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An unusually cold active nitroreductase for prodrug activations

Ayhan Çelik *, Gülden Yetiş

Gebze Institute of Technology, Department of Chemistry, 41400 Gebze-Kocaeli, Turkey

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ABSTRACT

A set of PCR primers based on the genome sequence were used to clone a gene encoding a hypothetical nitroreductases (named as Ssap-NtrB) from uropathogenic staphylococcus, *Staphylococcus saprophyticus* strain ATCC 15305, an oxygen insensitive flavoenzyme. Activity studies of the translation product revealed that the nitroreductase catalyses two electron reduction of a nitroaromatic drug of nitrofurazone (NFZ), cancer prodrugs of CB1954 and SN23862 at optimum temperature of 20 °C together with retaining its maximum activity considerably at 3 °C. The required electrons for such reduction could be supplied by either NADH or NADPH with a small preference for the latter. The gene was engineered for heterologous expression in *Escherichia coli*, and conditions were found in which the enzyme was produced in a mostly soluble form. The recombinant enzyme was purified to homogeneity and physical, spectral and catalytical properties were determined. The findings lead us to propose that Ssap-NtrB represents a novel nitro reductase with an unusual cold active property, which has not been described previously for prodrug activating enzymes of nitroreductases.

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1. Introduction

Activation of prodrugs by bacterial nitroreductases (NTRs) has attracted considerable interest in recent years because of having differential effects between tumour and normal cells in cancer prodrug therapy.[1] The basis of the activation is that NTRs catalyse the reduction of nitro groups in substrates to form the corresponding hydroxylamine derivatives. These derivatives can be further metabolised by cellular enzymes to form cytotoxic agents that cross link with DNA, resulting in rapid cell deaths.[2–4] Such reduction properties establish NTRs as prodrug activators. They have been therefore utilised in cancer prodrug therapy including antibody-directed enzyme prodrug therapy (ADEPT),[5] gene directed enzyme prodrug therapy (GDEPT),[6] clostridia-directed enzyme prodrug therapy (CDEPT)[7] and virus directed enzyme prodrug therapy (VDEPT).[8,9] In fact, FMN-dependent NTRs from *Escherichia coli* (nfsB) with combination of prodrug (CB1954) have been extensively studied for antitumourogenic properties[6,10–12] and reached the clinical trial stage in VDEPT.[13]

Nitroreductases are widely distributed among bacteria, but also found in archaea and eukaryotic species[1] and divided into two groups; oxygen insensitive (type I) and oxygen sensitive (type II). So far a number of oxygen insensitive NTRs have been identified from various bacterial sources, examples include *Escherichia coli* (NfsA, NfsB, YdjA),[14–16] *Salmonella typhimurium* (Cnr, SnrA),[17–19] *Pseudomonas putida* (PnrA),[20] *Vibrio harveyi* (FRP),[21] *Vibrio fischeri* (renamed as *Aliivibrio fischeri*) (FRase1),[22] *Rhodobacter capsulatus* (NprA, NprB),[1,23] *Bacillus subtilis* (NfrA1, YwrO)[24,25] and more recently *Bacillus licheniformis*.[26]

Members of this family utilise FMN as a cofactor and are often found to be homodimers. The structural aspects of nitroreductases and roles of key active site amino acids in binding FMN as well as substrates are now clearly defined, largely as results of extensive structural studies of *E. coli* NTRs.[27–31] This structural data combined with recent mechanistic studies[32] has allowed detailed understanding and the rationalisation of catalytic and prodrug binding properties of the enzymes. Based on such information, many CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) like prodrug have been designed and synthesised.[6,33,34] A subset of these compounds have demonstrated better binding and cytotoxic properties than CB1954, offering a great deal of opportunities in directed enzyme prodrug therapy (DEPT).

As prodrug activating enzymes, NTRs from bacterial sources have a number of advantages in DEPT strategy-the absence or low catalytic activities of mammalian equivalents and differences in substrate specificity prevent non specific prodrug activation at the sites other than the tumour. NTR, however are required to meet certain criteria to be used in DEPT strategy. Stability, the levels of enzymatic activity when conjugated to antibody, sufficient catalytic activity under physiological conditions and cofactor availability are among the factors for successful applications of NTRs. Therefore the search for ideal nitroreductases for therapeutic uses necessitates identifying new enzymes with desired properties from natural sources or by modification of existing enzymes by mutagenesis, or even combination of both.

* Corresponding author. Tel.: +90 262 605 3130; fax: +90 262 605 3101.
E-mail address: ayhancelik@gyte.edu.tr (A. Çelik).

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We now report the identification and characterisation of a novel nitroreductase from uropathogenic staphylococcus, *Staphylococcus saprophyticus*, which is frequently isolated from young female outpatients suffering from uncomplicated urinary tract infections.[35] The gene encoding the Ssap-NtrB from this bacterium was cloned and the recombinant protein overexpressed in *E. coli*. This functional nitroreductases was shown to be FMN containing flavoenzyme. At very low temperature, it catalyses reduction of various electron acceptors including an antibiotic of nitrofurazone, dinitrobenzamide family cancer prodrug of CB1954 and SN23862 and also an artificial electron acceptor of $K_3[Fe(CN)_6]$, in the expense of nicotinamide cofactors (NAD(P)H).

2. Materials and methods

2.1. Chemicals and reagents

FMN and nitrofurazone (NFZ), Coomassie brilliant blue R-250, a protease inhibitor mixture, PMSF (phenylmethylsulfonyl fluoride), SDS (sodium dodecyl sulphate) were supplied by Sigma–Aldrich. NADH and NADPH were obtained Roche Applied Science. IPTG (isopropyl-β-D-thiogalactopyranoside) was supplied by Qiagen (ATQ Biotechnology, TR). Restriction endonucleases, T4 DNA ligase, 2× Tag Master Mix™ were from New England BioLabs (NEB GmbH, Germany). Nitrobenzamide prodrug of CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) and SN23862 (5-[*N*,*N*-bis(2-chloroethyl)-amino]-2,4-dinitrobenzamide) were kind gift from Professor Richard Knox (Morvus Technology Limited, UK). BSA was obtained from Merck Biosciences. All other biochemical reagents were obtained from various sources at highest purity possible. Reagents from Merck, Fluka and Sigma–Aldrich were used as supplied without prior purification.

2.2. Sequence analysis

Protein sequence of Ssap-NtrB was obtained from GeneBank hosted in the National Centre for Biotechnology Information (NCBI) (Accession no: gi73661517). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 software.[36] Neighbor-Joining (NJ) method was used for phylogenetic tree searching and inference. Multiple sequence alignment was performed using CLUSTRAL W program with the default parameters.[37]

2.3. Bacterial strains, plasmids, culture media

The source of chromosomal DNA for the cloning of Ssap-NtrB was *Staphylococcus saprophyticus* (strain DSM 20229/ATCC 15305), obtained from DSMZ culture collection (DSMZ GmbH, Braunschweig, Germany). The *E. coli* host used for cloning experiments was TOP10 [F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G], and the host used for protein expression studies was *E. coli* BL21(DE3) [F-*omp*T *hsd*SB(rB-, mB-) *gal dcm* (DE3)] (both from Invitrogen). The plasmid employed for both cloning and expression was pET14b (Novagen). All organisms were cultivated in LB broth or on LB-agar at 30 or 37 °C and supplemented with antibiotics if necessary.

2.4. Cloning of Ssap-NtrB gene

S. saprophyticus was grown in LB medium overnight at 37 °C and genomic DNA was obtained using phenol/chloroform extraction method[38] with minor modifications. Bacterial pellets were initially re-suspended in 250 μl of a lysis solution containing 200 μg of lysostaphin per ml, 20 mM Tris–Cl, 2 mM EDTA, and 1.2% Triton X-100 and incubated for 30 min at 37 °C. Purified genomic DNA was diluted at a concentration of 7.6 ng/μl in TE buffer (10 mM Tris–Cl, pH 8.0, 1 mM EDTA).

A 627 bp portion of Ssap-NtrB gene was amplified from *S. saprophyticus* genomic DNA using the following forward and reverse primers. To facilitate cloning *Nde*I and *Xho*I restriction sites were incorporated at 5′-end of each primer and underlined respectively (supplied by IDT, Leuven, Belgium): FP (5′-CGGTGTCCATATGATA-AATAATAATTTTGAAG-3′) and RP (5′-CACCTCGAGTTAAACGTATT-TAGTCACAG-3′). The PCR reaction mixture consisted of 2× Tag Master Mix™ (25 μL), forward and reverse primers (4 μL, 1.6 pmol/μL), genomic DNA (2 μL, 7.6 ng/μL) and distiled water (15 μL) in a reaction volume of 50 μL. The PCR reaction, carried out in MJ Mini Thermal Cycler™ (BioRad), involved an initial denaturation step of 5 min at 95 °C, and then subjected to 29 cycles of amplification (95 °C, 1 min; 52 °C, 1 min; 68 °C, 1.5 min) followed by a final extension at 68 °C for 10 min. The resulting 627 bp PCR product was gel purified using QIAprep Kit (Qiagen, ATQ Biotechnology, Ankara, TR). The PCR product and pET14b vector were digested with *Nde*I/*Xho*I and gel purified using QIAprep Kit (Qiagen). Ligation was achieved using T4 DNA ligases according to the manufacturer instructions (NEB). Five microlitre of ligation mixture was transferred into 50 μL *E. coli* TOP10 competent cell lines according to the manufacturer instructions (Invitrogen). Overnight cultures were prepared each of resulting colonies on LB-Agar medium containing 100 μg/ml ampicillin. Restriction analysis was performed using the same restriction enzymes to confirm successful ligation of the gene into the vector. This construct contains the complete nitroreductase coding region and N-terminal His-tag together with a thrombin cleavage site, and named as pSsap-NtrB. The plasmid DNA was purified from transformants using a Roche High Pure™ plasmid isolation kit. A sample of the purified DNA was sequenced (RefGen, Ankara, TR) to confirm the identity of the amplification product on both strands. DNA sequences were determined using T7 promoter and T7 terminator primers. Nucleotide sequence data were assembled and analysed using the BioEdit software packages (Ibis Therapeutics, CA).

Expression of the gene was under the control of IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible T7 RNA promoter. For heterologous gene expression, the resulting construct (pSsap-NtrB) was transformed into expression strain of *E. coli* BL21(DE3) (Invitrogen).

2.5. Expression and purification of Ssap-NtrB

For protein expression, *E. coli* BL21 (DE3) containing the expression construct of pSsap-NtrB was grown in LB medium containing 100 μg/ml ampicillin at 37 °C in a orbital shaker at 180 rpm. After induction with 0.1 mM IPTG at an optical density (OD600) of 0.5, growth was continued for up to 3 h at 20 °C before harvesting. The cell pellet was obtained via centrifugation at 8000×*g* for 10 min and stored −20 °C until use. Aliquots were withdrawn at regular time points. Expression was assessed by comparing the banding pattern obtained by SDS–PAGE analysis of whole cell extracts with that of a negative control (i.e., *E. coli* BL21 (DE3) containing pET14b plasmid only).

For the auto induction, the procedure was adapted from Studier.[39] Briefly, a single colony was used to inoculate a 10 ml of starter culture for 1 L of auto induction medium, which was incubated at 30 °C and 180 rpm for at least 16 h, until stationary phase was reached. The cell pellet was obtained via centrifugation at 8000×*g* for 10 min and stored −20 °C until use. Approximately every gram of wet cell paste from either induction procedures was suspended in 4 ml of ice-cold buffer A (20 mM Tris–Cl, pH 7.8, 0.5 M NaCl, 5 mM imidazole) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μl of the protease inhibitor mixture

(Sigma–Aldrich). The cells were incubated with 80 μl of lysozyme (10 mg/ml) for 30 min in ice at an angle of 45° for mixing readily, before disruption by sonication using a Soniprep 150 sonicator (Sanyo, Tokyo, Japan) fitted with a 3-mm diameter probe. The cell suspension was kept on ice and sonicated with a 15 s burst followed by a 45 s interval for six times. The resulting cell extract was centrifuged at 28,000×*g* for 30 min at 4 °C. The cell-free extract was carefully removed and filtered through a 0.45-micron filter unit (Sartorius, Goettingen, Germany). The clarified extract was then loaded onto a 1-ml Nickel HiTrap™ column (Amersham Biosciences), equilibrated in buffer A, at a flow rate of 1 ml/min. Column chromatography was performed manually at 4 °C. The column was washed with several times with buffer A (10 column volumes) to remove unbound material. The majority of contaminating proteins were then removed by washing with 10 mM imidazole in buffer A (10 column volumes) before elution of the recombinant His-tagged Ssap-NtrB by 100 mM imidazole in buffer A. The buffer was exchanged to buffer B (50 mM sodium phosphate, pH 7.5) using a PD-10 desalting column (Amersham Biosciences) at 4 °C. The sample volume was reduced using a spin concentrator with a 10 kDa cut-off membrane (Millipore, Germany). Glycerol was added to a final concentration of 50% (v/v) and the sample stored at −20 °C until use. The protein sample was saturated by commercially available FMN followed by gel filtration chromatography using PD-10 desalting column, when necessary.

2.6. SDS–PAGE analysis and protein concentration determination

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a Mini-PROTEAN II electrophoresis cell (Bio-Rad). Protein bands were visualised with Coomassie brilliant blue R-250. Enzyme concentrations were determined using the conventional dye-binding assay using Bradford procedure.[40] Bovine serum albumin (BSA) was used for generation of standard curve. Spectroscopic determination of the enzyme concentration was also determined for confirmation using the calculated (based on protein sequence) extinction coefficient value of 13370 M^{-1} cm^{-1} at 280 nm.[41] Bradford procedure was primarily used for standardisation of the enzyme assays. Since FMN is also absorbs at 280 nm, the amount of FMN bound to the protein was taken into account if the enzyme concentration was to be determined spectrophotometrically at 280 nm.

2.7. Protein mass spectrometry of Ssap-NtrB

Mass spectrometry of the recombinant His_6-tagged Ssap-NtrB was performed by MALDI-TOF MS using MicroFlex instruments (Bruker Daltonics, Billerica, MA). A protein sample (50 μl of 9 mg/ml) was concentrated by precipitation using freshly prepared 500 μl of 20% TCA (trichloroacetic acid). The resulting pellet was washed twice with 250 μl of cold acetone, and centrifuged at 10000×*g*. The pellet was air dried, and re-dissolved in 40 μl of formic acid/dH_2O/isopropanol (1:3:2) mixture. The mixture was mixed with 2× equivalent volume of matrix (saturated solution of sinapinic acid in 50% isopropanol), and applied on a sample target surface. The sample was air dried at room temperature to crystallise. The MS was operated in positive ion mode (20 kV, 50 shots) and calibrated with the Bruker's protein standards II kit including cytochrome c (12,360 Da), myoglobin (16,952 Da), trypsinogen (23,982 Da), protein A (44,613 Da) and bovine serum albumin (BSA, 66,527 Da).

2.8. Flavin cofactor analysis

The amount of Ssap-NtrB bound FMN was determined as described before.[23] The purified enzyme (800 μl, 1.6 mg/ml) was heated to 120 °C for 20 min. The samples was then centrifuged at 13,500×*g* before ultrafiltration using a Vivaspin™ micro centrifugal filter with 10 kDa molecular cut off (Sartorius). The released flavin content was identified via HPLC equipped with C18 RP column (ACE3, 150 mm × 4.6 mm × 3 μm). The mobile phase was methanol and 10 mM sodium phosphate buffer, pH 7.0 with linear gradient of 1% methanol for 5 min, up to 50% methanol for 10 minutes with flow rate of 0.7 ml/min and monitored at 375 and 445 nm with PDA detector (Agilent HPLC 1100). For the purpose of determination of flavin: protein ratio, the concentration of flavin was determined by spectroscopic method at 445 nm using absorption coefficients of 12,500 M^{-1} cm^{-1} (for FMN).[26] The protein content was determined as described previously using both Bradford procedure[40] and spectroscopic methods at 280 nm for confirmation. The FMN concentration was then simply divided by protein concentration to obtain the flavin: protein ratio. The absorption coefficients calculated for the protein were 13,370 M^{-1} cm^{-1} in *apo* form and 24,701 M^{-1} cm^{-1} for *halo* form, in which absorption at 280 nm due to FMN was included in all calculation.

2.9. pH and temperature optimum, and thermal stability determinations

The pH-activity profile of Ssap-NtrB was determined using NFZ. The buffers used were 50 mM citric acid–sodium citrate (pH 5.4–6.2), 50 mM sodium phosphate (pH 6.0–7.9), 50 mM Tris–Cl (pH 7.4–9.0) and 50 mM glisin-NaOH (pH 8.6–10.6) containing 4% DMSO. Activity values were determined in each of these buffers using 0.150 mM NFZ, 9.4 μg/ml Ssap-NtrB and 200 μM NADPH at 15 °C. Temperature profile of Ssap-NtrB was determined at a series of temperatures ranged from 3 to 40 °C by NFZ assay. Activity values were determined using 0.150 mM NFZ, 12.5 μg/ml Ssap-NtrB, and 200 μM NADPH in 50 mM sodium phosphate buffer containing 4% DMSO at pH 7.4. In a typical experiment, the buffer solution (800 μl) containing NFZ was incubated at desired temperature until the equilibrium reached. The enzyme sample (20 μl) was added and the mixture was further incubated for 3 min. The reaction was then started by addition of NADPH. The NFZ reduction was followed spectrophometrically at 440 nm for 2 min. The initial rates of NFZ disappearance were calculated using extinction coefficient of 880 M^{-1} cm^{-1}[27,42] and expressed as a percentage of the maximum activity obtained at 15 °C. The temperature in Uv–vis spectrometer was maintained using a circulating cooled water bath and temperature in the cuvette was constantly monitored using a digital thermometer.

In the enzyme thermal stability studies, the protein samples (0.4 mg/ml, 400 μl) in 50 mM sodium phosphate buffer (pH 7.5) were pre-incubated at 20, 30, 35, 40 and 50 °C for 0–240 min and aliquots were taken at regular intervals and cooled. In all cases, the remaining enzyme activity was assayed against 0.150 mM of NFZ in 50 mM sodium phosphate buffer, pH 7.5 with 4% DMSO in a similar manner described previously and expressed as a percentage of the maximum activity, for which the was no heat treatment.

2.10. Enzyme assays with electron acceptors

Cofactor preference (NADPH or NADH) of Ssap-NtrB catalysed reactions was carried out using $K_3[Fe(CN)_6]$, as an artificial electron acceptor. In a typical reaction, 1 mM (saturating concentration) of $K_3[Fe(CN)_6]$ in 50 mM Tris–Cl buffer pH 7.0 was incubated with 50 μg/ml of Ssap-NtrB for 3 min at 15 °C and the reaction was initiated by addition of either NADPH or NADH at varying concentrations. The reaction was monitored spectrophotometrically at 340 nm for 10 min. Steady state enzyme kinetics with purified Ssap-NtrB as a prodrug activator enzyme was monitored using both CB1954 and SN23862. In a typical reaction, series of CB1954

in the range of 0.05–4 mM or SN23862 (0–165 μM) was prepared in 50 mM Tris–Cl (pH 7.0) buffer containing 4% DMSO. To this solution, Ssap-NtrB was added to give final concentrations of 12.5 μg/ml. The reaction was then started by addition of NADPH at final concentration of 200 μM and monitored at 420 nm (based on equal absorption of both 2- and 4-hydroxylamine reduction product of CB1954 at this wavelength; $\varepsilon_{420} = 1200\ M^{-1}\ cm^{-1}$)[43] for CB1954 and 340 nm (based on NADPH usage; $\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$)[44] for SN23862.

All kinetics measurements were performed using Uv–vis spectrophotometer (Shimadzu UV-3600) equipped with a circulating water bath (VWR model 1162A). Nonlinear regression analysis and Michaelis–Menten curve fitting was performed using Origin v.7 software (OriginLab, Northampton, MA). One unit (U) is defined as the amount of the enzyme that catalyses the conversion of 1 μmol of substrate per minute under the experimental conditions specified. The specific activity was expressed as μmol min^{-1} mg^{-1}active enzyme.

2.11. Metabolite analysis

Product profile studies of CB1954 and SN23862 catalysed by Ssap-NtrB were performed. In a typical reaction, CB1954 or SN23862 was prepared in 50 mM Tris–Cl (pH 7.0) buffer, filtered and degassed for 5 min, to give final concentration of 1 mM (80 μM for SN23862). All reactions were prepared anaerobically by repetitive bubbling with nitrogen for 10 min before and after addition of the enzyme. To this solution, Ssap-NtrB and NADPH were added to give final concentrations of 50 μg/ml and 3.4 mM respectively. The reactions were continued for at least 30 min at 18–20 °C before being stopped by addition of ice-cold methanol. Samples were immediately transferred to −80 °C for at least 1.5 h followed by centrifugation at 12,000×*g* for 10 min. The HPLC analyses for determinations of reaction products of CB1954 were performed using gradient HPLC system (Agilent HPLC1100) equipped with RPC18 column (ACE5, ACE-HPLC, UK) and Uv–visible detector at 260 and 420 nm (390 and 450 nm for SN23862). The mobile phase was acetonitrile–water mixture (v/v, 10% for 5 min, 10–60% for 20 min, 30% for 15 min.) with flow rate of 1 ml/min. For the quantification, the extinction coefficients were used as follows: $\varepsilon_{260} = 7880\ M^{-1}\ cm^{-1}$ for the 4-hydroxylamine and $\varepsilon_{260} = 5420\ M^{-1}\ cm^{-1}$ for the 2-hydroxylamine.[45]

3. Results

3.1. Cloning of Ssap-NtrB gene

Using BLAST search[46] with the sequence of *E. coli* nitroreductases (nfsB), a potential nitroreductase gene was identified from the genome sequence of *S. saprophyticus*. Oligonucleotide primers were designed from the sequence data covering the translational start region of the nitroreductase gene. The forward primer contained mismatches to generate an *Nde*I restriction site (underlined). Similarly, the reverse primers were designed to generate an *Xho*I restriction site (underlined) for direct cloning into the pET14b vector. The deduced product of *S. saprophyticus* NTR (Ssap-NtrB) without N-terminus modification is a protein of 208 amino acid residues, 23.4 kDa, and *p*I of 5.49. Addition of hexa histidine tag for the purpose of easy purification increased the amino acid residues to 228, 25.6 kDa and *p*I of 6.7. Protein BLAST database searches of the translated *S. saprophyticus* DNA sequence showed high scoring similarities with putative nitroreductases from *Staphylococcus epidermidis* and *E. sibiricum* (55% identity, 72% similarity and 47% identity and 68% similarity, respectively).

3.2. Production of His_6-Ssap-NtrB and SDS–PAGE analysis

The expression construct (pSsap-NtrB) was transformed into *E. coli* BL21(DE3), and the cell extract was analysed by SDS–PAGE. A protein of the expected size (~25 kDa) was readily detected on a Coomassie-stained gel from an extract of *E. coli* BL21 (DE3) harbouring pSsap-NtrB plasmid (Fig. 1). The recombinant protein was absent in the negative control of the same strain harbouring pET14b. No detectable difference in the observed levels of heterologous expression was noticed between IPTG-induced and noninduced cells, that suggest a degree of leakiness within the system. When the cells were grown at a temperature of 37 °C, most of the Ssap-NtrB accumulates in the insoluble fraction, whereas the protein was significantly soluble in the cells grown at 25 °C. His_6-Ssap-NtrB was purified using metal ion affinity chromatography. A single step purification protocol yields 40–50 mg protein from a litre culture with over 90% purity based on SDS–PAGE analysis (Fig. 1, lane 8).

3.3. Protein mass spectrometry of Ssap-NtrB

The calculated mass of His_6-tagged Ssap-NtrB from its amino acid sequences is 25,567 Da. The subunit molecular mass of the monomer of enzyme (single charged) was determined by MALDI-TOF and found to be 25,452 Da (Fig. 2). The calculated and measured masses are in agreement with each other and the difference in masses is well within the instrument's resolution power and experimental errors.

3.4. Flavin cofactor analysis

The yellow colour of purified Ssap-NtrB from *S. saprophyticus* indicates the presence of bound cofactor. The Uv–vis spectra of the purified Ssap-NtrB protein showed the characteristic two peaks at 368 and 456 nm (Fig. 3A) resembling an oxidised FMN cofactor, which is consistent with bacterial nitroreductases. The bands were quickly disappeared upon reduction of the flavoenzyme by addition of sodium dithionite in excess. The flavin content was subsequently analysed by RP-HPLC using commercial FMN as a standard and identified as flavin mononucleotide (FMN) (Fig. 3B

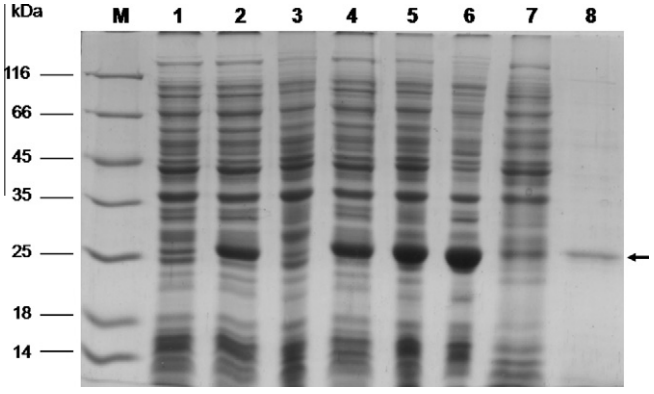

Figure 1. SDS–PAGE analysis of whole cell extracts of a culture of *E. coli* BL21(DE3)pSsap-NtrB following induction with IPTG. The arrow indicates the position of the recombinant protein. Lane M molecular mass standards (molecular masses in kDa are indicated on the left); lane 1 cell extracts of uninduced *E. coli* BL21(DE3)pET14b (control); lane 2 cell extract of uninduced *E. coli* BL21(DE3)pSsap-NtrB; lane 3 cell extract of IPTG induced *E. coli* BL21(DE3)pET14b after 2 h; lane 4 cell extract of IPTG induced *E. coli* BL21(DE3)pSsap-NtrB after 2 h; lane 5 cell extract of IPTG induced *E. coli* BL21(DE3)pSsap-NtrB after 5 h; lane 6 insoluble fraction of *E. coli* BL21(DE3)pSsap-NtrB; lane 7 cell free extract; lane 8 purified His_6-tagged Ssap-NtrB

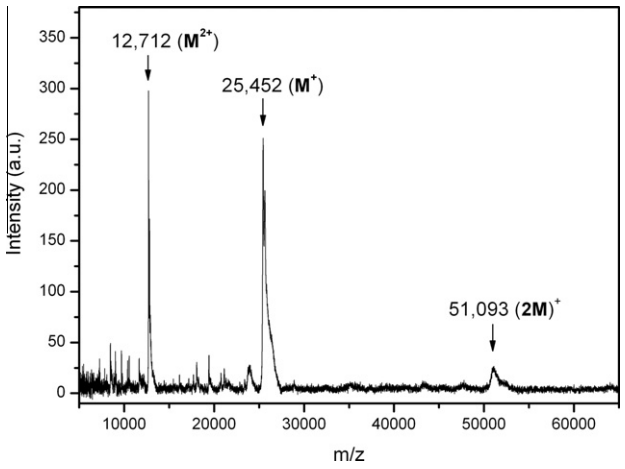

Figure 2. MALDI-TOF MS analysis of His_6-Ssap-NtrB. The experimentally determined mass along with the predicted masses are highlighted by arrows for the singly (M^+) and doubly (M^{2+}) charged monomers as well as singly charged dimer $(2M)^{2+}$ species. Mass for the singly monomer was found to be 25,452 Da.

and C). The flavin/protein ratio has been found to be 0.36 ± 0.04 indicating that for per mol of enzyme, there are 0.36 mol of FMN present. The ratio was raised to 1.10 ± 0.20 by incubation of excess FMN with the purified enzyme followed by gel filtration chromatography for removing the unbound FMN. Furthermore, an addition of 1 mM commercial FMN to growth media has resulted in about 1.2- and 2.3-fold increases in yield of purified protein and flavin/protein ratio, respectively, when compared to the growth media without external FMN supplements.

3.5. Cofactor preference of Ssap-NtrB

A steady state experiment was performed in order to determine the pyridine-nucleotide preference of Ssap-NtrB. Using the ferricyanide as an artificial electron acceptor, oxidation of pyridine-nucleotide was monitored and resulting plots of k_{obs} versus nucleotide concentration can be seen in Figure 4. The NADPH-dependent reduction of potassium ferricyanide gave a $k_{cat(app)}$ of 7.10 ± 0.45 $s^{-1}$and $K_{M(app)}$ of 71.27 ± 10.51 μM whereas NADH gave a $k_{cat(app)}$ of 4.58 ± 0.60 s^{-1} and $K_{M(app)}$ of 95.26 ± 25.52 μM. These results make it clear that Ssap-NtrB has similar preference for both nucleotides with a small favour towards to NADPH.

3.6. pH and temperature optimum, and thermal stability determinations

We have investigated the effect of pH and temperature on Ssap-NtrB by monitoring the change of enzymatic activity over pH range of 5.5–10.5 and temperature range of 3–40 °C as well as temperature stability of Ssap-NtrB. The activity-pH profile showed an optimum pH at between 6.58 (ionic strength, μ = 0.081 M) and 8.0 (μ = 0.142 M) (Fig. 5) for the reduction of NFZ at 15 °C in 50 mM phosphate buffer and in 50 mM Tris–Cl buffer (for pH 7.5, μ = 0.045 M). Observations of similar activities at pH 7.5 in both phosphate and Tris–Cl with different ionic strengths (μ = 0.128 M and μ = 0.045 M) could indicate that pH effect was not confounded by differences in ionic strength. Additionally, Ssap-NtrB lost its activity considerably outside pH 6.0 and 8.5.

The effect of temperature on Ssap-NtrB was investigated by monitoring the change of enzymatic activity over the range of 3–40 °C. The activity-temperature profile showed maximum specific activity of 6.36 ± 0.24 U/mg for the reduction of NFZ at 15–20 °C under the experimental conditions specified (Fig. 6A

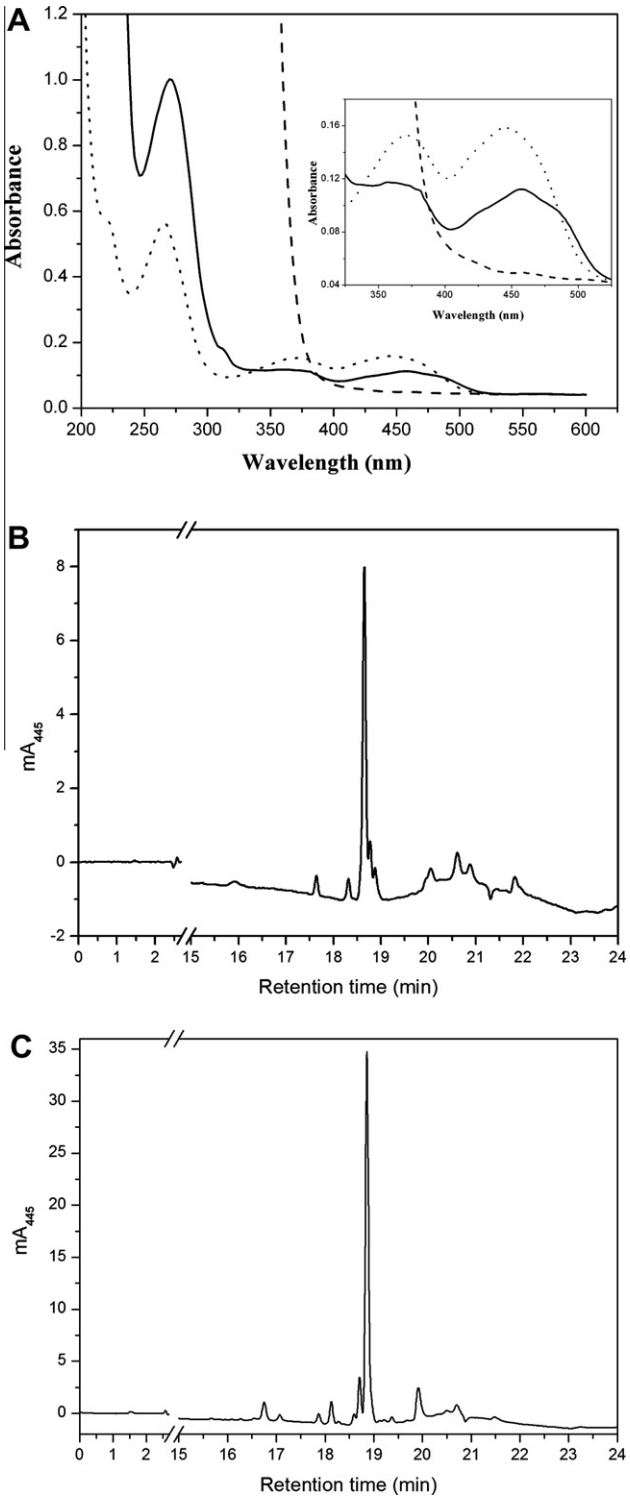

Figure 3. Flavin analysis of purified Ssap-NtrB. (A) Absorption spectra of the purified flavoprotein His_6-Ssap-NtrB from *S. saprophyticus* is shown (solid line). Loss of the FMN bands by reduction with an excess of sodium dithionite (dashed line) together with commercial FMN standard (dotted line) are shown. Insert, the characteristic part of the spectra (320–520 nm) was magnified for clarity. Protein concentration was 1.6 mg/ml (63 μM) in 50 mM Tris–Cl buffer, pH 7.4. (B) HPLC analysis of FMN extracted from His_6-Ssap-NtrB. (C) HPLC analysis of FMN standard obtained commercially.

(a)). (One unit is defined as the amount of the enzyme that catalyses the conversion of 1 μmol of substrate per minute under the experimental conditions specified.) Upon saturation the

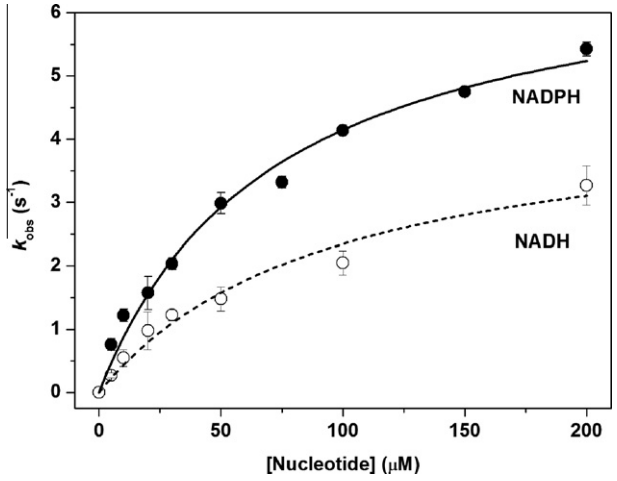

Figure 4. Michaelis–Menten curves for NADPH and NADH-dependent reaction of $K_3[Fe(CN)_6]$ catalysed by Ssap-NtrB. Each point is the mean of triplicate assays.

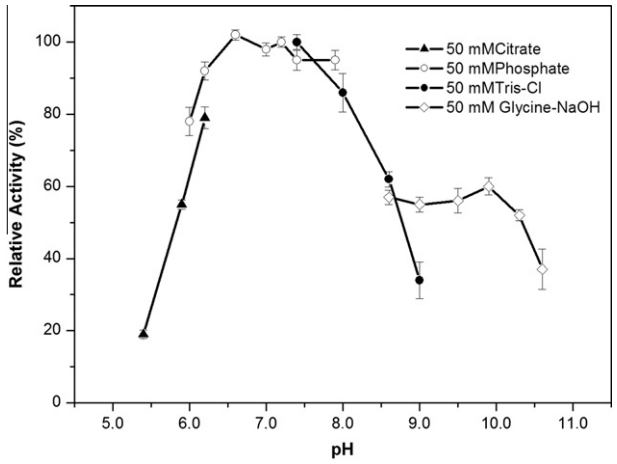

Figure 5. Activity-pH profile of Ssap-NtrB profiles were determined using 50 mM citrate (pH 5.4–6.2), 50 mM sodium phosphate (pH 6.0–7.9), 50 mM Tris–Cl (pH 7.4–9.0) and 50 mM glycine-NaOH (pH 8.6–10.6) buffers. The specific activity was measured using NFZ as substrate and expressed as μmol min^{-1} mg^{-1}active enzyme and the relative specific activity is expressed as the percentage of the maximum specific activity obtained under the experimental conditions.

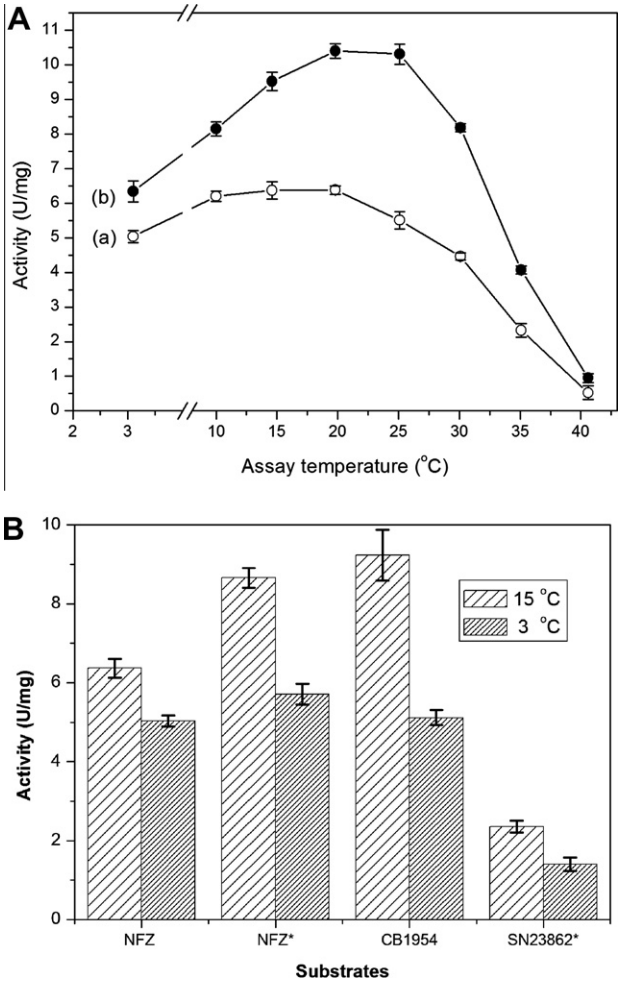

Figure 6. Activity-temperature profile of Ssap-NtrB catalysed reduction of NFZ, CB1954 and SN23862 (A) (a) NFZ reduction was measured by standard activity assay with Ssap-NtrB in 50 mM sodium phosphate buffer at pH 7.0 in the temperature range of 3–40 °C. (b) NFZ reduction was measured by standard activity assay with Ssap-NtrB saturated with commercial FMN in 50 mM sodium phosphate buffer at pH 7.0 in the temperature range of 3–40 °C. (B) NFZ, CB1954 and SN23862 reduction was measured in 50 mM sodium phosphate buffer at pH 7.0 at 3 and 40 °C. *Activities are based upon monitoring of NADPH turnover and have not been corrected for uncoupled oxidase activity and therefore they are upper estimates.

enzyme with flavin cofactor (FMN) by incubation purified enzyme with commercial FMN, followed by gel permeation chromatography to remove unbound flavin, gave rise to an increase in activity up to 10.40 ± 0.19 U/mg at 20–25 °C (Fig. 6A(b)). Surprisingly, unlike the other type I nitroreductases, over 95% and 80% activity values relative to the maximum activity (at 15 °C) were measured at 10 and 3 °C respectively for Ssap-NtrB without saturation. Upon saturation, over 76% and 62% activity values relative to the maximum activity (at 20 °C) were measured at 10 and 3 °C, respectively. At the other end of the lines, there were sharp decrease in activities beyond the room temperature for both cases; only a fraction of the maximum activities were obtained at 40 °C. In order to see the generalities of such observations, the temperature profile of substrates of prodrugs including CB1954 and SN23862 were investigated at both 15 and 3 °C monitoring either NFZ consumption and product formation for CB1954 or cofactor consumption (NADPH reduction) for NFZ and SN23862 (Fig. 6B). Activity profile of Ssap-NtrB against prodrug of CB1954 and SN23862 was similar with NFZ, retaining 55 and 60% of the maximum activities, respectively.

We investigated the kinetic stability of Ssap-NtrB by measuring its half life at the different temperature points expanding from 20 to 50 °C for incubation time of 0–240 min (Fig. 7). The remaining residual activity was determined at 20 °C using NFZ activity assay and expressed as a percentage of activity obtained with no heat treatment. Ssap-NtrB lost its activity notably at 30 °C within 60 min and almost complete thermo-inactivations were measured within 3 min at above 50 °C. Saturating protein samples with commercial FMN externally has raised enzyme activity 1.6-fold at optimum temperature, but there was no significant contribution has been observed for stability of Ssap-NtrB. The half life of the enzyme at 30, 35, 40 and 50 °C have been calculated to be 160, 28, 7 and <1 min, respectively.

3.7. Kinetic parameters and metabolite analysis

Preliminary activity experiments with fixed substrate concentration showed that the purified enzyme was shown to be active with NFZ, CB1954 and SN23862 (for the structure of substrates and its metabolites, see Supplementary Scheme 1). Therefore, the

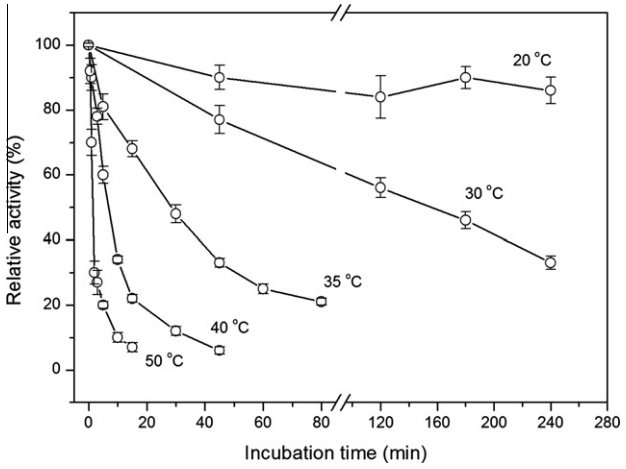

Figure 7. Thermal stability of Ssap-NtrB. The enzyme was dissolved in 50 mM Tris–Cl (pH 7.5) at a concentration of 0.4 mg/ml, and incubated for 0–240 min at the temperatures indicated. The remaining residual activity was determined at 20 °C using NFZ activity assay and expressed as a percentage of activity obtained without heat pre-treatment of the enzyme. Each point is the mean of triplicate assays.

reductive metabolism of NFZ, CB1954 and SN23862 by Ssap-NtrB were investigated. HPLC analysis followed by mass spectrometric analysis showed that the corresponding reduction products for all three substrates were obtained.

In the case of NFZ, a single reduction product was obtained in good yield under the experimental conditions (Supplementary Fig. S1). The product of the enzymatic reduction of NFZ was isolated using a reverse phase HPLC and analysed by ESI-MS in the negative mode with a molecular ion $[M-H]^-$ at $m/z = 183$, indicating a molecular mass of 184, this corresponds to a loss of an oxygen atom and the gain of two hydrogen atoms (a four-electron reduction) to form the hydroxylamine species as previously observed with other nitroreductases.[27] To determine the full kinetic parameters for NFZ reduction, the steady-state kinetic experiments were repeated at a series of 13 NFZ concentrations ranging from 0 to 300 μM. Ssap-NtrB catalysed reduction of NFZ obeyed Michaelis–Menten kinetics (Supplementary Fig. S4A). All the kinetic data were fitted to Michaelis–Menten equation by nonlinear regression and calculated steady state parameters, $k_{cat(app)}$ and $K_{M(app)}$ are given in Table 1. Steady state parameters for the reduction of NFZ, CB1954 and SN23862 using other well known nitroreductases were also shown in Table 1 for comparisons. HPLC analysis indicated that Ssap-NtrB was able to reduce the prodrug of CB1954 to mixture of the isomers **4HX** [5-(aziridin-1-yl)-4-hydroxyamino-2-nitrobenzamide] and **2HX** [5-(aziridin-1-yl)-2-hydroxyamino-4-nitrobenzamide] with around 1:1 molar ratio (Supplementary Fig. S2). Under the experimental conditions, almost all the substrate was converted to the metabolites within 30 min in the expense of NAD(P)H. Further studies by MS(ESP^-) showed that the first isomer with retention time of 4.95 min could be detected in the negative mode with a molecular ion $[M-H]^-$ at $m/z = 237$, indicating a molecular mass of 238 and confirming that it was indeed the **4HX** (based on the information available in the literature). Similarly, the second isomer with the retention time of 12.86 min had the same characteristics as the first one, $[M-H]^-$ at $m/z = 237$, indicating a molecular mass of 238 confirming that it was the **2HX**. Finally, very small amounts of metabolites (with retention times of 19.2 and 24.5 min) were also observed. Considering the retention times, we assumed that they were the further reduction products of CB1954; the corresponding amines (Supplementary Fig. S2). To examine the substrate specificity of the enzymes, similar steady-state kinetic experiments were done with prodrug of CB1954 and SN23862 as the substrates. High concentration of CB1954 was required to saturate the enzyme (4 mM). Due to the limited solubility of CB1954, it is only possible to obtain the initial part of the Michaelis–Menten curve for this substrate, giving a good estimate of the specificity constant, k_{cat}/K_M, but poor estimates of apparent k_{cat} or apparent K_M individually.

The reduction of SN23862 resulted in mixture of product with complex and unstable nature. Therefore, mass spectrometric analyses was not completed and being under investigation (Supplementary Fig. S3). The steady-state kinetic experiments were repeated at a series of nine SN23862 concentrations ranging from 0 to 160 μM. Any attempt to use higher concentration than this range resulted in diminished activity of the enzyme by probably substrate inhibition. Therefore, Michaelis–Menten curve was achieved using substrate concentration of up to 160 μM with a series of eight different concentrations.

4. Discussion

Homology based databank search identified a putative nitroreductase gene in *Staphylococcus saprophyticus*. PCR based amplification was performed and the resulting gene product was cloned into an expression vector for evaluation. Use of pET14b vector gave a construct that allowed production of fusion proteins with a polyhistidine (His_6) affinity tag site at their N-termini. *Escherichia coli* cells (BL21(DE3)) that was transformed with the resulting recombinant plasmid expressed the Ssap-NtrB protein upon induction with IPTG in soluble form at the efficient level of 40–50 mg/L. The resulting NTR was purified to homogeneity using metal-chelate affinity chromatography. Protein characterisation including sequencing, SDS–PAGE and protein mass spectrometry confirmed its identity.

Bacterial oxygen insensitive nitroreductases could be classified into two main groups that are representative of the *E. coli* NTRs: group A (*NfsA*) and group *B* (*NfsB*). The phylogenetic analysis suggested Ssap-NtrB to be relatively close to minor the *E. coli* nitroreductase (*NfsB*) (Fig. 8). However, amino acids sequence comparison analysis revealed that only 20% identity and 25% similarity with NfsB.

Similar to other type I nitroreductases, a concentrated solution of Ssap-NtrB is yellow and resistant to decolouration when the enzyme solution was dialysed. This indicates that strong binding of flavin to the enzyme. Spectroscopic analysis followed by HPLC analysis proved that it is FMN that receives the reducing equivalents from the pyridine nucleotides. Together, these results indicate that Ssap-NtrB is a flavoprotein tightly associated with FMN. Recombinant expression of His_6-tagged Ssap-NtrB in *E. coli* yielded high level of enzyme expression, but only about 30% of enzyme contains FMN. The molar ratio of FMN:protein however can easily be raised to 1.1 by addition of FMN externally.

Direct measurements of the reduction of the FMN group by pyridine nucleotides showed that the enzyme displays no marked preference for any of nucleotide used. The ratio of catalytic efficiencies (k_{cat}/K_M) for NADPH over that of NADH for the reduction of artificial electron acceptor (ferricyanide) is only 2.1-fold, showing only a small favour towards to NADPH. This result indicates that Ssap-NtrB has similar nucleotide preference with group B of oxygen insensitive nitroreductases (type I),[1] therefore it is named as Ssap-NtrB. In *E. coli*, for examples, the *nfsB* uses both NADPH and NADH to reduce NFZ and other nitro compounds, while *nfsA* uses NADPH exclusively to catalyse similar reactions.[14,47,48]

The nitroreductase purified in this work showed maximal activity at between pH 6.0–8.0 regardless of the buffers used. This is consistent with several well characterised bacterial nitroreductases, having an optimal pH around 7 (reviewed in[1]). The Ssap-NtrB displayed, however distinct unusual characteristics in temperature profiles with other nitroreductases. Among all major oxygen

Table 1
Kinetic parameters for the reduction of NFZ, CB1954 and SN23862 by purified Ssap-NtrB. Kinetic parameters for the reduction of NFZ, CB1954 and SN23862 using the major nitroreductases have also provided for comparisons

Enzyme	Substrates	Cofactor	K_M[a] (μM)	k_{cat}[a] (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
Ssap-NtrB (This work)	NFZ	NADPH	49.8 ± 3.9	2.20 ± 0.05	44180
Ssap-NtrB (This work)	CB1954	NADPH	1065.2 ± 53.1	2.26 ± 0.07	2120
Ssap-NtrB (This work)	SN23862	NADPH	82.4 ± 16.9	0.77 ± 0.07	9350
NfsB[b]	NFZ	NADPH	170 ± 13	10.5 ± 0.1	61764
NfsB[b]	NFZ	NADH	160 ± 6	10.7 ± 0.2	66875
NfsA[c]	CB1954	NADPH	220 ± 18	16 ± 0.48	73000
NfsB[c]	CB1954	NADPH	3600 ± 660	26 ± 2.6	7300
NfsB[c]	CB1954	NADH	11000 ± 2600	62 ± 11	5600
AzoR[c]	CB1954	NADPH	1400 ± 160	0.15 ± 0.0077	110
AzoR[c]	CB1954	NADH	6600 ± 1000	0.15 ± 0.015	23
ywrO[d]	CB1954	NADPH	618 ± 169	8.2 ± 1.1	13268
ywrO[d]	SN23862	NAD(P)H	Inactive	ND	–

[a] These are apparent K_M and k_{cat} values at 200 μM NADPH (for Ssap-NtrB), 250 μM NAD(P)H (for NfsA, NfsB and AzoR) and 500 μM NADPH (for ywrO).
[b] Data was taken from.[27]
[c] Data was taken from.[68]
[d] Data was taken from.[24]

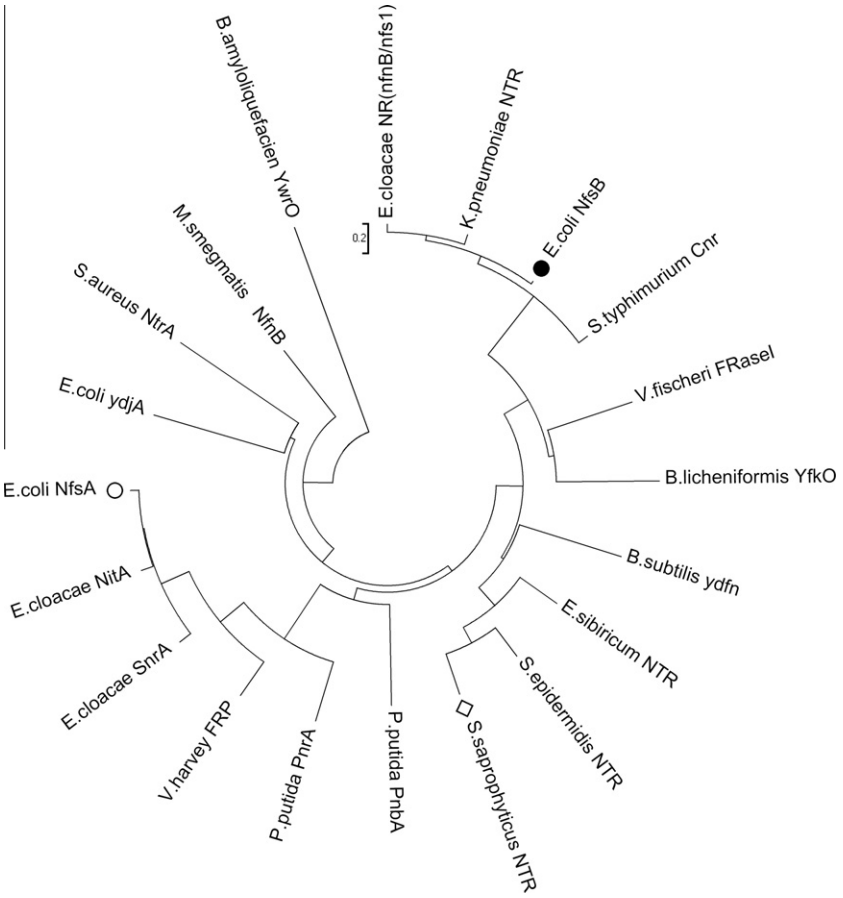

Figure 8. Pylogenetic tree based on deduced amino acids sequences of the type I (oxygen insensitive) nitroreductases superfamily including new member, (♦) SsapNTR (Ssap-NtrB) from *S. saprophyticus*. There are two main groups in the type I nitroreductases; group A and B, which includes subgroups of B_1 and B_2. Members of the group A enzymes includes the major *E. coli* nitroreductases of nfsA (○),[61] *E. cloacae* NitA,[62] *E. cloacae* SnrA,[17] *V. harveyi* FRP,[21] *P. putida* PrnA,[20] *Mycobacterium smegmatis* NfnB,[63] and *P. putida* PnbA.[20] The group B_1 includes the minor *E. coli* nitroreductases of nfsB (●)[48], *Klebsiella pneumoniae* NTR,[64] *E. cloacae* NR (nfnB/nfs1),[62] *S. typhimurium* Cnr,[17] *V. fischeri* FRaseI,[22] a hypothetical nitroreductases from *Exiguobacterium sibiricum* NTR,[65] a hypothetical nitroreductases from *S. epidermidis* NTR[66] and *S. saprophyticus* SsapNTR (Ssap-NtrB). The group B_2 includes *E. coli* nitroreductases of ydjA(◇).[30] and *Staphylococcus aureus* NtrA.[67] Ungrouped nitroreductases from *Bacillus amyloliquefaciens* YwrO[24] is also included.

insensitive nitroreductases characterised so far, Ssap-NtrB is probably the first example that shows uncharacteristic temperature-activity profile; showing optimum activity at 15–20 °C and retaining up to 85% of its optimum activity at 3 °C for the reduction of NFZ and other substrates (3 °C was the minimum temperature at which experiments were conducted properly. Below this temperature, the activity measurements were hampered due to excess condensation of water on the cell surface in the Uv–vis spectrophometer). Saturation the enzyme with external FMN, however, resulted in an increase in activity at about 1.6-fold, giving specific activity of 10.40 ± 0.19 U/mg at 20–25 °C due to probably generating more active enzymes upon incorporation of flavin

(FMN). The saturated enzyme has again retained over 62% of its optimum activity at 3 °C for the reduction of NFZ. It was noticed that the temperature optimum was shifted slightly from 15–20 to 20–25 °C, for that we have no logical reasoning at present or the shift may be not significant at all. Both *E. coli* nitroreductases (NfsA and NfsB), for example, has optimum activity at 40 °C and they lose their activity dramatically below 15 °C.[49,50] Similar activity-temperature profiles to *E. coli* nitroreductases have been observed with other nitroreductases; examples include *B. licheniformis* (30 °C),[26] *Enterobacter cloacae* NR (45 °C),[51] *Klebsiella* sp. C1 Ntr (30–40 °C),[52] *V. fischeri* FraseI (26 °C),[22] *R. capsulatus* NprA and NprB (30 °C)[23] and *Clostridium acetobutilicum* NitA and NitB (30 °C)[53] (the temperature optimum were given in parentheses). Although Ssap-NtrB shows some similar biochemical properties with type I (group B) nitroreductases, it differs dramatically in this aspect; being a cold active nitroreductase. The rapid temperature dependent inactivation of the enzyme above the optimum, strengths further the cold active nature of Ssap-NtrB. The existing data concerning cold-adopted enzymes indicates that a high specific activity is almost always coupled with a low thermostability.[54] Saturating protein samples with commercial FMN added externally has raised enzyme activity noticeably at optimum temperature, but we have not seen any significant improvement in stability upon saturating the enzyme with externally added FMN. No clear evidence was found to support the idea that FMN interacts with both monomers and likely to contribute to thermal stability. The finding, therefore, ruled out that low stability was due to lack of FMN (or that the enzyme was being mainly in *apo* form) when external FMN addition was omitted. The cold active nature of Ssap-NtrB is quite surprising since the bacterium, from which the nitroreductase cloned (*S. saprophyticus*) is known to belong to the mesophilic bacteria, which grows best in moderate temperature between 25 and 40 °C. Having active enzymes at low temperature might be related to the bacterium ability for surviving in a cold temperature environment. Finding cold-adopted nitroreductases might, therefore shed a light on biological functions of nitroreductases in general and represent great potentials in many biotechnological areas of interests.

We have examined the kinetics and metabolite profiles of Ssap-NtrB with nitrofurazone, CB1954 and SN23862. Nitroreductases catalysed bioconversion of nitroaromatics to highly toxic corresponding nitroso and hydroxyl amine metabolites have been exploited in the use of nitrofuran derivatives as antibiotics. NFZ, for example, is bactericidal for many Gram-positive and Gram-negative bacteria and this antibacterial is effective for control of *Aeromonas*, *Vibrio* and related species. The study of Ssap-NtrB catalysed NFZ reduction yielded a four-electron reduction product of the hydroxylamine derivative ((E)-2-(5-hydroxyamino furan-2-yl-methylene) hydrazinecarboxamide). The steady state kinetics revealed comparable values with *E. coli* NfsB.[27,43] Since type I nitroreductases follows ping-pong bi-bi mechanism, only specificity constants (the ratio k_{cat}/K_M) are comparable meaningfully. The catalytic efficiency of *E. coli* NfsB with NFZ is slightly better than Ssap-NtrB under the experimental conditions (about 1.4-fold). This finding is, however, not totally unexpected as Ssap-NtrB share low amino acid sequence homology with *E. coli* NfsB (20% identity and 24% similarity) and the flavin (FMN) content of the enzyme in the recombinant form (His_6-Ssap-NtrB), that is mandatory for the enzyme reaction was found to be approximately 30%. An increase of FMN/protein ratio would contribute positively in the specificity constant.

NAD(P)H-dependent nitroreductases are of importance due to their potential in biomedicinal applications. In particular, several cancer therapies, *E. coli* NfsB with cancer prodrug of CB1954 have reached the clinical trial stages. On the other hand, poor catalytic efficiency limits the activation of CB1954 by NfsB at clinically relevant doses. We have evaluated Ssap-NtrB ability for activation of CB1954 and another bioreductive prodrug called SN23862. CB1954 was converted into two hydroxylamine metabolites with equal amount as it was reported for *E. coli* NfsB catalysed reaction.[55] The product distribution or regioselectivity was postulated to be dependent on solvent accessibility of the two nitro groups.[32] Because, in silico studies using NfsB as model enzyme suggested that the mechanism for reduction of CB1954 is by electron transfer from FMN to the nitroaromatic ring, followed by protonation from solvent. Although the formation of highly toxic metabolite (**4HX**) is desirable as it is generally regarded as the principal cytotoxin to form a bifunctional DNA cross-linking agent, the production of both metabolites is also equally important. First of all, it has been suggested that the mixture in CB1954 metabolites vary in cytotoxicity toward different cell types. For instance, SKOV-3 cells are more sensitive than HCT-116 to the 2-HX reduced metabolite.[56] Secondly, the 2HX derivative of CB1954 was also shown to have a greater bystander effect via its amine derivative in GDEPT, killing neighbouring cells where the enzyme was not expressed.[56] This is particularly important since not all tumour cells will be infected with the vector containing the gene of NTR. Finally, Ssap-NtrB (similar to *E. coli* NfsB) can utilise both NADH and NADPH efficiently as electron donors (whereas NfsA has a sole preference for NADPH). *E. coli* NfsB shows small preference for former for the reduction of both NFZ[27] and CB1954[57] while Ssap-NtrB showed a small favour towards to NADPH. Considering the intracellular concentrations of reduced nicotinamide cofactors it could present a small advantage over *E. coli* NfsB for EPT. (NADPH is usually present at higher concentration than NADH in mammalian cells. Even though the total amount of NAD^+ and NADH is about 10 times higher than that of $NADP^+$ and NADPH, the ratio of NADH/NAD^+ is much lower than the ratio of NADPH/$NADP^+$; NADPH is being the dominant form of reduced nicotinamide cofactor in the cells).[58]

Type I nitroreductases preferentially perform two-electron chemistry with a ping-pong bi-bi kinetic mechanism.[1] The steady state kinetics of Ssap-NtrB with CB1954 are therefore, tentatively compared with $K_{M(app)}$ and $k_{cat(app)}$ values with other nitroreductases[1] and are summarised in Table 1 (Note that the concentration of NADPH were 200 and 250 μM for Ssap-NtrB and NfsB assays, respectively). Even though the turnover number is lower than *E. coli* NfsB, about 20-fold differences have been observed in $K_{M(app)}$ indicating that Ssap-NtrB has higher affinity towards CB1954 than NfsB. The flavin (FMN) content of the recombinant enzyme (approximately 30%) again contributed significantly for obtaining lower level of activity as it was explained for NFZ bioconversion. However the specificity constant k_{cat}/K_M, which is the key parameter determining the rate of reaction at low substrate concentration, yielded similar values; only twofold lower specificity constant obtained in comparison to that for NfsB.

The reduction of SN23862 yielded a mixture of products with some complexity (Supplementary Fig. S3) and characterisation of the metabolites were not completed and being under investigation. The steady state kinetics of Ssap-NtrB with SN23862, however, signifies the extent of substrate specificity of the enzyme.

In conclusion, we have purified and characterised a nitroreductase enzyme from *S. saprophyticus*. Even though there are 20% identity and 25% similarity between this enzyme and the well known nitroreductase; *E. coli* Ntr (NfsB), they share several biochemical properties including reducing NFZ, CB1954 and SN23862 in the expense of either NADH or NADPH cofactors, pH activity-profiles and molecular sizes. What they do not share is the activity-temperature profiles, in which *E. coli* Ntr (NfsB) has optimum activity at 30–40 °C with no reported activities below 15 °C, whereas Ssap-NtrB retains considerable enzymatic activities at 15 and 3 °C. Such unique property makes Ssap-NtrB as a versatile prodrug activating enzyme. As in the enzyme prodrug therapy (EPT), use of *E. coli* NfsB

in combination with prodrug of CB1954 has used to kill tumour cells. To develop EPT further, a useful direction could be a combination of EPT with crymotheraphy[59], a local treatment or refrigeration in which the tissues are exposed to a temperature of 4–10 °C. In this case, cold-adopted nitroreductases such as Ssap-NtrB could be an ideal candidate due to its significant activity at lowered temperature. The use enzyme at lowered temperature could serve as a trigger mechanism for the enzyme activity as well as reducing risk of damaging healthy tissues surrounding tumour cells. In addition, Ssap-NtrB can easily be utilised to access sufficient amount of temperature sensitive drug candidates and intermediates, enzymatically from prodrugs and other compounds for purpose of pharmacological studies in vitro. Furthermore, cell ablation can be utilised as a cure for certain disorders such as cancer and leukaemia. Cold active nature of Ssap-NtrB could possibly be used as a tool to study conditional cell ablation[60] in cold-blooded animals (poikilotherms), which are becoming useful as experimental subjects and are attracted the attention of scientists in a variety of biological disciplines, including space biology and medical research. Finally, application of organisms with degradative capabilities, or their enzyme for eradicating pollutants from the environment is of importance. Among the pollutants, nitroaromatic compounds including TNT are widely used as recalcitrant explosives and are required to be eliminated from the environment. Our preliminary work indicated that Ssap-NtrB acts on simple nitroaromatic tested so far (data not shown). As a result of this preliminary work, it could be foreseen that Ssap-NtrB could therefore be used in areas where high activity at low temperatures is required for bioremediation of compounds of explosive nature and volatile toxic compounds.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.04.004.

References and notes

1. Roldán, M. D.; Pérez-Reinado, E.; Castillo, F.; Moreno-Vivián, C. *FEMS Microbiol. Rev.* **2008**, *32*, 474.
2. Knox, R. J.; Boland, M. P.; Friedlos, F.; Coles, B.; Southan, C.; Roberts, J. J. *Biochem. Pharmacol.* **1988**, *37*, 4671.
3. Knox, R. J.; Friedlos, F.; Jarman, M.; Roberts, J. J. *Biochem. Pharmacol.* **1988**, *37*, 4661.
4. Roberts, J. J.; Friedlos, F.; Knox, R. J. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 1073.
5. Knox, R. J.; Friedlos, F.; Sherwood, R. F.; Melton, R. G.; Anlezark, G. M. *Biochem. Pharmacol.* **1992**, *44*, 2297.
6. Friedlos, F.; Denny, W. A.; Palmer, B. D.; Springer, C. J. *J. Med. Chem.* **1997**, *40*, 1270.
7. Fox, M. E.; Lemmon, M. J.; Mauchline, M. L.; Davis, T. O.; Giaccia, A. J.; Minton, N. P.; Brown, J. M. *Gene Ther.* **1996**, *3*, 173.
8. McNeish, I.; Green, N.; Gilligan, M. E. A. *Gene Ther.* **1998**, *1061*, 5.
9. Weedon, S. J.; Green, N. K.; McNeish, I. A.; Gilligan, M. G.; Mautner, V.; Wrighton, C. J.; Mountain, A.; Young, L. S.; Kerr, D. J.; Searle, P. F. *Int. J. Cancer* **2000**, *86*, 848.
10. Anlezark, G. M.; Melton, R. G.; Sherwood, R. F.; Wilson, W. R.; Denny, W. A.; Palmer, B. D.; Knox, R. J.; Friedlos, F.; Williams, A. *Biochem. Pharmacol.* **1995**, *50*, 609.
11. Cobb, L. M.; Connors, T. A.; Elson, L. A.; Khan, A. H.; Mitchley, B. C. V.; Ross, W. C. J.; Whisson, M. E. *Biochem. Pharmacol.* **1969**, *18*, 1519.
12. Friedlos, F.; Court, S.; Ford, M.; Denny, W. A.; Springer, C. *Gene Ther.* **1998**, *5*, 105–112.
13. Djeha, A. H.; Thomson, T. A.; Leung, H.; Searle, P. F.; Young, L. S.; Kerr, D. J.; Harris, P. A.; Mountain, A.; Wrighton, C. J. *Mol. Ther.* **2001**, *3*, 233.
14. Bryant, D. W.; McCalla, D. R.; Leeksma, M.; Laneuville, P. *Can. J. Microbiol.* **1981**, *27*, 81.
15. Zenno, S.; Koike, H.; Kumar, A. N.; Jayaraman, R.; Tanokura, M.; Saigo, K. *J. Bacteriol.* **1996**, *178*, 4508.
16. Zenno, S.; Koike, H.; Tanokura, M.; Saigo, K. *J Biochem* **1996**, *120*, 736.
17. Watanabe, M.; Ishidate, M., Jr.; Nohmi, T. *Nucleic Acids Res.* **1990**, *18*, 1059.
18. Watanabe, M.; Nishino, T.; Takio, K.; Sofuni, T.; Nohmi, T. *J. Biol. Chem.* **1998**, *273*, 23922.
19. Nokhbeh, M. R.; Boroumandi, S.; Pokorny, N.; Koziarz, P.; Paterson, E. S.; Lambert, I. B. *Mutat. Res.* **2002**, *508*, 59.
20. Caballero, A.; Lázaro, J. J.; Ramos, J. L.; Esteve-Núñez, A. *Environ. Microbiol.* **2005**, *7*, 1211.
21. Lei, B.; Liu, M.; Huang, S.; Tu, S. C. *J. Bacteriol.* **1994**, *176*, 3552.
22. Zenno, S.; Saigo, K.; Kanoh, H.; Inouye, S. *J. Bacteriol.* **1994**, *176*, 3536.
23. Blasco, R.; Castillo, F. *Appl. Environ. Microbiol.* **1993**, *59*, 1774.
24. Anlezark, G. M.; Vaughan, T.; Fashola-Stone, E.; Paul Michael, N.; Murdoch, H.; Sims, M. A.; Stubbs, S.; Wigley, S.; Minton, N. P. *Microbiol* **2002**, *148*, 297.
25. Zenno, S.; Kobori, T.; Tanokura, M.; Saigo, K. *Biosci., Biotechnol., Biochem.* **1978**, *1998*, 62.
26. Emptage, C.; Knox, R.; Danson, M.; Hough, D. *Biochem. Pharmacol.* **2009**, *77*, 21.
27. Race, P.; Lovering, A.; Green, R.; Ossor, A.; White, S.; Searle, P.; Wrighton, C.; Hyde, E. *J. Biol. Chem.* **2005**, *280*, 13256.
28. Lovering, A.; Hyde, E.; Searle, P.; White, S. *J. Mol. Biol.* **2001**, *309*, 203.
29. Parkinson, G.; Skelly, J.; Neidle, S. *J. Med. Chem.* **2000**, *43*, 3624.
30. Choi, J.-W.; Lee, J.; Nishi, K.; Kim, Y.-S.; Jung, C.-H.; Kim, J.-S. *J. Mol. Biol.* **2008**, *377*, 258.
31. Skelly, J. V.; Collins, P. J.; Knox, R. J.; Anlezark, G. M.; Melton, R. G. *J. Mol. Biol.* **1994**, *238*, 852.
32. Christofferson, A.; Wilkie, J. *Biochem. Soc. Trans.* **2009**, *37*, 413.
33. Anlezark, G.; Melton, R.; Sherwood, R.; Wilson, W.; Denny, W.; Palmer, B.; Knox, R.; Friedlos, F.; Williams, A. *Biochem. Pharmacol.* **1995**, *50*, 609.
34. Hu, L.; Yu, C.; Jiang, Y.; Han, J.; Li, Z.; Browne, P.; Race, P. R.; Knox, R. J.; Searle, P. F.; Hyde, E. I. *J. Med. Chem.* **2003**, *46*, 4818.
35. Kuroda, M.; Yamashita, A.; Hirakawa, H.; Kumano, M.; Morikawa, K.; Higashide, M.; Maruyama, A.; Inose, Y.; Matoba, K.; Toh, H.; Kuhara, S.; Hattori, M.; Ohta, T. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13272.
36. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. *Mol. Biol. Evol.* **2011**, *28*, 2731.
37. Thompson, J. D.; Higgins, D. G.; Gibson, T. J. *Nucleic Acids Res.* **1994**, *22*, 4673.
38. Sambrook, J.; Russell, D. W. *Cold Spring Harb. Protoc.* **2006**, *2006*. pdb.prot4455.
39. Studier, F. *Protein Exp. Purif.* **2005**, *41*, 207.
40. Bradford, M. *Anal. Biochem.* **1976**, *72*, 248.
41. Gill, S. C.; von Hippel, P. H. *Anal. Biochem.* **1989**, *182*, 319.
42. Jarrom, D.; Jaberipour, M.; Guise, C. P.; Daff, S.; White, S. A.; Searle, P. F.; Hyde, E. I. *Biochemistry* **2009**, *48*, 7665.
43. Race, P.; Lovering, A.; White, S.; Grove, J.; Searle, P.; Wrighton, C.; Hyde, E. *J. Mol. Biol.* **2007**, *368*, 481.
44. Galante, Y.; Hatefi, Y. In *Methods in Enzymology*; Sidney, F., Lester, P., Eds.; Academic Press, 1978; Vol. 53, p 15.
45. Knox, R.; Friedlos, F.; Biggs, P.; Flitter, W.; Gaskell, M.; Goddard, P.; Davies, L.; Jarman, M. *Biochem. Pharmacol.* **1993**, *46*, 797.
46. Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. *Nucleic Acids Res.* **1997**, *25*, 3389.
47. Whiteway, J.; Koziarz, P.; Veall, J.; Sandhu, N.; Kumar, P.; Hoecher, B.; Lambert, I. B. *J. Bacteriol.* **1998**, *180*, 5529.
48. Zenno, S.; Koike, H.; Tanokura, M.; Saigo, K. *J. Biochem.* **1996**, *120*, 736.
49. Zenno, S.; Koike, H.; Tanokura, M.; Saigo, K. *J. Bacteriol.* **1996**, *178*, 4731.
50. Zenno, S.; Kobori, T.; Tanokura, M.; Saigo, K. *J. Bacteriol.* **1998**, *180*, 422.
51. Bryant, C. The University of California 1990.
52. Kim, H.; Song, H. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 766.
53. Kutty, R.; Bennett, G. *Arch. Microbiol.* **2005**, *184*, 158.
54. Gerday, C.; Aittaleb, M.; Bentahir, M.; Chessa, J.-P.; Claverie, P.; Collins, T.; D'Amico, S.; Dumont, J.; Garsoux, G.; Georlette, D.; Hoyoux, A.; Lonhienne, T.; Meuwis, M.-A.; Feller, G. *Trends Biotechnol.* **2000**, *18*, 103.
55. Knox, R.; Boland, M.; Friedlos, F.; Coles, B.; Southan, C.; Roberts, J. *Biochem. Pharmacol.* **1988**, *37*, 4671.
56. Helsby, N. A.; Ferry, D. M.; Patterson, A. V.; Pullen, S. M.; Wilson, W. R. *Br. J. Cancer* **2004**, *90*, 1084.
57. Vass, S. O.; Jarrom, D.; Wilson, W. R.; Hyde, E. I.; Searle, P. F. *Br. J. Cancer* **1903**, *2009*, 100.
58. Williamson, D. H.; Lund, P.; Krebs, H. A. *Biochem. J.* **1967**, *103*, 514.
59. Varon, J.; Acosta, P. *Chest* **2008**, *133*, 1267.
60. Curado, S.; Stainier, D. Y. R.; Anderson, R. M. *Nat. Protoc.* **2008**, *3*, 948.
61. Zenno, S.; Koike, H.; Kumar, A.; Jayaraman, R.; Tanokura, M.; Saigo, K. *J. Bacteriol.* **1996**, *178*, 4508.
62. Koder, R.; Miller, A. *Protein Exp. Purif.* **1998**, *13*, 53.

63. Manina, G.; Bellinzoni, M.; Pasca, M. R.; Neres, J.; Milano, A.; De Jesus Lopes Ribeiro, A. L.; Buroni, S.; Škovierová, H.; Dianišková, P.; Mikušová, K.; Marák, J.; Makarov, V.; Giganti, D.; Haouz, A.; Lucarelli, A. P.; Degiacomi, G.; Piazza, A.; Chiarelli, L. R.; De Rossi, E.; Salina, E.; Cole, S. T.; Alzari, P. M.; Riccardi, G. *Mol. Microbiol.* **2010**, *77*, 1172.
64. Bryant, C.; Hubbard, L.; McElroy, W. *J. Biol. Chem.* **1991**, *266*, 4126.
65. Joint Center for Structural Genomics (Jcsg), (Pdb code: 3GE6), 2009.
66. Zhang, Y. Q.; Ren, S. X.; Li, H. L.; Wang, Y. X.; Fu, G.; Yang, J.; Qin, Z. Q.; Miao, Y. G.; Wang, W. Y.; Chen, R. S.; Shen, Y.; Chen, Z.; Yuan, Z. H.; Zhao, G. P.; Qu, D.; Danchin, A.; Wen, Y. M. *Mol. Microbiol.* **2003**, *49*, 1577.
67. Streker, K.; Freiberg, C.; Labischinski, H.; Hacker, J.; Ohlsen, K. *J. Bacteriol.* **2005**, *187*, 2249.
68. Prosser, G. A.; Copp, J. N.; Syddall, S. P.; Williams, E. M.; Smaill, J. B.; Wilson, W. R.; Patterson, A. V.; Ackerley, D. F. *Biochem. Pharmacol.* **2010**, *79*, 678.