37-fold higher than those previously reported for NfsA_Ec and NfsB_Ec (Rich et al, 2018). NfsB_Vv was particularly

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

<Insert Table 3 here>

Conclusions

We have previously used activation of the *E. coli* SOS response to report on genotoxicity as a measure of nitroreductase-mediated activation of prodrug substrates. Here, to monitor activity with dinitrotoluene substrates that did not yield a clear SOS signal upon reduction, we implemented a more generally applicable screen for nitroreductase activity based on consumption of exogenously added NADPH that provided a dominant electron source for the reaction. In addition to providing much higher throughput than IC_{50} assays, we showed that our screen could provide relative activity data with substrates such as 2,6-dinitrotoluene that are insufficiently toxic at achievable concentrations for IC_{50} values to be calculated. The linear range of the assay was insufficient to discriminate between the more active NfsB family nitroreductases while still detecting the less active NfsA and AzoR family enzymes. However, by reducing both the substrate concentration and duration of incubation we were able to discriminate between the different NfsB family nitroreductases and identified *V. vulnificus* NfsB and *B. subtilis* YfkO as two of the most active dinitrotoluene reducing enzymes in our collection. Purification of these nitroreductases as His_6-tagged proteins enabled steady state kinetic assays to be performed, demonstrating *V. vulnificus* NfsB to be over 5-fold more active with 2,4-dinitrotoluene and over 26-fold more active with 2,6-dinitrotoluene than either of the canonical nitroreductases *E. coli* NfsA and NfsB.

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Table 1. Nitroreductase candidate library

Bacterial strain nitroreductase sourced from	**Nitroreductase gene**	**UniProt identifier**
Bacillus amyloliquefaciens CAMR 0454	*ywrO*	Q8VSR5
Bacillus coagulans ATCC 7050	*nfsA*	G2TLJ0
Bacillus licheniformis ATCC14580	*nfrA*	Q65DM9
	ycnD	Q65ND8
	yfkO	Q65MG6
Bacillus subtilis NZ isolate	*nfrA*	P39605
	ycnD	P94424
	ydgI	P96707
	yfkO	O34475
	ywrO	P80871
Bacillus thuringiensis serovar konkukian str. 97-27	*nfsA*	Q6HKT9
Citrobacter koseri ATCC 27156	*nfsA*	A8AIR6
	nfsB	A8AJP2
Cronobacter (previously *Enterobacter*) *sakazakii* ATCC 29544	*nfsA*	A7MF39
	nfsB	A7MK61
Erwinia carotovora subsp*. atroseptica* SCRI1043	*nfsA*	Q6D3R4
Escherichia coli W3110	*azoR*	P41407
	mdaB	P0AEY5
	nemA	P77258
	nfsA	P17117
	nfsB	P38489
	wrbA	A1A9Q9
	ycaK	P43340
	ycdI	P75894
	ydjA	P0ACY1
	yieF	P0AGE6
Klebsiella pneumoniae ATCC 13883	*nemA*	A6T9Z9
	nfsA	A6TT6U7
	nfsB	A6T5Y2
	ycdI	A6T798
Lactobacillus sakei subsp*. sakei* 23K	*nfsA*	Q38UW5
Listeria innocua Clip11262	*nfsA*	Q92D83
	ywrO	Q92AM4
Listeria welshimeri ATCC 35897	*nfsA*	A0AH51
Mycobacterium smegmatis mc^2155	*nfsA*	A0QWF2
Neisseria meningitidis strain H44/76	*nfsB*	E6N0E6
Nostoc punctiforme PCC73102	*nfsA*	B2J8C5
Pseudomonas aeruginosa PAO1	*ycaK*	Q9I4B3
	ydgI	Q9HTZ9
	yieF	O31038
Pseudomonas putida KT2440	*azoR*	Q88EC8
	nfsA	Q88K03

	nfsB	Q88K59
Pseudomonas savastanoi pv. phaseolicola	*mdaB*	Q48NL7
	wrbA	E7PSG4
Salmonella typhi ATCC l9430	*azoR*	P63462
	nemA	Q8Z6P3
	nfsA	Q8Z848
	nfsB	Q8Z8M5
Vibrio fischeri ATCC 7744	*fraseI*	P46072
	nfsA	B5EVB7
Vibrio harveyi ATCC 14126	*frp*	Q56691
	nfsB	A6AP86
Vibrio harveyi KCTC 2720	*frp,* herein referred to as *frp2*	AAM73721 (Genbank)
Vibrio vulnificus ATCC 27562	*azoR*	Q8DA68
	nfsA	Q8D4B5
	nfsB	Q7MCD2
	nemA	Q8D549

Table 2. Dinitrotoluene IC_{50} values for *E. coli* 7NT pUCX:*nitroreductase* strains

	IC_{50} (µM)	
Nitroreductase	**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
FraseI_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 His_6-tagged nitroreductases

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

[1]Apparent kinetic parameters as measured at 250 μM NADPH

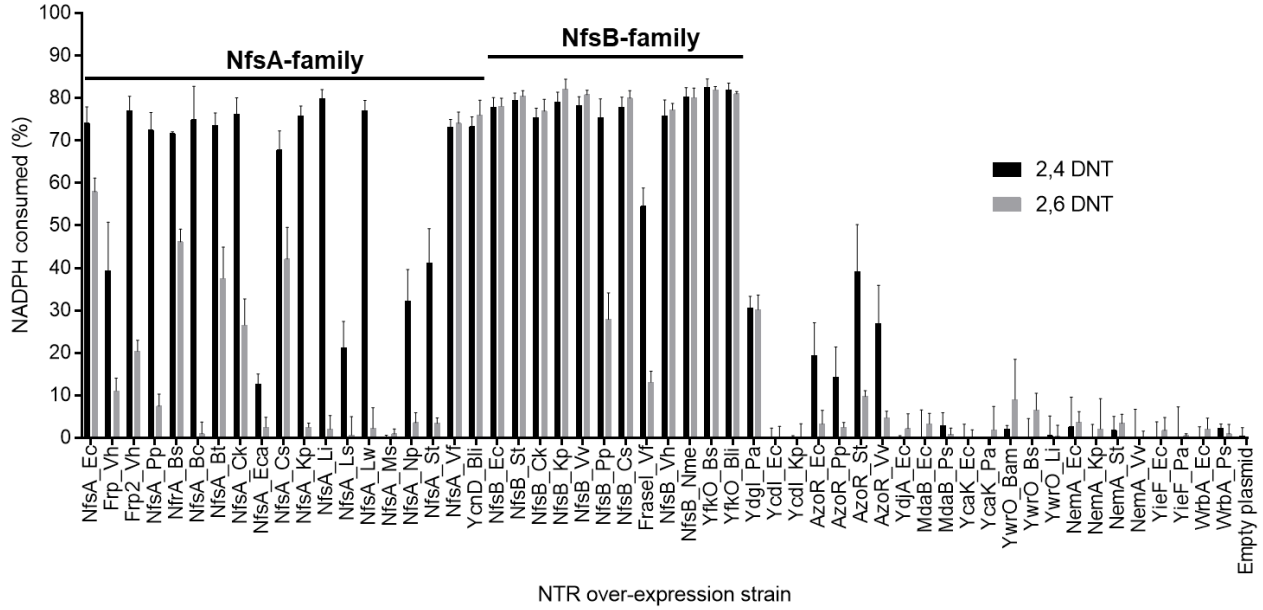
Figure Legends

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 µl) of nitroreductase-expressing *E. coli* 7NT strains were incubated with 200 µM NADPH and 150 µM 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 ± 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 µl) 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 IC_{50} assays. Correlation analysis showed that 2,4-dinitrotoluene (2,4-DNT) IC_{50} 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

A.


NfsA-family
NfsB-family
NADPH consumed (%)
2,4 DNT
2,6 DNT
NfsA_Ec
Frp_Vh
Frp2_Vh
NfsA_Pp
NfrA_Bs
NfsA_Bc
NfsA_Bt
NfsA_Ck
NfsA_Eca
NfsA_Cs
NfsA_Kp
NfsA_Li
NfsA_Ls
NfsA_Lw
NfsA_Ms
NfsA_Np
NfsA_St
NfsA_Vf
YcnD_Bli
NfsB_Ec
NfsB_St
NfsB_Ck
NfsB_Kp
NfsB_Vv
NfsB_Pp
NfsB_Cs
FraseI_Vf
NfsB_Vh
NfsB_Nme
YfkO_Bs
YfkO_Bli
Ydgl_Pa
YcdI_Ec
YcdI_Kp
AzoR_Ec
AzoR_Pp
AzoR_St
AzoR_Vv
YdjA_Ec
MdaB_Ec
MdaB_Ps
YcaK_Ec
YcaK_Pa
YwrO_Bam
YwrO_Bs
YwrO_Li
NemA_Ec
NemA_Kp
NemA_St
NemA_Vv
YieF_Ec
YieF_Pa
WrbA_Ec
WrbA_Ps
Empty plasmid
NTR over-expression strain

B.

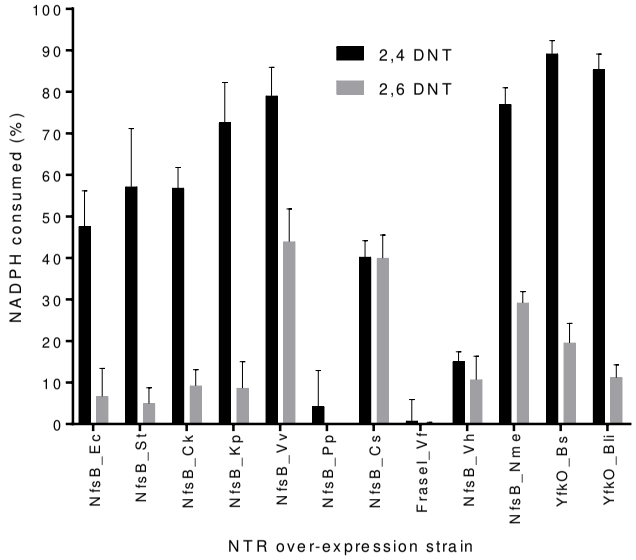
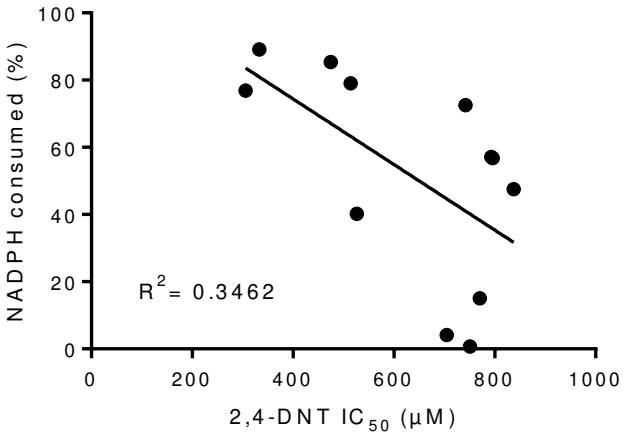

2,4 DNT
2,6 DNT
NADPH consumed (%)
NfsB_Ec
NfsB_St
NfsB_Ck
NfsB_Kp
NfsB_Vv
NfsB_Pp
NfsB_Cs
FraseI_Vf
NfsB_Vh
NfsB_Nme
YfkO_Bs
YfkO_Bli
NTR over-expression strain

Figure 2


NADPH consumed (%)
100
80
60
40
20
0
R^2= 0.3462
0
200
400
600
800
1000
2,4-DNT IC_{50} (µM)

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David Ackerley 25-MAY-2019