Bacillus amyloliquefaciens orthologue of Bacillus subtilis ywrO encodes a nitroreductase enzyme which activates the prodrug CB 1954

Gill M. Anlezark, Thomas Vaughan,† Elizabeth Fashola-Stone, N. Paul Michael,‡ Heather Murdoch, Meg A. Sims, Simon Stubbs,‡ Stuart Wigley§ and Nigel P. Minton

A nitroreductase with distinct properties that can activate the prodrug 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) was isolated from Bacillus amyloliquefaciens. The encoding gene was identified as a homologue of the ywrO of Bacillus subtilis, and was obtained as a PCR product by reverse genetics, cloned and the entire nucleotide sequence determined. The gene was found to reside between homologues of the B. subtilis alsD and yswB genes; however, the ywrO and yswB genes of B. amyloliquefaciens were not separated by a fourth gene, ywsA. The B. amyloliquefaciens ywrO gene was overexpressed, the recombinant protein purified and its properties were compared with those of two CB 1954-activating enzymes, Escherichia coli B nitroreductase (NTR) and Walker DT-diaphorase (DTD). In common with these enzymes menadione was an electron acceptor (\(K_m\) 3 \(\mu\)M) and activity with this substrate was inhibited by the presence of dicoumarol (\(K_i\) 1 \(\mu\)M). In contrast, YwrO showed a marked preference for NADPH as a cofactor (\(K_m\) 40 \(\mu\)M) and therefore could not be classified as a DTD (EC 1.6.99.2). The flavin FMN was an acceptor with high affinity. B. amyloliquefaciens YwrO was shown to be a flavoprotein with a monomeric molecular mass of 21.5 kDa by calculation and SDS-PAGE. The cytotoxic 4-hydroxylamine derivative was the single CB 1954 reduction product, but B. amyloliquefaciens YwrO was inactive with the bischloroethyl analogue of CB 1954, SN 23862. In both of these properties B. amyloliquefaciens YwrO more closely resembles DTD than NTR. Its \(K_m\) for CB 1954 was lower than that of NTR (617 \(\mu\)M compared to 862 \(\mu\)M). Enhanced in vitro cytotoxicity of CB 1954 was demonstrated on incubation of V79 cells with prodrug, NADPH and B. amyloliquefaciens YwrO. The work has led to the identification of a previously unknown nitroreductase, B. amyloliquefaciens YwrO, with distinct properties which will aid the rational selection of appropriate genes for applications in directed enzyme prodrug therapy (DEPT).

Keywords: prodrug activation, DEPT, cancer therapy, flavoprotein, in vitro cytotoxicity
INTRODUCTION

The monofunctional prodrug 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) can be activated to form a cytotoxic hydroxylamine derivative by nitroreductase enzymes (Fig. 1) (Anlezark et al., 1995; Knox et al., 1988b). The difunctional alkylating agent formed upon reduction with these enzymes can cross-link DNA via a non-enzymic combination with cellular thiols (Knox et al., 1991). Therefore, CB 1954 has potential applications in cancer therapy. CB 1954 is toxic to Walker rat carcinoma cells due to the presence of a form of NAD(P)H oxidoreductase, DT-diaphorase (DTD; EC 1.6.99.2) (Knox et al., 1988b), in these cells. The enzyme DTD was originally defined as having the ability to use the cofactors di- and triphosphopyridine nucleotide (NADH and NADPH) equally well. Although other mammalian cells, including human cells, do not possess an equivalent DTD activity (Boland et al., 1991) the conversion of CB 1954 to hydroxylamine can be exploited in cancer therapy using directed enzyme prodrug therapy (DEPT) approaches.

DEPT involves directing an exogenous enzyme with the required properties to the target tumour as a fusion protein, as part of an antibody–enzyme conjugate (antibody-directed enzyme prodrug therapy, ADEPT) (Bagshawe, 1987; Bagshawe et al., 1988), or by delivering the encoding gene to the target cells. The presence of the functional enzyme will eliminate the targeted cells and, through a bystander effect, will also eliminate cells in the immediate vicinity of the targeted cells on administration of the prodrug (gene-/viral-directed enzyme prodrug therapy, GDEPT/VDEPT, or clostridial-directed enzyme prodrug therapy, CDEPT) (Dachs et al., 1997; Minton et al., 1995; Lemmon et al., 1997).

Bacterial enzymes offer a number of advantages in DEPT strategies in that there is often no mammalian equivalent and their substrate specificity may differ substantially from that of mammalian enzymes so that non-specific prodrug activation, at sites other than the tumour, can be avoided. A ‘classical’ nitroreductase (NTR) isolated from Escherichia coli B activates CB 1954, and its bischloroethyl analogues and orthologues, to form cytotoxic derivatives (Anlezark et al., 1992, 1995). The use of NTR in ADEPT is, however, limited by the requirement for a cofactor to be co-administered with the prodrug and by the achievable level of enzyme activity in the tumour when directed there by chemical conjugation with antibody fragments (Anlezark et al., 1996; G. M. Anlezark, unpublished data). However, NTR has been used in gene therapy approaches with some success in vitro (Bailey et al., 1996; Bridgewater et al., 1997; Searle et al., 1998; Latham et al., 2000) and in animal models (Clark et al., 1997; Drabek et al., 1997; McNeish et al., 1998). Nevertheless, in common with Walker DTD, the kinetics of the enzyme–substrate interaction are suboptimal (Knox et al., 1992). Furthermore, in contrast to the single cytotoxic product formed by the Walker enzyme, the reaction catalysed by NTR results in the formation of two products, the 4-hydroxylamine (4HX) and the relatively non-toxic 2-hydroxylamine (2HX) derivatives (Fig. 1; Knox et al., 1992; Anlezark et al., 1992). This limits the use of NTR in gene therapy, where the efficiency of transfection and the expression of proteins from transfected genes can be low, particularly in vivo.

Ideally, a DEPT enzyme should fulfill a number of criteria for its successful use including a high affinity and specificity for the substrate, and a high turnover so that sufficient cytotoxin is generated within the tumour for clinical efficacy even if transfection and expression efficiency is low. We have previously screened a number of bacterial classes for prodrug-activating activity and these studies have indicated that an enzyme with the desired properties is present in some Bacillus spp. (G. M. Anlezark, unpublished data). The present study describes the isolation of a novel nitroreductase from Bacillus amyloliquefaciens, and its purification, characterization and evaluation as a candidate for DEPT strategies.

METHODS

Bacterial strains, plasmids and culture media. The source of chromosomal DNA for the cloning of ywrO was B. amyloliquefaciens (CAMR strain 0454). The E. coli host used for cloning experiments was Top10 [F' mcrA Δ(mrr–hsdRMS–mcrBC) 80lacZΔM15 lacX74 recA1 deoR araD139 Δ(ara–leu)7697 galU galK rpsL (Str8) endA1 nupG]. Plasmids employed for cloning and expression studies, respectively, were pCR2.1-TOPO (Invitrogen) and pET21b (Novagen). All organisms were cultivated in L-broth or on L-agar at 37 °C and supplemented where necessary with antibiotics (100 µg ampicillin ml⁻¹ or 30 µg kanamycin ml⁻¹).

For enzyme purification and DNA preparation, 20 ml L-broth was inoculated with a single colony of B. amyloliquefaciens and grown at 37 °C with gentle shaking for 6–8 h. A 1 ml aliquot of the starter culture was thereafter used to inoculate 200 ml L-broth in a 1 l baffled flask which was then incubated overnight at 37 °C with shaking at 150 r.p.m. Cells
were harvested by centrifugation and washed with 20 mM potassium phosphate buffer, pH 7.0, before use, or stored at −20 °C until required.

All chemicals and reagents used throughout this study were purchased from Sigma unless otherwise indicated.

**General recombinant methods.** The pCR2.1-TOPO cloning vector was used in accordance with the manufacturer’s (Invitrogen) instructions. All recombinant methods including isolation of plasmid DNA, cultivation and transformation of *E. coli*, and all molecular manipulations of DNA were performed as described by Sambrook et al. (1989). All restriction endonuclease and DNA-modifying enzymes were used in accordance with the supplier’s instructions (NBL). For nucleotide sequencing 1–2 µg template DNA was mixed with 4.5 pmol oligonucleotide primer and the sequencing reaction was completed using AmpliTag FS dye terminator chemistry in a robotic workstation. DNA sequence analysis was performed using an ABI Prism DNA sequencer (Perkin Elmer). Oligonucleotides were synthesized on an ABI synthesizer (Perkin Elmer). Primary data were manipulated using software in the Lasergene program (DNA STAR). Inverse PCR was performed as described by Whelan et al. (1992).

**Cloning of the *ywrO* gene.** A 230 bp portion of *ywrO* was amplified from the *B. amyloliquefaciens* chromosome using the two degenerate primers P1 (5′-GTNCAYCCNGATAGGARAA-3′) and P2 (5′-CCRTANGCCANNCCRTG-3′); P1 was capable of encoding an heptapeptide motif from the determined N-terminal sequence of the purified enzyme (VHPDMEN) and P2 could encode an internal sequence motif (HGWAYG) found to be entirely conserved between the hypothetical bacterial proteins YrkL (*Bacillus subtilis*) and YabF (*E. coli*). The 230 bp fragment was cloned into pCR2.1-TOPO to yield pBAM1 (Fig. 2). The remainder of the gene was isolated by using inverse PCR. *B. amyloliquefaciens* chromosomal DNA was cleaved with *StyI*, and the fragments generated were circularized through their subsequent incubation with DNA ligase. The ligated DNA was then used as the template for a PCR employing two divergent primers based on the sequenced 230 bp fragment: primers P3 (5′-GGTTATTGCCTGAG-3′) and P4 (5′-GTGACCTGTCGCCCCG-3′). The 2.9 kb fragment was generated and cloned into pCR2.1-TOPO (Invitrogen), and the sequence of the insert of the recombinant plasmid pBAM2 was determined. This allowed the identification of the nucleotide sequence of the remaining parts of the *B. amyloliquefaciens* gene. Using this information, a contiguous copy of the entire structural gene was amplified from the *B. amyloliquefaciens* chromosome using primer P5 (5′-GGTTGAGTACATGAGAATTATT-3′) and the active fractions eluted at 120–140 mM KCl were concentrated and desalted before reapplying to Mono Q equilibrated in 20 mM Bistris, pH 6. Elution was again by KCl gradient, and the active fractions eluted at 120–140 mM KCl were pooled and concentrated.

**Electrophoresis.** Homogeneity of the purified protein was assessed in SDS- or native-PAGE in 8–25% gradient Phastgel (Amersham), or in 4–12% gradient NuPage BisTris gels (Novex) according to the manufacturers’ instructions. Visualization of the protein bands was by Coomassie staining (Phastsystem) or silver staining (Bio-Rad) according to the protein concentration. For final identification of the active protein in IEF or the native-PAGE gels, 4–6% 8.5 M Phastgels or Novex 4–20% gradient Tris/glycine gels were used. Activity staining was performed using o-methyl red (200 µM in 20 mM BisTris, pH 7.0, 1% DMSO) and NAD(P)H (500 µM) as a cofactor. The gels were placed in lidded Petri dishes, and the mixture was incubated at 37 °C for 1 h before addition of the substrate and detection of the reaction.
Purification of a nitroreductase from
B. amyloliquefaciens strain 0454

Following the procedures outlined in Methods, SDS-
PAGE on an 8–25% gradient Phastgel indicated the presence of one major band and several minor bands in
the concentrate. To confirm that the major band was the
nitroreductase, the concentrate was applied to an IEF gel
and, after focusing, the gel was sliced to allow Coomassie
blue staining of one half and an activity stain (o-
methyl red, which is decolourized by the enzyme) to be
applied to the other half. A clear band of decolourized
methyl red, which is decolourized by the enzyme) to be
observed. These bands were excised and putative bands
in the vicinity were also excised. Differential staining of
these with NADPH (Biedler et al., 1981) and NAD 33
(P)H (Biedler et al., 1981) was followed by two hypo-
thetical genes encoding proteins, YabF and YheR (28 and
33% identity, respectively). In view of this observation,
a strategy was formulated whereby sequence homology
between the identified bacterial proteins, together with
the determined N-terminal amino acid sequence of the
discovered B. amyloliquefaciens enzyme, was used to
amplify a region of the desired encoding gene from the
B. amyloliquefaciens genome (see Methods). Nucleotide
sequencing of the insert of the plasmid obtained
(pBAM1) revealed the presence of an ORF which encoded
a polypeptide that shared 66% sequence

iso the corresponding DNA. To facilitate subsequent expression studies, a NdeI site
was created over the translational start codon of the gene during its amplification by PCR. This NdeI site,
Fig. 2. Molecular cloning of the ywrO gene. The region of the B. amyloliquefaciens genome in the vicinity of ywrO is shown above the four plasmid maps. The arrangement in B. subtilis is identical except that a hypothetical gene, yswA, is present in the indicated position. Only an internal 1.5 kb fragment (indicated by \ in the map) of the 2.9 kb StyI fragment was sequenced. The region of the ywrO gene originally amplified (cloned into pCR2.1 to generate pBAM1), using the primers P1 and P2, has been shaded black. The position of the primers P3 and P4 used to generate the inverse PCR fragment cloned in pBAM2 is also shown beneath the shaded region of ywrO. Plasmids pBAM3 and pBAM4 were made by inserting a DNA fragment generated by PCR using the primers P5 and P6 into pCR2.1-TOPO and pET21b between the vector NdeI and EcoRI sites (see Methods). A prime (') before or after the gene indicates either a 5'- or 3'-truncation, respectively. Also marked are the following vector-borne elements: lac, the lacZ promoter; T7, the T7 polymerase promoter; ori, the ColE1 origin of replication; KmR, the kanamycin resistance gene; ApR, the ampicillin resistance gene.

together with an EcoRI site created at the 3’-end of the gene, was used to subclone ywrO into the equivalent sites of the expression vector pET21b. This placed the translational start codon of ywrO in the resultant plasmid, pBAM4, at the optimal distance from the vector-encoded ribosome binding site. E. coli novablue cells carrying pBAM4 were grown overnight at 37 °C following induction with IPTG. A crude extract was prepared in 20 mM potassium phosphate buffer, pH 7, by sonication with the addition of DNAase and RNAase before centrifugation to aid clarification. Purification was essentially as described above for the native enzyme, omitting the initial ammonium sulphate fractionation step. The supernatant was buffer-exchanged into 20 mM Tris, pH 7–6, for separation on FPLC Mono Q. A second anion-exchange step was performed in 20 mM Bistris, pH 6, and following this the protein appeared essentially homogeneous on SDS-PAGE (not reduced) (Fig. 3b). The yield from the small-scale process was approximately 25% with a 24-fold increase in specific activity (Table 1). The pure protein was also electrophoresed on native NuPage 4–12% gradient gels and activity-stained

Fig. 3. Absorption spectrum of B. amyloliquefaciens YwrO, SDS-PAGE analysis and activity staining in native-PAGE. (a) Absorption spectrum showing peak maxima at 381 and 451 nm. (b) SDS-PAGE in 4–12% gradient NuPage Bistris gel, 25 µl YwrO sample or 10 µl molecular mass marker per lane. Lanes: 1, crude extract of E. coli novablue bearing pBAM4; 2, partially purified B. amyloliquefaciens YwrO in 20 mM Tris, pH 7.6; 3, YwrO purified to homogeneity in 20 mM Bistris, pH 6.0; 4, blank; 5, molecular mass marker (SeeBlue Plus2, Invitrogen). (c) Native PAGE in 4–20% Novex Tris/glycine gel with samples and markers loaded at 10 µl per lane. Molecular mass markers do not show true mobility in native-PAGE and so are not annotated. Lanes 1–7 were stained with Coomassie blue; lanes 8–14 were activity-stained with o-methyl red as described in Methods. Lanes: 1, markers (SeeBlue Plus 2); 2, blank; 3, crude extract as in Fig. 3(b); 4, partially purified YwrO; 5, blank; 6, purified YwrO; 7 and 8, blank; 9, purified YwrO; 10, partially purified YwrO; 11, blank; 12, crude extract; 13, blank; 14, molecular mass markers.
lines indicate that the inhibition was uncompetitive.

Table 1. Purification scheme for recombinant B. amyloliquefaciens YwrO

<table>
<thead>
<tr>
<th>Fraction</th>
<th>CB 1954 activity (µmol min⁻¹ ml⁻¹)</th>
<th>Protein (mg ml⁻¹)</th>
<th>Specific activity (µmol min⁻¹ ml⁻¹)</th>
<th>Total activity (µmol min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1·19</td>
<td>9·40</td>
<td>0·13</td>
<td>5·95</td>
</tr>
<tr>
<td>Crude extract in 20 mM Tris pH 7·6</td>
<td>1·14</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mono Q in Tris pH 7·6 Pooled active fractions</td>
<td>0·84</td>
<td>3·19</td>
<td>0·26</td>
<td>2·08</td>
</tr>
<tr>
<td>20 mM Bistris starting material</td>
<td>0·70</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mono Q in Bistris pH 6·0 (final product)</td>
<td>0·74</td>
<td>0·24</td>
<td>3·08</td>
<td>1·48</td>
</tr>
</tbody>
</table>

ND, Not determined.

Table 2. Substrate and cofactor specificity for recombinant B. amyloliquefaciens YwrO

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>Cofactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB 1954</td>
<td>618 ± 169</td>
<td>8·2 ± 1·1</td>
<td>NADPH</td>
</tr>
<tr>
<td>SN 23862</td>
<td>Inactive</td>
<td>ND*</td>
<td>NAD(P)H</td>
</tr>
<tr>
<td>Menadione</td>
<td>3·4 ± 0·5</td>
<td>1750 ± 9·7</td>
<td>NADPH</td>
</tr>
<tr>
<td>(K_m: 8·7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMN</td>
<td>53·0 ± 6·0</td>
<td>87·3 ± 1·8</td>
<td>NADPH</td>
</tr>
<tr>
<td>FMN</td>
<td>104·7 ± 35·6</td>
<td>54·2 ± 2·4</td>
<td>NADH</td>
</tr>
<tr>
<td>FAD</td>
<td>209·0 ± 27·0</td>
<td>78·2 ± 2·0</td>
<td>NADPH</td>
</tr>
<tr>
<td>NADPH (substrate 5 µM menadione)</td>
<td>40·0 ± 3·8</td>
<td>169·4 ± 3·3</td>
<td>NAD(P)H</td>
</tr>
<tr>
<td>Azodyes</td>
<td>Active with o-methyl red</td>
<td>ND</td>
<td>NAD(P)H</td>
</tr>
</tbody>
</table>

ND, Not determined.

* SN 23862 $k_{cat}$ value could not be determined because YwrO is inactive with this substrate.

Fig. 4. The inhibition of menadione reduction by dicoumarol.

The concentration of dicoumarol (µM) was plotted against substrate concentration/rate of reaction (i.e. [menadione]/ν) for each of the five menadione concentrations tested: ●, 1 µM; ○, 1·5 µM; ▽, 2 µM; ▽, 3 µM; ■, 4 µM. Lines of best fit were constructed for each substrate concentration and the point of intersection indicates the inhibition constant ($-K_i$). Converging lines indicate that the inhibition was uncompetitive.

using NAD(P)H and o-methyl red. Decolourized bands were clearly seen in lanes containing the YwrO protein (Fig. 3b).

The estimated molecular mass from comparison with low-molecular-mass standards was 21·5 kDa, which agrees well with the value calculated from the deduced amino acid sequence. By gel filtration (Superdex 75), however, the molecular mass was estimated to be approximately 88 kDa, suggesting that the protein exists as a tetramer in the native state. The spectrum of the pure protein was typical of a flavoprotein with λ_max at 381 and 451 nm (Fig. 3a).

Kinetic parameters with respect to CB 1954 were determined by HPLC assay using 10 µl of the final product in a 500 µl assay mix containing 100–1000 µM CB 1954, 500 µM NADPH and incubating the mixture at 37 °C for 10 min. Reduction of CB 1954 was determined by comparison of peak areas at 325 nm in standard and enzyme tubes. $K_m$ was calculated to be 618 ± 169 µM with a $V_{max}$ of 5·5 µmol min⁻¹ mg⁻¹, giving a $k_{cat}$ of 8·2 s⁻¹. The single product formed was found to be exclusively the cytotoxic 4HX derivative. As described in Methods, reduction product peaks were baseline-separated from each other and from those of
the cofactor and substrate, and very small peaks could be detected. The only peaks detected in any sample were for CB 1954, NADPH and the 4HX product. There was no activity with the bischloroethyl analogue of CB 1954, SN 23862, with either NADPH or NADH as the cofactor.

B. amyloliquefaciens YwrO could use FMN and FAD as substrates and acted as a menadione reductase (Table 2). With all of these substrates the rate of reaction was 2–10-fold faster with NADPH than with NADH (data shown for FMN and NAD(P)H only). When menadione was used as the substrate the rate of reaction with NADH as cofactor was very low (< 5 μmol min⁻¹ ml⁻¹ with 10 μM menadione against > 40 μmol min⁻¹ ml⁻¹ with NADPH as cofactor) and kinetic parameters with this cofactor were not determined. The cofactor concentration in all cases was at least 300 μM to ensure saturation. Substrate inhibition was seen with concentrations of menadione greater than 10 μM and therefore the kinetic parameters were determined using the Cornish-Bowden method (Cornish-Bowden, 1979) for substrate inhibition. B. amyloliquefaciens YwrO could not use nitrofurazone or SN 23862, the bischloroethyl mustard analogue of CB 1954, as substrates with either NADH or NADPH as cofactor. The reduction of menadione was potently inhibited uncompetitively by dicoumarol with a Kᵢ value of 1 μM (Fig. 4).

In vitro cytotoxicity tests

The sulphorhodamine B assay was used to determine the cytotoxicity of the combination of enzyme, cofactor and CB 1954 on V79 cells of NTR and B. amyloliquefaciens YwrO. Exposure of the cells for 3 h to the various treatments was carried out in serum-free medium to avoid activation of CB 1954 by endogenous enzymes. The results are shown in Fig. 5. Control curves are shown for prodrug alone or in combination with either NADH or NADPH. Other controls were performed concurrently in each experiment (enzyme alone or with prodrug or cofactor, cofactor alone or with prodrug, DMSO alone) but none of the median absorbance values differed significantly from those of untreated cells (P > 0.05, Mann–Whitney test).

Under the conditions used CB 1954 (3–9–500 μM) was not cytotoxic either alone or in combination with NADH or NADPH. However, in the presence of 4 μg B. amyloliquefaciens YwrO or NTR there was a dose-related cytotoxic effect. The ED₅₀ values for the two enzymes were calculated by probit analysis and were 6.3 ± 0.5 μM (NTR) (95% confidence intervals 5.4–7.3) and 137.1 ± 8.2 μM (B. amyloliquefaciens YwrO) (95% confidence intervals 121.9–154.4).

**DISCUSSION**

The aim of this study was to isolate and characterize a novel enzyme for DEPT strategies. For nitroreductases the strategies of choice are likely to be of the GDEPT or VDEPT type because of the necessity and inherent difficulty of providing exogenous cofactor for ADEPT approaches (Knox et al., 1995; Anlezark et al., 1996). Whilst the requirements for prodrugs for the alternative approaches may be different (Connors, 1995; Denny & Wilson, 1998), the requisite properties of the enzymes are probably substantially alike in that selectivity, a high affinity for the substrate and a high turnover are the principal considerations. Additionally, if CB 1954 is to be used as a prodrug, consideration needs to be given to product formation. The E. coli B NTR reduces CB 1954 to produce a mixture of the two hydroxylamine reduction products (Fig. 1) (Knox et al., 1992), one of which (the 2HX) is substantially less cytotoxic. Therefore, it is considered an advantage for a DEPT enzyme to produce the 4HX derivative only and certainly not solely the 2HX. Alternative prodrugs also merit attention, including analogues of CB 1954 (Palmer et al., 1992; Anlezark et al., 1995) and other compounds where nitroreduction is a ‘trigger’ for activation (Mauger et al., 1994; Siim et al., 1997). An individual enzyme might not possess all the desired properties or substrate specificity, but if a portfolio of enzymes could be

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**Fig. 5.** Enhanced cytotoxicity in V79 cells of CB 1954 in the presence of B. amyloliquefaciens YwrO and E. coli B NTR. Cytotoxicity was assessed in 96-well plates seeded with V79 cells in DMEM + 10% (v/v) fetal calf serum at 10000 cells ml⁻¹, by sulphorhodamine B assay 4 d after a 3 h exposure to: ●, CB 1954 alone; ○, CB 1954 + NADH; ▲, CB 1954 + NADPH; ▼, CB 1954 + NADH + NTR; ■, CB 1954 + NADPH + YwrO. Cells were exposed to CB 1954 alone (doubling dilutions from 500 μM), in combination with CB 1954 plus NAD(P)H (125 or 250 μM), or CB 1954 plus NAD(P)H and enzyme (4 μg) in a total volume of 200 μl DMEM. Results are expressed as a percentage of A₅₃₀ values for the control cells, mean ± SD (untreated, A₅₃₀: 1 ± 0.05; n = 6) of at least six wells in two independent experiments. All treatments were carried out in serum-free DMEM. For wells exposed to prodrug, cofactor and enzyme, five concentrations of CB 1954 in doubling dilutions from 500 μM (B. amyloliquefaciens YwrO) or 50 μM (NTR) were used. Statistical analysis was performed using the Mann–Whitney test comparing the median values for prodrug + cofactor with those for prodrug + cofactor + enzyme at each dose of prodrug. The asterisks denote significant differences between the medians, P < 0.05.
identified with specificity for one or more classes of prodrugs, exploitation of nitroreduction for prodrug activation in cancer therapy (Connors, 1995) could find applications in a wide spectrum of disease.

The *B. amyloliquefaciens* YwrO protein described in the present study possesses at least two of the desired attributes for DEPT. Firstly, it produces only 4HX from the reduction of CB 1954 and secondly, it has a higher affinity for the prodrug than either NTR or Walker DTD (*Kₐ values of 617, 862 and 826 µM, respectively*), with its *kₑₐₚ* being comparable to that of NTR (*and greater than that of Walker DTD*). However it cannot use the mustard analogue of CB 1954 as a substrate. As yet few enzymes with optimal properties have been available, with the only published alternatives to NTR being the Walker rat carcinoma DTD and human NQ02 (Knox et al., 1988a; Wu et al., 1997).

As well as being able to reduce CB 1954, *B. amyloliquefaciens* YwrO has a number of other properties in common with DTD or NTR. It is a flavin reductase and can use either FAD or FMN as substrates, although the affinity for the latter is greater. NTR has flavin reductase activity, but the flavins are relatively poor substrates and reduction rates are low (G. M. Anlezark, unpublished data), as they are for a highly related protein NfsB, compared with proteins from other related species (Zenno et al., 1996). *B. amyloliquefaciens* YwrO is also an azoreductase, rapidly reducing the azodye o-methyl red.

The *B. amyloliquefaciens* protein described in this work has been identified with the aid of the complete genome sequence of a highly related organism. The value of sequence homology in these circumstances for identifying and screening enzymes for specific properties, such as prodrug activation, is clear. In the present work we have cloned and overexpressed the previously unknown product of a gene which was identified by sequence homology to be an orthologue of the *ywrO* of *B. subtilis*. In *Bacillus* spp., and amongst members of other genera (Bryant et al., 1981; Kinouchi & Ohnishi, 1983; Rosenkranz et al., 1982; G. M. Anlezark, unpublished data), total nitroreductase activity assayed in crude cell extracts often represents activity of at least three enzymes which can be purified individually only with difficulty (G. M. Anlezark, unpublished data). In the course of this study we have also cloned and overexpressed the *B. subtilis* gene (G. M. Anlezark, unpublished results), but the protein had relatively little activity with the prodrugs CB 1954 or SN 23862. This demonstrates that sequence homology may not always indicate structural homology or similar enzyme–substrate interactions. Nevertheless, searching genomes and databases for homologous sequences offers a relatively rapid screening method for identification of potentially useful enzymes for DEPT strategies. Using the entire genome sequence of *B. subtilis* and database mining, we have already identified further genes potentially encoding prodrug-reducing enzymes which will form the basis of further publications.

*B. amyloliquefaciens* YwrO shows no sequence homology with NfsB or other *Enterobacteriaceae* nitroreductases, but shares a degree of homology with Walker DTD, other mammalian diaphorases and NQ02. Sequence differences may contribute to the greater ability of the bacterial enzyme and NQ02 to turn over CB 1954 compared to other DTDs. In particular, the Walker enzyme and NQ02 (active with CB 1954) have a Tyr residue at position 104, whereas NQ01 (DTD) and the mouse enzymes (low CB 1954 activity) do not. This residue has been shown to have a significant effect on the affinity of the proteins for CB 1954, with the substitution of Tyr for Gln shifting the properties of NQ01 and mouse enzymes towards those of the Walker enzyme and NQ02 (Chen et al., 1997) with respect to CB 1954. *B. amyloliquefaciens* and *B. subtilis* YwrO share the Tyr in the equivalent position in their sequences, but these differences may not represent all of the critical residues involved in prodrug reduction because *B. subtilis* YwrO has very low activity with CB 1954, reducing the prodrug too slowly to be of clinical use, despite its high degree of homology with *B. amyloliquefaciens* YwrO. Further studies will be necessary to elucidate which parts of the molecules are essential for efficient production of the 4HX cytotoxin.

Although YwrO produces only the cytotoxic 4HX derivative of CB 1954, in contrast to the 2HX and 4HX products of the prodrug reduction by NTR, its effects in cytotoxicity assays are less than those of NTR under the same conditions. It is difficult to say how an *in vitro* experiment will relate to an *in vivo* situation where the gene product is being expressed in mammalian cells. The *kₑₐₚ* value of the novel protein is lower than that of the NTR and it is not yet known which of the kinetic parameters will prove to be crucial in an *in vivo* setting. Nevertheless, the *kₑₐₚ* value of *B. amyloliquefaciens* YwrO is greater than that of the Walker enzyme, which is able to achieve efficient *in vitro* cytotoxicity using CB 1954 and NADH (Knox et al., 1988b). It may be that a critical concentration of the toxic product is needed *in vitro*, where a relatively low initial cell density will allow diffusion of the product away into the surrounding medium. In a dense tumour mass diffusion will be restricted and an enzyme working relatively slowly will not be a disadvantage. Three-dimensional tumour models may provide a better estimate of *in vivo* efficacy (Hoffman, 1993; Santini & Rainaldi, 1999) and it remains to be seen which enzyme will be effective when expressed in *in vivo* models.

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