Nitroreductase from *Salmonella typhimurium*: characterization and catalytic activity

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The biocatalytic activity of nitroreductase from *Salmonella typhimurium* (NRSal) was investigated for the reduction of α,β-unsaturated carbonyl compounds, nitroalkenes, and nitroaromatics. The synthesized gene was subcloned into a pET28 overexpression system in *E.coli* BL21 strain, and the corresponding expressed protein was purified to homogeneity with 15% protein mass yield and 41% of total activity recovery. NRSal showed broad substrate acceptance for various nitro compounds such as 1-nitrocyclohexene and aliphatic nitroalkenes (alkene reductase activity), as well as nitrobenzene (nitroreductase activity), with substrate conversion efficiency of > 95%. However, the reduction of enones was generally low, proceeding albeit with high stereoselectivity. The efficient biocatalytic reduction of substituted nitroalkenes provides a route for the preparation of the corresponding nitroalkanes. NRSal also demonstrated the first single isolated enzyme-catalyzed reduction of nitrobenzene to aniline through the formation of nitrosobenzene and phenylhydroxylamine as intermediates. However, chemical condensation of the two intermediates to produce azoxybenzene currently limits the yield of aniline.

Introduction

Flavoenzymes are biocatalysts that catalyze a wide range of biochemical reactions ranging from dehydrogenation, one- and two-electron transfer redox reactions, oxygen activation during oxidation and hydroxylation reactions, to light emission in bioluminescent bacteria.[1–2] As part of flavoenzymes, the recently growing "Old Yellow Enzyme" (OYE) family was reported to catalyze the reduction of various substrates such as α,β-unsaturated aldehydes, ketones, imides, esters, nitriles, and carboxylic acids.[3–6] The asymmetric bioreduction of C=C bonds employing OYE family proteins can create up to two chiral centers and therefore constitutes an interesting route towards enantiomerically pure compounds. The C=C bond reduction catalyzed by enoate reductases proceeds *via* a ping-pong mechanism, where the flavin is first reduced at the expense of a nicotinamide cofactor in the reductive half-reaction, followed by the asymmetric reduction of the substrates through *trans*-hydrogenation (oxidative half-reaction).[7–9] The OYE family also features nitroreductase activity through biodegradation of explosive compounds such as PETN, TNT, and nitroglycerin. Several members of the OYE family such as XenA and XenB from *Pseudomonas sp.*,[10] PETN reductase from *Enterobacter cloacae*,[11] OYEs from *Saccharomyces sp.*,[12] YqjM from *Bacillus subtilis*,[13] and OPRs from *Arabidopsis thaliana*[14] have been characterized for their biocatalytic activity on both nitroaromatics and aliphatic nitro compounds.

In our previous work, we have characterized three un-related enoate reductases: XenA from *Pseudomonas putida*, KYE1 from *Kluyveromyces lactis*, and Yers-ER from *Yersinia bercovieri*. We have shown that these three enoate reductases feature broad substrate specificity but different substrate preferences on a set of various α,β-unsaturated carbonyl compounds.[6] Since enoate reductases often possess nitroreductase activity, we initiated a new search for a candidate that possesses broad substrate specificity for nitro compounds, however, applying a different strategy: the investigation of nitroreductases for their enoate reductase activity, with specific emphasis on the biocatalytic reduction of nitroalkenes to provide an efficient route for the synthesis of chiral substituted nitroalkanes. This strategy was based on the observation that some nitroreductases had shown reductase activity on quinones.[57,58,59]

Nitroalkanes are potential intermediates in the synthesis of amines, aldehydes, carboxylic acids or denitrated compounds.[15] The traditional organic synthesis of enantiomerically pure nitroalkanes occurs through asymmetric conjugate addition to nitroalkenes,[17–19] reduction of nitroalkenes with transition metal catalyst,[20] hydrogenation with Jacobsen-type organocatalyst,[21] or addition of α,β-unsaturated bonds onto nitroalkanes.[22] Due to various restrictions on these organic synthesis processes (such as growing environmental restrictions on the type of catalyst used, narrow substrate acceptance), an efficient biocatalytic route for chiral nitroalkanes is highly desirable. Recently, several published works have demonstrated stereoselective reduction of nitroalkenes using: OPR1 and OPR3 from *Lycopersicon esculentum*,[13] OYE from *Saccharomyces carlsbergensis* and OYE2-3 from *Saccharomyces cerevisiae*,[12] NCR from *Zymomonas mobilis*,[12] YqjM from *Bacillus subtilis*,[13] PETN reductase from *Enterobacter cloacae*,[11] and crude enzyme preparation from *Clostridium sporogenes*.[16] These enzymatic processes are a valuable addition to the established synthetic toolbox and inspire the search for new candidates.

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Table 1 Purification of wild type NRSal monitored with nitrofurazone activity[a]

Purification step	Total protein/mg[b]	Total activity [U]	Specific activity [U/mg]	Yield (%)
Lysate	53.3	285	5.34	100
$(NH_4)_2SO_4$	22.4	136	5.02	48
Dialysis	22.0	149	6.78	52
DEAE	8.20	116	14.2	41

[a] Dry cell weight 1.1 g L^{-1}. [b] Nitrofurazone activity assay: 50 mM TrisHCl buffer pH 7.2, 0.05 mM Nitrofurazone, 0.1 μM NRSal, GDH recycling system: (8 μM NADPH, 2 mM Glucose, 10 U GDH). The activity was calculated by monitoring the decrease of nitrofurazone wavelength at 375 nm with extinction coefficient of $\varepsilon_{375nm} = 15{,}000$ $(M\ cm)^{-1}$.[30]

In this context, we came across a homolog of nitroreductase from *Enterobacter cloacae*: nitroreductase from *Salmonella typhimurium* (NRSal). NRSal was first discovered in 1989 from *Salmonella typhimurium* strain TA1538NR,[23] and represents a new class of flavin-dependent nitroreductase enzymes.[24–27] The nucleotide sequence of this oxygen-insensitive FMN-containing NRSal encodes for 217 amino acids, with a calculated molecular weight of 23,955 Da. This enzyme has been shown to reduce some nitroaromatic compounds, such as *p*-nitrophenol or *p*-nitrobenzoic acid.[28] Since then however, there has been no published work about any further characterization of NRSal, and especially its catalytic activity, which renders it an interesting candidate for us to explore. In this paper, we examine the ability of NRSal to catalyze the reduction of α,β-unsaturated carbonyl compounds, nitroalkenes, and nitroaromatics.

Results & discussion

Cloning, overexpression, and purification

The gene coding for NRSal was purchased from BioBasic Inc (Ontario, Canada) according to the published sequence (UnitProtKB accession # P15888).[23,29] Sequence analysis revealed that NRSal from *Salmonella typhimurium* shares high amino acid identity and similarity with nitroreductase from *Enterobacter cloacae* with 89% identity and 93% similarity (Fig. 1). The gene product was cloned into vector pET28a(+) (Novagen, San Diego, CA) at the following restriction sites: NcoI and HindIII with stop codon for expression of wild type NRSal, NcoI and HindII for expression of NRSal with C-terminal polyhistidine tag, and NdeI and HindIII with stop codon for expression of NRSal with N-terminal polyhistidine tag. The addition of the polyhistidine tag was for ease of enzyme purification.

The purification of wild-type NRSal was optimized compared to previously published method.[28] The original six steps of NRSal purification were reduced to two steps, with first ammonium sulfate precipitation to remove the majority of non-proteinaceous biological material, followed by anion exchange column chromatography with DEAE-Sepharose column to isolate pure NRSal with 15% protein mass recovery from the cell lysate sample, and with total activity recovery of 41%. The purification of wild-type NRSal was monitored with the standard activity assay on nitrofurazone[28] (Table 1) followed by SDS-PAGE analysis (Fig. 2). The final elution showed a single band upon SDS-PAGE analysis. The NRSal with N- or C-terminal polyhistidine tag was purified according to the standard Ni^{2+}-NTA bead protocol from Qiagen (Valencia, CA). However, the activity of NRSal with polyhistidine tag was found to be much lower compared to the wild type. Activity check was performed by monitoring the reduction of nitrofurazone (known substrate for NRSal[28]) and cyclohexenone (common substrate for enoate reductases). NRSal with N- or C-terminal polyhistidine tag showed specific activity of 4.40 U/mg and 3.27 U/mg respectively for nitrofurazone reduction, which was much lower compared to wild type specific activity of 14.2 U/mg. An analogous pattern was observed for the reduction of cyclohexenone, where wild type NRSal showed significantly higher specific activity (0.39 U/mg) compared to NRSal with N-terminal (0.16 U/mg) and C-terminal (0.17 U/mg) polyhistidine tag. Since the presence of the polyhistidine tag considerably decreased enzymatic activity of NRSal, further characterization was continued only with wild-type, untagged-NRSal.

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NR_SAL   1   MDIVSVALQRYSTKAFDPSKKLTAEEADKIKTLLQYSPSSTNSQPWHFIV    50
             |||:||||:|:||||||.|||||||||:||||||||||||||||||||||
NR_ENT   1   MDIISVALKRHSTKAFDASKKLTAEEAEKIKTLLQYSPSSTNSQPWHFIV    50
NR_SAL  51   ASTEEGKARVAKSAAGNYTFNERKMLDASHVVVFCAKTAMDDAWLERVVD   100
             ||||||||||||||||.|.|||||||||||||||||||||||||||||||||
NR_ENT  51   ASTEEGKARVAKSAAGTYVFNERKMLDASHVVVFCAKTAMDDAWLERVVD   100
NR_SAL 101   QEDADGRFATPEAKAANDKGRRFFADMHRVSLKDDHQWMAKQVYLNVGNF   150
             ||:|||||.|||||||.|||.:||||||||.||||.|||||||||||||||
NR_ENT 101   QEEADGRFNTPEAKAANHKGRTYFADMHRVDLKDDDQWMAKQVYLNVGNF   150
NR_SAL 151   LLGVAAMGLDAVPIEGFDAEVLDAEFGLKEKGYTSLVVVPVGHHSVEDFN   200
             ||||.|||||||||||||.:||.||||||||:||||||||||||||||||||
NR_ENT 151   LLGVGAMGLDAVPIEGFDAAILDEEFGLKEKGFTSLVVVPVGHHSVEDFN   200
NR_SAL 201   AGLPKSRLPLETTLTEV    217
             |.|||||||.|.:||.
NR_ENT 201   ATLPKSRLPLSTIVTEC    217
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Fig. 1 Amino acid alignment of nitroreductase NRSal from *Salmonella typhimurium* (P15888, NR_Sal) and nitroreductase from *Enterobacter cloacae* (Q01234, NR_ENT) using EMBOSS Pairwise Alignment Needle Algorithm (http://www.ebi.ac.uk).

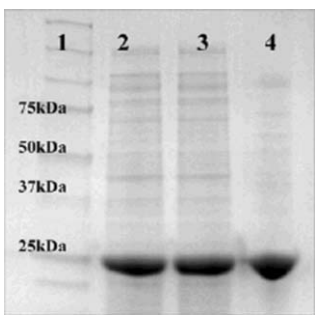


Fig. 2 SDS-PAGE analysis for purification of wild type NRSal: lane 1 protein ladder, lane 2 cell lysate sample, lane 3 70% $(NH_4)_2SO_4$ precipitation, lane 4 DEAE-Sepharose column.

Enzymatic activity & stability study

We first investigated the effect of temperature and pH on NRSal by monitoring the change of enzymatic activity over the range of 10–60 °C (Fig. 3), and pH range of 4–9 (Fig. 4). The activity-temperature profile showed maximum specific activity of 13.6 U/mg for reduction of nitrofurazone at 45 °C and pH 7.5, which was 3.2-fold higher than the activity at room temperature (20 °C). Arrhenius plots yield an activation energy E_a of 42.7 kJ mol^{-1} for the temperature range of 10–45 °C (r^2 = 0.963), and a deactivation energy E_d of −31.5 kJ mol^{-1} for the temperature range of 45–60 °C (r^2 = 0.965). The activity-pH profile showed an optimum pH at 7.2 with highest specific activity of 8.10 U/mg for reduction of nitrofurazone at 20 °C in 50 mM TrisHCl buffer. We also found that NRSal had lower activity (but similar activity–pH pattern with a maximum activity at pH 7.2) in 50 mM phosphate buffer compared to 50 mM TrisHCl buffer. The activity of NRSal is therefore buffer dependent and TrisHCl is the optimum buffer for reactions run between pH 6–7.5. Additionally, NRSal considerably lost its activity beyond pH 8.

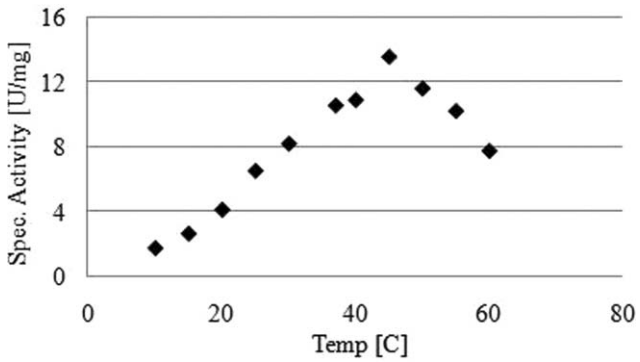


Fig. 3 Activity–temperature profile of NRSal. Activity was measured with standard nitrofurazone activity assay in 50 mM phosphate buffer at pH 7.5.

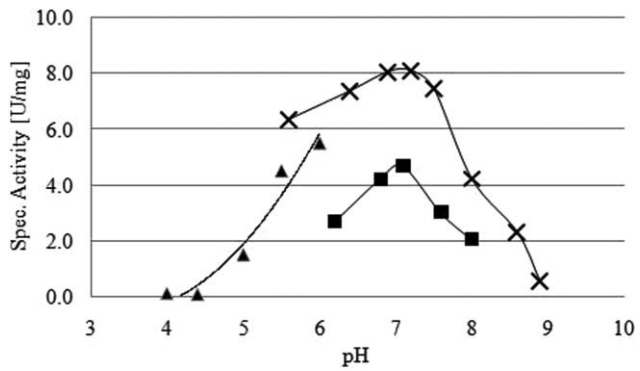


Fig. 4 Activity–pH profile of NRSal in three different buffers: 50 mM TrisHCl (x), 20 mM citrate buffer (▲), and 50 mM phosphate buffer (■). Activity was measured with standard nitrofurazone activity assay at 20 °C.

Since thermal stability of enzymes is a key consideration for application in larger scale processes, we investigated the kinetic stability of NRSal by measuring its half-life at three different temperature points of 37, 45, and 50 °C (Table 2). The deactivation of NRSal at all temperatures followed first order deactivation kinetic (data not shown). Also, NRSal seemed reasonably stable with shortest half-life of 2.3 h measured at 50 °C, and half-life decreased exponentially with increasing temperatures.

Table 2 Half-life of NRSal measured at various temperatures using 1-nitrocyclohexene as substrate

T/°C	Half-life	$k_{d,obs}$ [a]
37	365 h	0.0019 h^{-1}
45	17.9 h	0.0388 h^{-1}
50	2.3 h	0.3100 h^{-1}

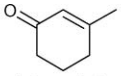
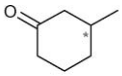
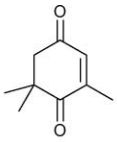
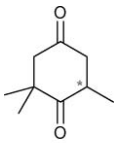
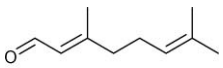
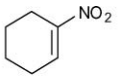
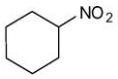
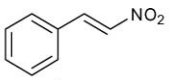
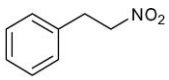
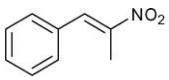
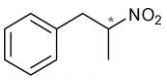
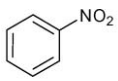
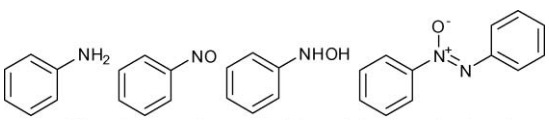
[a] $k_{d,obs}$ is the observed deactivation rate constant

Substrate spectrum characterization

With finding of a broad enoate reductase activity in a nitroreductase as one of our goals, we investigated the catalytic activity of purified NRSal with several common α,β-unsaturated carbonyl substrates for enoate reductases (Table 3. Entries **1**–**4**). We first tested NRSal activity on cyclohex-2-enone (**1**) and related substrate 3-methyl-2-cyclohexenone (**2**).[31–33] The nitroreductase enzyme displayed enoate reductase activity on both substrates. Both compounds **1** and **2** were reduced, albeit with maximum substrate conversion of only 12% and 5.2% respectively. NRSal showed strong stereospecificity in the reduction of **2** and yielded (*R*)-3-methyl-2-cyclohexanone with 84% e.e. Substrates ketoisophorone (**3**) and citral (**4**) were also tested due to their large-scale industry application to produce the corresponding levodione and citronellal, respectively.[34–37] NRSal showed modest maximum conversion of 12% with **3** to produce (*R*)-levodione with 87% e.e, and did not show any biocatalytic reduction of aldehyde **4**. This remarkable stereoselectivity for a nitroreductase in reducing C=C bonds may point at a mechanism similar to that of OYEs, as OYEs proceed *via* a ping pong Bi Bi mechanism, as do nitroreductases in the reduction of the nitro group.[7–9,57,58]

Despite the low conversions with α,β-unsaturated carbonyl substrates, we hypothesized that NRSal, as a nitroreductase, would display stronger alkene reductase activity in the presence of a nitro group. Indeed, NRSal showed broader substrate acceptance for nitro related compounds (Table 3. Entries **5**–**8**). First, we observed the reduction of C=C bond on nitro compounds **5** to **7**. Maximum conversion of 1-nitrocyclohexene (**5**) was 97% to produce the reduced product 1-nitrocyclohexane. Aliphatic nitroalkenes (**6**–**7**) were also shown to be favored substrates of NRSal with overall conversion efficiency of > 95%. Both compounds **6** and **7** were reduced to the corresponding alkanes, in case of compound **7** to the racemic product; either the enzyme was not stereoselective for that compound, or alternatively the potential enantiomeric product could have racemized due to the acidity of the proton on C_α. Finally, we observed a more typical behavior for a nitroreductase in the reduction of aromatic nitro compound such as nitrobenzene (**8**), which was reduced with conversion of > 99% to produce nitrosobenzene, phenylhydroxylamine, but also aniline and the chemical condensation product azoxybenzene. To our knowledge, this is the first report of a single enzyme-catalyzed reduction of nitrobenzene to aniline (reaction detailed in the next section).

Table 3 Biocatalytic conversion of various α,β-unsaturated carbonyl compounds, nitroalkenes, and nitroaromatic substrates with NRSal (* represents a center of chirality)

Entry	Substrate	Product	Conversion (NADPH/NADH)
1	 2-Cyclohexen-1-one	 2-Cyclohexanone	12%/12%
2	 3-Methyl-2-cyclohexenone	 3-Methyl-2-cyclohexanone	5.2%/4.8% (*R*) 84% e.e
3	 Ketoisophorone	 Levodione	12%/10% (*R*) 87% e.e
4	 Citral	N A^{-1}	No conversion
5	 1-Nitrocyclohexene	 1-Nitrocyclohexane	95%/97%
6	 Trans-β-nitrostyrene	 Phenylnitroethane	95%/95%
7	 1-Phenyl-2nitropropene	 1-Phenyl-2nitropropane	Both >99% Racemic mixture
8	 Nitrobenzene	 Aniline / Nitrosobenzene / Phenyl-hydroxylamine / Azoxybenzene	>99%/>99% Overall nitrobenzene conversion

Overall, NRSal accepts both cofactors NADPH/NADH equally well, with higher substrate acceptance for compounds containing the electron withdrawing nitro group. Importantly, the diverse catalytic activity of NRSal was reflected in the broad substrate spectrum and range of conversion efficiency.

Enzymatic reduction of nitrobenzene to aniline

The enzymatic reduction of nitroaromatic compounds can follow two pathways: the reduction of the nitro group or the reduction of the aromatic ring. The nitro group reduction is either a one-electron or two-electron reduction mechanism.[38–40] Both mechanisms involve the reduction of the nitro group to the amine through highly reactive nitroso and hydroxylamino intermediates. The aromatic ring in nitroaromatic compounds can also be reduced, resulting into the corresponding Meisenheimer complex, followed by subsequent release of nitrite.[41–42] The formation of the Meisenheimer complex has been observed for several members of the OYE family such as PETN reductase from *Enterobacter cloacae*,[43] XenB from *Pseudomonas fluorescens*,[44] and NemA from *Escherichia coli*,[45] which also display nitroreductase activity on the nitro group.

Published work on the reduction of nitrobenzene to aniline was mainly accomplished with metal support catalysts (e.g Ru/SBA, Ni, Pt/C catalysts) or whole-cell biocatalysts (e.g *Rhodotorula mucilaginosa*, *Arracacia xanthorrhiza*, *Candida guilliermondii*, *Saccharomyces cerevisiae*).[46–50,55] The single enzyme-catalyzed reduction of nitrobenzene (e.g with nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45) was reported to produce phenylhydroxylamine, a highly reactive intermediate.[51–52]

To the best of our knowledge, the discovery of NRSal reduction of nitrobenzene represents the first single isolated enzyme-catalyzed reduction of nitrobenzene to aniline. The mechanism for NRSal nitrobenzene reduction was shown to follow the two-electron reduction pathway of the nitro-group: nitrobenzene was reduced to form nitrosobenzene first and phenylhydroxylamine next, followed by subsequent reduction of phenylhydroxylamine to aniline. At the same time, chemical condensation between the nitrosobenzene and phenylhydroxylamine resulted in the production of azoxybenzene (Fig. 5).[53–54] Control experiments were performed to demonstrate the condensation reaction by mixing of these standards under similar reaction conditions but without enzyme present. Real-time analysis was performed to quantify the reduction of 1 mM nitrobenzene over time course of 160 min: nitrobenzene was completely converted within 40 min (with conversion of > 99%), along with the formation of 0.26 mM nitrosobenzene, 0.09 mM phenylhydroxylamine, 0.06 mM aniline, and 0.35 mM azoxybenzene (Fig. 6). Nitrobenzene and phenylhydroxylamine were found to be chemically unstable and were completely degraded after 6 h, with no further increase of aniline as final product. In an effort to boost the percentage formation of aniline in the overall reaction, one possible method was to limit the condensation between nitrosobenzene and phenylhydroxylamine. Experiments were performed to remove molecular oxygen from the reaction system as initial hypothesis that O_2 was essential in the condensation reaction. However, azoxybenzene formation was still detected at similar concentration level, implying that its formation is a condensation reaction without oxidation.

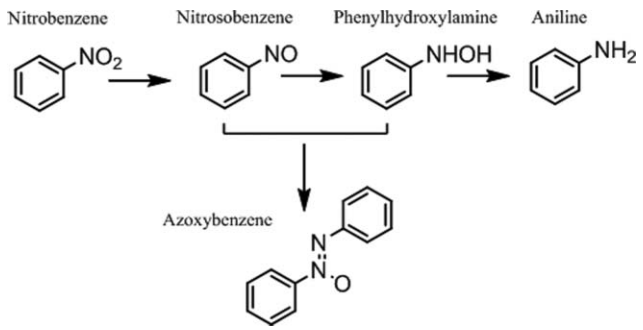


Fig. 5 Proposed nitrobenzene reduction pathway to aniline and azoxybenzene by NRSal.

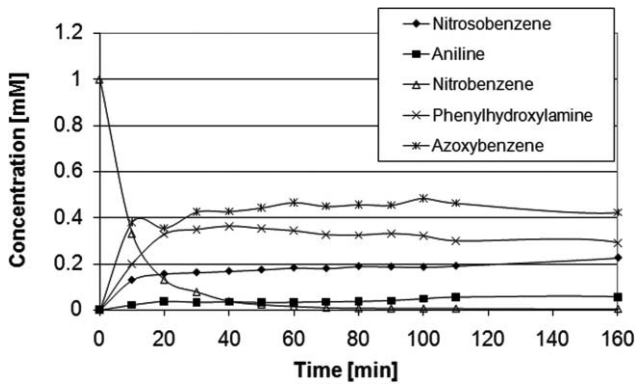


Fig. 6 Conversion-time plot for the reduction of nitrobenzene by NRSal in the presence of NADH.

3. Conclusion

In summary, nitroreductase NRSal from *Salmonella typhimurium* is a versatile biocatalyst that displays both nitroreductase and enoate reductase activity. The enzyme showed strong preference in the reduction of C=C bonds for nitro-containing substrates, with less satisfactory biocatalytic activity towards the reduction of C=C bonds in enones, still with a high stereoselectivity. It is interesting that a true nitroreductase presents strong enoate reductase activity with substrate stereopreference, is an FMN-bound enzyme and is still not associated with OYE family. The feasibility of biocatalytic reduction of nitroalkenes to nitroalkanes provides an addition as part of the synthetic toolbox to obtain various intermediates of interest. This is also the first isolated single enzyme-catalyzed reduction of nitrobenzene to aniline, which provides a possible perspective for a green production process of anilines other than traditional synthesis through chemical hydrogenation and metal-supported catalyst.[46] Although overall aniline formation is still limited due to condensation reaction in the system, we are currently investigating for optimized reaction conditions and larger scale reaction system.

Experimental section

NRSal primer sequences, cloning, and expression

The DNA sequence of NRSal from *Salmonella thypimurium* was synthesized by BioBasic Inc (Ontario, Canada) according to UnitProtKB accession # P15888. The corresponding specific 5′- and 3′-primers were synthesized at Eurofins-mwg|operon Biosciences (Huntville, AL). The primers contain a gene-encoding segment (italic), a restriction site (underlined), and an overhang. The 5′-primers of wild type and C-terminal polyhistidine tagged introduced a NcoI restriction site (5′-CAT G**CC ATG** ***G****CA ATG GAT ATT GTT TCC GTA GCT CTG C*-3′), and N-terminal tagged introduced a NdeI restriction site (5′-GGG AAT TC**C ATA** ***TG****A TGG ATA TTG TTT CCG TAG CTC TGC*-3′) into the PCR fragment. The 3′-primer introduced a HindIII restriction site in to the PCR fragment (with stop codon: 5′-CCC **AAG CTT** *TTA CAC TTC AGT CAG GGT GGT TTC CA*-3′, and without stop codon: 5′- CCC AAG CTT CAC TTC AGT CAG GGT GGT TTC CA-3′).

DNA amplification was performed with standard PCR protocol with Pfu polumerase from New England Biolabs (Marlborough, MA) and PCR buffer from Stratagene (La Jolla, CA). DNA was amplified for 30 cycles in an Eppendorf Gradient Thermocycler (Eppendorf, Hamburg, Germany) followed by gel purification with standard Qiagen gel kit (Qiagen, Valencia, CA). PCR assay: 100 ng of DNA, 200 μM of each dNTP, 10 μM of each primers, 1 U of Pfu polymerase, and 5 μL of Pfu buffer in a final volume of 50 μL. Each DNA amplification cycle: 1 min denaturation at 95 °C, 1 min annealing step at 50, 55, and 60 °C, 3.5 min extension step at 68 °C. The final PCR products were digested with restriction enzymes and ligated into pET28a (+) (Novagen, San Diego) and transformed into competent *E. coli* XL1-Blue cells. The NRSal genes were sent for DNA sequencing at Eurofins-mwg|operon Biosciences (Huntville, AL).

The NRSal genes were transformed into *E. coli* BL21 for protein expression. Starter culture consisted of 5 ml LB_{Kan} inoculated from

frozen stock and grown overnight at 37 °C. The starter culture were used for inoculation of 300 ml culture (0.1% v/v), which was gently aerated until OD_{600} reached 0.5 for addition of 0.1 mM of IPTG and left overnight for protein expression. Harvested cell pellets were kept for −80 °C for protein purification.

Purification of NRSal

The cell pellets of wild type NRSal were re-suspended in 50 mM TrisHCl buffer pH 7.5 (buffer A) and disrupted by sonication. The supernatant was separated after centrifugation, where NRSal was precipitated between 40–70% of saturated ammonium sulfate. The precipitate was dissolved again in buffer A and dialyzed over 6 h. The dialyzed sample was applied to DEAE-Sepharose™ FF (GE Healthcare, Sweden) column and washed with 25 mM step increment of NaCl in buffer A. Purified NRSal was collected at elution of 150 mM NaCl with 0.1 mM of free FMN added to account for the loss of FMN during the purification (only 20% FMN occupancy measured before addition of free FMN). Although experiments were performed to measure for the FMN occupancy at the end of purification, free FMN in the sample seemed to saturate the spectrophotometer measurement even with multiple wash cycles. The NRSal with N- or C-terminal polyhistidine tag were purified with Ni^{2+}-NTA beads according to the standard protocols of Qiagen (Valencia, CA). The protein concentration was determined by the Bradford method with Coomassie Plus Protein assay reagent and BSA assay (Pierce Chemical) as the calibration curve. SDS-PAGE analysis was performed with 50 µg protein sample elution into each well. The protein samples were mixed with 2X sample buffer (125 mM TrisHcl, pH 6.8, 4% SDS, 50% glycerol. 0.02% bromophenol blue, and 10% 2-mercaptoethanol). The mixed samples were incubated at 100 °C for 5 min and loaded onto 12% PAGE™ Gold precast gel and run in BioRad Mini Protean chamber (BioRad, Hercules CA) at 150 V for 45 min with Tris-HEPES-SDS running buffer (12.1 g L^{-1} Tris, 23.8 g L^{-1} HEPES, 1 g L^{-1} SDS). Biorad Precision Plus protein ladder was used as standards in the gel analysis. GelCode® Blue Stain Reagent (Pierce, Rockford IL) was used for final gel staining.

Enzyme Assay

NRSal kinetic measurement were performed by monitoring the oxidation of NAD(P)H at 340 nm using ε_{340nm} of 6.22 $mM^{-1}cm^{-1}$. Reaction assays were analyzed with Shimadzu GC-2010 using RT-BDEXcsf chiral column (30 m 0.32 mm 0.25 µm, Restek) for entry # **1–4**; Cyclosil-B chiral column (30 m 0.32 mm 0.25 µm, J&W Scientific) for entry # **6–8**; and SHRX5 column (15 m 0.25 mm 0.25 µm Shimadzu) for entry # 5. All substrates and products were purchased commercially from Sigma Aldrich or VWR with highest purity available except products of entry **6–8**, and phenyl-hydroxylamine. Prof. Jim Spain Research Group (Georgia Institute of Technology) kindly provided synthesized phenylhydroxylamine.[56] Products of **6–7** were synthesized according to previous published protocols.[16]

Gas chromatography analytical procedures

GC-FID analyses were performed on a Shimadzu GC-2010 instrument with helium as carrier gas. Conversions of compounds **1–8** and ee values of the corresponding products were determined with the columns and methods described below.

2-Cyclohexen-1-one (**1**) and nitrobenzene (**8**) were analyzed using Shimadzu SHRX5 column (15 m, 0.25 mm, 0.25 µm). Temperature program for **1**: injector and detector temperature at 220 °C; split ratio 25 : 1; start at 100 °C, hold 2 min, 10 °C min^{-1} to 160 °C, hold 2 min. Retention time: 2-cyclohexen-1-one 6.3 min, cyclohexanone 4.8 min. Temperature program for **8**: injector and detector temperature at 300 °C; split ratio 25 : 1; start at 80 °C, hold 5 min, 10 °C min^{-1} to 290 °C, hold 5 min. Retention time: nitrobenzene 5.8 min, aniline 2.9 min, nitrosobenzene 2.0 min, phenylhydroxylamine 3.9 min, azoxybenzene 14.8 min. Compounds 3-methyl-2-cyclohexenone (**2**), ketoisophorone (**3**), citral (**4**), 1-nitro-cyclohexenone (**5**), and *trans*-β-nitrostyrene (**6**) were analyzed using RESTEK Rt-BDEXcst chiral column (30 m, 0.32 mm, 0.25 µm). Temperature program for **2**: injector and detector temperature at 200 °C; split ratio 20 : 1; start at 90 °C, 1 °C min^{-1} to 130 °C, 5 °C min^{-1} to 180 °C. Retention time: 3-methyl-2-cyclohexenone 14.9 min, (*R*)-3-methyl-2-cyclohexanone 13.0 min, (*S*)-3-methyl-2-cyclohexanone 13.1 min. Temperature program for **3**: injector and detector temperature at 210 °C; split ratio 25 : 1; start at 100 °C, 3 °C min^{-1} to 130 °C, 20 °C min^{-1} to 200 °C, hold 3 min. Retention time: ketoisophorone 11.0 min, (*R*)-levodione 6.8 min, (*S*)-levodione 7.1 min. Temperature program for **4**: injector and detector temperature at 220 °C; split ratio 6.4 : 1; start 80 °C, 2.5 °C min^{-1} to 130 °C, hold 10 min, 5 °C min^{-1} to 180 °C, hold 5 min. Retention time: citral 20.1 min. Temperature program for **5**: injector and detector temperature at 220 °C; split ratio 5 : 1; start 120 °C, 10 °C min^{-1} to 210 °C; hold 5 min. Retention time: 1-nitrocyclohexenone 6.8 min, 1-nitrocyclohexanone 5.7 min. Temperature program for **6**: injector and detector temperature at 220 °C; split ratio 25 : 1; start 120 °C, hold 3 min, 10 °C min^{-1} to 210 °C; hold 5 min. Retention time: *trans*-β-nitrostyrene 7.2 min, phenylnitroethane 3.6 min. Compound 1-phenyl-2-nitropropene (**7**) was analyzed using Agilent Cyclosil-B chiral column (30 m, 0.32 mm, 0.25 µm). Temperature program for **7**: injector and detector temperature at 220 °C; split ratio 25 : 1; start at 100 °C, hold 10 min, 5 °C min^{-1} to 130 °C, hold 15 min, 5 °C min^{-1} to 220 °C, hold 2 min. Retention time: phenyl-2-nitropropene 11.5 min, racemic phenyl-2-nitropropane 9.6 min and 10.5 min.

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Notes and references

1 M. W. Fraaije and A. Mattevi, *Trends Biochem. Sci.*, 2000, **25**, 126–132.
2 V. Massey, *Biochem. Soc. Trans.*, 2000, **28**, 283–296.
3 R. E. Williams, D. A. Rathbone, N. S. Scrutton and N. C. Bruce, *Appl. Environ. Microbiol.*, 2004, **70**, 3566–3574.
4 R. Stuermer, B. Hauer, M. Hall and K. Faber, *Curr. Opin. Chem. Biol.*, 2007, **11**, 203–213.
5 B. Kosjek, F. J. Fleitz, P. G. Dormer, J. T. Kuethe and P. N. Devine, *Tetrahedron: Asymmetry*, 2008, **19**, 1403–1406.

6 J. F. Chaparro-Riggers, T. A. Rogers, E. Vazquez-Figueroa, K. M. Polizzi and A. S. Bommarius, *Adv. Synth. Catal.*, 2007, **349**, 1521–1531.
7 K. Stott, K. Saito, D. J. Thiele and V. Massey, *J Biol Chem*, 1993, **268**, 6097–6106.
8 C. E. French, A. M. Hailes, D. A. Rathbone, M. T. Long, D. L. Willey and N. C. Bruce, *Bio-Technol*, 1995, **13**, 674–676.
9 B. J. Brown, J. W. Hyun, S. Duvvuri, P. A. Karplus and V. Massey, *J. Biol. Chem.*, 2002, **277**, 2138–2145.
10 D. S. Blehert, B. G. Fox and G. H. Chambliss, *J Bact*, 1999, **181**, 6254–6263; J. J. Griese, R. P. Jakob, S. Schwarzinger and H. Dobbek, *J. Mol. Biol.*, 2006, **361**, 140.
11 H. S. Toogood, A. Fryszkowska, V. Hare, K. Fisher, A. Roujeinikova, D. Leys, J. M. Gardiner, G. M. Stephens and N. S. Scrutton, *Adv. Synth. Catal.*, 2008, **350**, 2789–2803; H. Nivinskas, J. Sarlauskas, Z. Anusevicius, H. S. Toogood, N. S. Scrutton and N. Cenas, *FEBS J.*, 2008, **275**, 6192–6203.
12 M. Hall, C. Stueckler, B. Hauer, R. Stuermer, T. Friedrich, M. Breuer, W. Kroutil and K. Faber, *Eur. J. Org. Chem.*, 2008, 1511–1516; R. E. Williams, D. A. Rathbone, N. S. Scrutton and N. C. Bruce, *Appl. Environ. Microbiol.*, 2004, **70**, 3566–3574.
13 M. Hall, C. Stueckler, H. Ehammer, E. Pointner, G. Oberdorfer, K. Gruber, B. Hauer, R. Stuermer, W. Kroutil, P. Macheroux and K. Faber, *Adv. Synth. Catal.*, 2008, **350**, 411–418; T. B. Fitzpatrick, N. Amrhein and P. Macheroux, *J. Biol. Chem.*, 2003, **278**, 19891–19897.
14 E. R. Beynon, Z. C. Symons, R. G. Jackson, A. Lorenz, E. L. Rylott and N. C. Bruce, *Plant Physiol.*, 2009, **151**, 253–261; J. Straβner, A. Fürholz, P. Macheroux, N. Amrhein and A. Schaller, *J. Biol. Chem.*, 1999, **274**, 35067–35073.
15 R. Ballini, D. Fiorini, M. V. Gil and A. Palmieri, *Tetrahedron Lett.*, 2004, **45**, 1311–1311.
16 A. Fryszkowska, K. Fisher, J. M. Gardiner and G. M. Stephens, *J. Org. Chem.*, 2008, **73**, 4295–4298.
17 D. M. Mampreian and A. H. Hoveyda, *Org. Lett.*, 2004, **6**, 2829–2832.
18 J. Wu, D. M. Mampreian and A. H. Hoveyda, *J. Am. Chem. Soc.*, 2005, **127**, 4584–4585.
19 A. Côté, V. N. G. Lindsay and A. B. Charette, *Org. Lett.*, 2007, **9**, 85–87.
20 C. Czekelius and E. M. Carreira, *Angew. Chem., Int. Ed.*, 2005, **44**, 612–615.
21 N. J. A. Martin, L. Ozores and B. List, *J. Am. Chem. Soc.*, 2007, **129**, 8976.
22 R. Ballini, G. Bosica, D. Fiorini, A. Palmieri and M. Petrini, *Chem. Rev.*, 2005, **105**, 933–971.
23 M. Watanabe, M. Ishidate and T. Nohmi, *Nucleic Acids Res.*, 1990, **18**, 1059–1059.
24 S. Zenno, H. Koike, M. Tanokura and K. Saigo, *J Biochem*, 1996, **120**, 736–744.
25 C. Bryant and M. Deluca, *J Biol Chem*, 1991, **266**, 4119–4125.
26 M. R. Nokhbeh, S. Boroumandi, N. Pokorny, P. Koziarz, E. S. Paterson and I. B. Lambert, *Mutat. Res., Fundam. Mol. Mech. Mutagen.*, 2002, **508**, 59–70.
27 R. E. Williams, D. A. Rathbone, P. C. E. Moody, N. S. Scrutton and N. C. Bruce, *Biochem Soc Symp*, 2001, 143–153.
28 M. Watanabe, T. Nishino, K. Takio, T. Sofuni and T. Nohmi, *J. Biol. Chem.*, 1998, **273**, 23922–23928.
29 M. McClelland, K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Y. Du, S. F. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston and R. K. Wilson, *Nature*, 2001, **413**, 852–856.
30 F. J. Peterson, J. L. Holtzman, P. H. Duquette, R. E. Erickson, J. T. Callaghan and R. P. Mason, *Pharmacologist*, 1978, **20**, 157–157.
31 K. M. Fox and P. A. Karplus, *Structure*, 1994, **2**, 1089–1105.
32 P. A. Karplus, K. M. Fox and V. Massey, *Faseb J*, 1995, **9**, 1518–1526.
33 A. D. N. Vaz, S. Chakraborty and V. Massey, *Biochemistry*, 1995, **34**, 4246–4256.
34 M. Wada, A. Yoshizumi, Y. Noda, M. Kataoka, S. Shimizu, H. Takagi and S. Nakamori, *Appl. Environ. Microbiol.*, 2003, **69**, 933–937.
35 E. M. Buque-Taboada, A. J. J. Straathof, J. J. Heijnen and L. A. M. van der Wielen, *Adv. Synth. Catal.*, 2005, **347**, 1147–1154.
36 A. Müller, B. Hauer and B. Rosche, *Biotechnol. Bioeng.*, 2007, **98**, 22–29.
37 A. Muller, B. Hauer and B. Rosche, *J. Mol. Catal. B: Enzym.*, 2006, **38**, 126–130.
38 J. C. Spain, *Annu. Rev. Microbiol.*, 1995, **49**, 523–555.
39 C. Bryant and M. Deluca, *J Biol Chem*, 1991, **266**, 4119–4125.
40 C. Bryant, L. Hubbard and M. Deluca, *Faseb J*, 1988, **2**, A1778–A1778.
41 P. van Dillewijn, R. M. Wittich, A. Caballero and J. L. Ramos, *Appl. Environ. Microbiol.*, 2008, **74**, 6703–6708.
42 C. E. French, S. Nicklin and N. C. Bruce, *Appl Environ Microb*, 1998, **64**, 2864–2868.
43 C. E. French, S. J. Rosser, G. J. Davies, S. Nicklin and N. C. Bruce, *Nat. Biotechnol.*, 1999, **17**, 491–494.
44 J. W. Pak, K. L. Knoke, D. R. Noguera, B. G. Fox and G. H. Chambliss, *Appl. Environ. Microbiol.*, 2000, **66**, 4742–4750.
45 M. M. González-Pérez, P. van Dillewijn, R. M. Wittich and J. L. Ramos, *Environ. Microbiol.*, 2007, **9**, 1535–1540.
46 K. Chary and C. Srikanth, *Catal. Lett.*, 2009, **128**, 164–170.
47 A. F. Cunha, J. J. M. Orfao and J. L. Figueiredo, *Appl. Catal., A*, 2008, **348**, 103–112.
48 C. L. Zheng, B. C. Qu, J. Wang, J. T. Zhou, J. Wang and H. Lu, *J. Hazard. Mater.*, 2009, **165**, 1152–1158.
49 C. L. Zheng, J. T. Zhou, J. Wang, J. Wang and B. C. Qu, *J. Hazard. Mater.*, 2008, **160**, 194–199.
50 A. Agrawal and P. G. Tratnyek, *Environ. Sci. Technol.*, 1996, **30**, 153–160.
51 L. J. Nadeau, Z. He and J. C. Spain, *J. Ind. Microbiol. Biotechnol.*, 2000, **24**, 301–305.
52 A. Schenzle, H. Lenke, J. C. Spain and H. J. Knackmuss, *Appl Environ Microb*, 1999, **65**, 2317–2323.
53 W. Baik, J. L. Han, K. C. Lee, N. H. Lee, B. H. Kim and J. T. Hahn, *Tetrahedron Lett.*, 1994, **35**, 3965–3966.
54 A. Agrawal, P. G. Tratnyek, P. Stoffynegli and L. Y. Liang, *Abstr Pap Am Chem S*, 1995, **209**, 143.
55 V. Höller, D. Wegricht, I. Yuranov, L. Kiwi-Minsker and A. Renken, *Chem. Eng. Technol.*, 2000, **23**, 251–255.
56 J. K. Davis, G. C. Paoli, Z. Q. He, L. J. Nadeau, C. C. Somerville and J. C. Spain, *Appl. Environ. Microbiol.*, 2000, **66**, 2965–2971.
57 S. Zenno, H. Koike, M. Tanokura and K. Saigo, *J Biochem*, 1996, **120**, 736–744.
58 S. Zenno, H. Koike, A. N. Kumar, R. Jayaraman, M. Tanokura and K. Saigo, *J Bacteriol*, 1996, **178**, 4508–4514.
59 H. Nivinskas, S. Staskeviciene, J. Sarlauskas, R. L. Koder, A. F. Miller and N. Cenas, *Arch. Biochem. Biophys.*, 2002, **403**, 249–258.

