Chapter 2

PARALOGOUS GENE ANALYSIS REVEALS A HIGHLY ENANTIOSELECTIVE 1,2-O-ISOPROPYLIDENEGLYCEROL CAPRYLATE ESTERASE OF *Bacillus subtilis*

Melloney J. Dröge, Rein Bos and Wim J. Quax

Paralogous gene analysis reveals a highly enantioselective 1,2-O-isopropylideneglycerol caprylate esterase of *Bacillus subtilis*

Carboxylesterase NP of *Bacillus subtilis* Thai I-8, characterised in 1992 as a very enantioselective (S)-naproxen esterase, was found to show no enantiopreference towards (+)-1,2-O-isopropylideneglycerol esters. The *ybfK* gene was identified by the *B. subtilis* genome project as an unknown gene with homology to carboxylesterase NP. The purpose of the present study was to characterise the *ybfK* gene product in order to determine whether this paralogue of carboxylesterase NP had an altered or enhanced stereospecificity. The *ybfK* gene was cloned and expressed in *B. subtilis* using a combination of two strong promotors in a multi-copy vector. The enzyme was purified from the cytoplasm of *B. subtilis* by means of anion exchange and hydrophobic interaction chromatography. The purified YbfK is an enzyme of 296 amino acids and shows an apparent molecular mass of 32 kDa (SDS-PAGE). Comparison of the activities of YbfK and carboxylesterase NP towards caprylate esters of IPG revealed that YbfK produces (+)-IPG-caprylate with 99.9% enantioselectivity. Therefore, we conclude that we have isolated a paralogue of carboxylesterase NP that can be used for the enantioselective production of (+)-IPG.

**Introduction**

Administration of single enantiomers is associated with an improved potency and selectivity of the drug and decreased adverse events as a result of activity of the wrong enantiomer. In addition, in 1999, chiral drug market reached $100 billion for the first time, being close to one-third of all drug sales world-wide. It is estimated that the sales of chiral drugs will increase by 8% annually. Since chiral compounds represent more than 50% of the world-wide most frequently prescribed drugs, the interest in the preparation and isolation of chiral drugs has dramatically increased. More drugs are marketed as single enantiomers instead of a racemic mixture, a process known as ‘chiral switching’ (Stinson, 1998; 2000; Tucker, 2000).

In the last decade, the use of bacterial enzymes, such as lipases (E.C. 3.1.1.3.) and esterases (E.C. 3.1.1.1.), to produce chiral drugs has become an alternative to chemical synthesis, since they are capable of enantioselective hydrolysis and esterification in a cheap and environmentally friendly process. In the past, several examples of kinetic resolution experiments using lipases and esterases have been described (Bornscheuer & Kazlauskas, 1999). For example, lipases have been used for the production of enantiopure
intermediates that are important in the synthesis of pharmaceuticals, such as diltiazem, taxol and thromboxane antagonists (Matsumae et al., 1993; Patel et al., 1992; 1994). Unfortunately, wild-type enzymes are seldomly suited for the production of stereospecific compounds, due to a low activity and enantioselectivity, a poor robustness and problems with production (Bornscheuer & Kazlauskas, 1999). These problems can sometimes be solved after engineering of these enzymes (Jaeger & Reetz, 2000; Liebeton et al., 2000; Petrounia & Arnold, 2000; Quax & Broekhuizen, 1994).

A large number of microbial esterases have been described and only a few of them are synthetically useful enzymes, with the exception of carboxylesterase NP (Bornscheuer & Kazlauskas, 1999). Interestingly, the carboxylesterase NP of B. subtilis Thai I-8, encoded by the nap gene, appeared to be an enantioselective catalyst (enantiomeric excess, ee, > 99% at 40% conversion) for the conversion of racemic naproxen methyl ester (a 2-arylpropionate ester) to (S)-naproxen, a widely used non-steroidal anti-inflammatory drug (NSAID) (Azzolina et al., 1994; 1995; Mutsaers & Kooreman, 1991; Quax & Broekhuizen, 1994; Smeets & Kieboom, 1992; Van der Laan et al., 1993). Carboxylesterase NP has excellent characteristics for industrial application since the enzyme can be produced in large amounts. After chemical engineering, variants of carboxylesterase NP with an improved stability were obtained, resulting in a very robust enzyme (Quax & Broekhuizen, 1994). This enzyme had some enantioselectivity on 1,2-O-isopropylideneglycerol esters (IPG esters) unfortunately, the observed enantioselectivity, 4% ee at 20% conversion, was insufficient for an industrialised process. IPG is an interesting chiral intermediate in the pharmaceutical industry, since it is a starting compound in the synthesis of ß-adrenoceptor antagonists (Smeets & Kieboom, 1992). Therefore, we have initiated a search for homologues of carboxylesterase NP of B. subtilis Thai I-8 with a better enantioselectivity on IPG esters.

Homologues of carboxylesterase NP were not found until the genome sequence of B. subtilis 168 was elucidated in 1997 (Kunst et al., 1997). In the genome of B. subtilis 168, an esterase was identified showing 98% identity to carboxylesterase NP (carboxylesterase, encoded by the nap gene). Surprisingly, a second esterase with 64% identity (YbfK, encoded by the ybfK gene) to carboxylesterase NP was found. A Gly – Xaa – Ser – Xaa – Gly motif is present in all three carboxylesterases, which is a consensus for active site serines and is a characteristic pentapeptide for most lipases and esterases. It is generally assumed that this serine is the nucleophilic residue involved in the catalysis. From this, we expected that YbfK would also display an esterolytic activity. The fact that B. subtilis 168 contains two similar esterases is in line with the observation that B. subtilis has many paralogous genes: for instance, B. subtilis also contains two homologues lipases, LipA and LipB, with an identity of 74% on protein level. However, both enzymes have different biochemical characteristics (Eggert et al., 2000).
In the current study, we present the isolation and cloning of the \textit{ybfK} gene and the overproduction and purification of the enzyme. Subsequently, we compare the enantioselective esterolytic activity of this enzyme with its paralogue, carboxylesterase NP, towards IPG esters.

**Material and methods**

**Plasmids, bacterial strains and media**

The plasmids and bacterial strains that were used in the present study are listed in table 1. The following media were used: 2xTY medium containing: Bactotrypton (1.6%), Bacto yeast extract (1%) and sodium chloride (0.5%); medium to prepare \textit{B. subtilis} competent cells containing: 100 mM potassium phosphate buffer, pH 7, 1% glucose, 0.4% potassium l-glutamate, 3 mM trisodium citrate, 3 mM MgSO$_4$, 0.0022 % ferric ammonium citrate, 0.1% casein hydrolysate, 0.002% l-tryptophane (Kunst & Rapoport, 1995). Antibiotic agents were used in the following concentrations: ampicillin 100 µg.ml$^{-1}$, kanamycin 20 µg.ml$^{-1}$, and chloramphenicol 5 µg.ml$^{-1}$.

**Chemicals**

The methyl ester of (S)-naproxen was provided by H.V. Wikström (Department of Medicinal Chemistry, University of Groningen, Groningen, the Netherlands). (-)-IPG-acetate, (-)-IPG-butyrate, (-)-IPG-caprylate, (+)-IPG-acetate, (+)-IPG-butyrate and (+)-IPG-caprylate were kindly provided by M.T. Reetz (Max-Planck Institut für Kohlenforschung, Mülheim, Germany).

**Oligonucleotides**

To construct the plasmid, the following primers were used (Life Technologies, UK): 
\textit{ybfKfor1}: 5'-CTTAGTTATGCTGGTACCTATCAATCAAACCGGA-3' (KpnI); 
\textit{ybfKrev1}: 5'-CAGAAGCTTTGCCAATCTTTTCGTATTTCC-3' (HindIII); 
\textit{Pnapfor1}: 5'-CTTAGGTACCAGGATCCCTCCATTGTGGCTGTCTTT-3' (KpnI, BamHI); 
\textit{Pnaprev1}: 5'-CCGCGGCAAACTGCATTGAATCTTTGTATCATACAAATGCCTCCCTCCATTCG GGAATACTAGATGAATGGTTTGACATACAAATGCCTCCCTCC-3' (SacI). Newly created restriction sites are indicated in bold italics.

**DNA techniques**

Recombinant DNA techniques were performed as described by Sambrook et al. (1989). Enzymes were from Life Technologies. Plasmid DNA was prepared as described by Birnboim & Doly (1979). DNA purification was performed by using the Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany).
TABLE I: Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain/plasmids</th>
<th>Genotype/properties</th>
<th>Reference/source</th>
</tr>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> TG-1</td>
<td><em>Sup E, K 12 (∆(lac-pro)), thi, bsdD5/F</em>, traD36, proAB, laqIq, lacZΔ-M15*</td>
<td>Amersham Uppsala, Sweden</td>
</tr>
<tr>
<td><em>B. subtilis</em> Thai-8</td>
<td></td>
<td>CBS 679.85</td>
</tr>
<tr>
<td><em>B. subtilis</em> 168</td>
<td><em>TipC2</em></td>
<td>Kunst et al., 1997</td>
</tr>
<tr>
<td><em>B. subtilis</em> 1050</td>
<td><em>NprR2, aprE18, aprA3, est::Cm, ΔLipA</em></td>
<td>Dartois et al, 1992; 1993</td>
</tr>
<tr>
<td>Bs1050(pMA)</td>
<td><em>B. subtilis</em> 1050 transformed with pMA, a pMA5 derivative, containing the <em>HpaII</em> and <em>nap</em> promotor</td>
<td>This work</td>
</tr>
<tr>
<td>Bs1050(pMA*ybfK)</td>
<td><em>B. subtilis</em> 1050 transformed with pMA*ybfK</td>
<td>This work</td>
</tr>
<tr>
<td>Bs1050(pMA*thai)</td>
<td><em>B. subtilis</em> 1050 transformed with pMA*thai</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td><em>Pem, ColE1, φ/f80dlacZ, Amp'</em></td>
<td>Norrander et al., 1983</td>
</tr>
<tr>
<td>pMA5</td>
<td><em>ColE1, npB, Neo', Amp', P</em>tipall*</td>
<td>Brückner et al., 1984; Dartois et al., 1994; Zyprian &amp; Matzura, 1986</td>
</tr>
<tr>
<td>pUCybfK</td>
<td>pUC18 derivative, contains the <em>ybfK</em> gene of <em>B. subtilis</em> 168</td>
<td>This work</td>
</tr>
<tr>
<td>pUCp*nap</td>
<td>pUC18 derivative, contains the promotor sequence of the <em>nap</em> gene of <em>B. subtilis</em> 168</td>
<td>This work</td>
</tr>
<tr>
<td>pMA*ybfK</td>
<td>pMA5 derivative, containing the <em>B. subtilis</em> 168 <em>ybfK</em> gene, downstream of the <em>HpaII</em> and <em>nap</em> promotor</td>
<td>This work</td>
</tr>
<tr>
<td>pMA*thai</td>
<td>pMA5 derivative, containing the <em>B. subtilis</em> Thai I-8 <em>nap</em> gene, downstream of the <em>HpaII</em> and <em>nap</em> promotor</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Construction of the plasmids**

The *ybfK* gene was amplified from the chromosomal DNA of *B. subtilis* 168 using the primers *ybfKf*or1 and *ybfKrev1*. The promotor of the *nap* gene (*B. subtilis* 168) was amplified using the primers *Pnapf*or1 and *Pnaprev1*. All PCRs were performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The PCR protocol had low annealing and extension temperatures: 4 min at 94°C, followed by 10 cycles of 30 s at 94°C, 1 min 37°C and 10 min at 55°C, followed by 30 cycles in which the extension time was at first 8 min, but increased by 15 s in each cycle (first cycle: 30 s 94°C, 1 min 55°C and then 8 min 72°C). At the end, DNA production was finished with 10 min at 72°C. The amplified gene
fragments were cloned into the *Hin*II site of pUC18. After digestion with *Kpn*I and *Sac*I (*nap* promotor sequence) and *Sac*I and *Hin*dIII (*ybf*K gene), the products were cloned by a triple ligation in pMA5, an *Escherichia coli/B. subtilis* shuttle vector using the *Kpn*I and *Hin*dIII restriction sites. In the plasmid pMA*ybf*K, the *ybf*K gene became located downstream of the *nap* promotor. *Bam*HI digestion removed the *E. coli* replicon and positioned the gene downstream of the strong Gram positive *Hpa*II promotor. The shortened plasmid was used to transform BCL1050. Restriction analysis and DNA sequencing verified the sequence of the constructs.

**Growth of the bacteria**

To produce enzymes, *B. subtilis* was grown in 2 litre shake flasks containing 500 ml 2xTY medium. The flasks were incubated at 37°C at 300 rpm with good aeration for 16 h.

**Enzyme purification**

After harvesting, the cells were resuspended in 0.1 M Tris HCl, pH 8, containing 20% sucrose, 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 mg.ml⁻¹ lysozyme. After an incubation of 30 min at 37°C, the spheroplasts were lysed by sonication (Van Dijl et al., 1991). The suspension was centrifuged and the supernatant was treated with 0.01 mg.ml⁻¹ DNAse in the presence of 3.5 mM MgCl₂ for 1 h at room temperature. The supernatant was loaded on 3 coupled HitrapQ columns (1.6 × 2.5 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated with 10 mM Tris HCl, pH 8, containing 1 mM EDTA, and using a flow of 5 ml.min⁻¹. Elution was performed with a combination of a stepwise and linear gradient from 0 to 1 M of sodium chloride in 10 mM Tris HCl, pH 8, containing 1 mM EDTA. The collected fractions (10 ml) were screened on the presence of YbfK using a combination of SDS-PAGE and the naproxen methyl ester assay. The fractions containing YbfK were pooled and 0.5 mM ammonium sulphate was added. This solution was loaded on a MT20 column (15 × 110 mm; Bio-Rad, Hercules, CA, USA) packed with 20 ml phenylsepharose HP (Amersham Pharmacia Biotech, Uppsala, Sweden), and equilibrated with 10 mM phosphate buffer, pH 8, containing 0.5 M ammonium sulphate. Elution was performed using a combination of a stepwise and linear gradient from 0.5 M to 0 M ammonium phosphate in 10 mM phosphate buffer, pH 8, collecting fractions of 10 ml. Fractions were screened for activity and the YbfK containing fractions were pooled. The solution was concentrated using a column with a cut-off of 10 kDa (3.5 Microsep; Filtron Technology Corporation, Northborough, MA, USA) and stored at -20°C. Protein concentration was determined by the Bradford and Lowry method (Pierce, Rockford, Illinios, USA).

**Electrophoresis**

SDS-PAGE was performed on a 12% separating and a 4% stacking gel (Laemmlli, 1970). Molecular mass markers were purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA).
Proteins were stained by the silver staining procedure of Pierce (Pierce, Rockford, Illinois, USA) or by Coomassie Brilliant Blue R-250 (Pierce, Rockford, Illinois, USA).

**Determination of the carboxylesterase activity**

Esterase activity was determined by using the naproxen methyl ester assay. 13 mg (S)-naproxen methyl ester was dissolved in 10 ml 14.3% w/v Tween 80 in 0.07 M MOPS buffer, pH 7.5. The ester was dissolved at 60°C in an ultrasonic bath (60 min). The solution was diluted to 50 ml with 0.07 M MOPS buffer, pH 7.5 (Mutsaers & Koo reman, 1991; Quax & Broekhuizen, 1994).

Samples were diluted with 0.1 M MOPS buffer containing 0.2% w/v BSA to a volume of 250 µl. 10 mM phosphate buffer, pH 8, was diluted correspondingly and was used as a reference. The sample solution and the substrate solutions were preincubated at 32°C in a water bath. 750 µl substrate solution was added to the sample solution and the final solutions were incubated in a water bath at 32°C, the optimal temperature for YbfK and carboxylesterase NP. The samples were analysed by HPLC. HPLC was performed using an Isco pump 2350, an Isco gradient mixer 2360 (ISCO Inc., Lincoln, NE, USA), a Kontron autosampler 360 (Kontron Instruments SpA, Milan, Italy), and a Shimadzu SPDM6A-Diode Array detector (Shimadzu Europe GmbH, Duisburg, Germany). The chromatographic conditions were: analytical column, LiChrospher 100 RP-18 (5 µm; LiChrocart 250-4); guard column, LiChrospher 100 RP-18 (5 µm; LiChrochart 4-4, Merck Darmstadt, Germany); eluens methanol : 10% acetic acid (90:10 v/v); isocratic flow of 0.75 ml.min⁻¹; pressure 1500 psi; injected volume 20 µl; DAD wave length 239 nm, band width 2 nm; spectrum absolute scale (mAbs) –10-1000; normalisation threshold 10 mABS. The capacity factor (k') for naproxen and the methyl ester of naproxen was 1.43 and 1.82, respectively. One unit (U) is defined as the amount of enzyme that hydrolyses 1 µmol (S)-naproxen methyl ester per minute. The hydrolysis by the blanks was always zero.

1,2-O-isopropylidene glycerol (IPG) ester assay

The esters of IPG were dissolved in 10 ml 14.3% w/v Tween 80 in 0.07 M MOPS buffer, pH 7.5 and diluted to 50 ml with 0.07 M MOPS buffer, pH 7.5. Samples were diluted with 0.1 M MOPS buffer containing 0.2% w/v BSA to a volume of 150 µl. 10 mM phosphate buffer, pH 8, was diluted correspondingly and was used as a reference. The sample solution and the substrate solutions were preincubated at 32°C in a water bath. 500 µl substrate solution was added to the sample solution and the final solutions were incubated in a water bath at 32°C. After incubation, 500 µl saturated NaCl solution was added and the aqueous solution was extracted twice with 1 ml ethylacetate.

GC analysis was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with a 7673 injector and a Hewllet Packard 3365 Chemstation under the following conditions: column WCOT fused-silica CP-wax 52 CB (10 m × 0,25 mm id, film
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thickness 0.25 µm, Chrompack International, Middelburg, The Netherlands), oven temperature programme 50-125°C at 3°C.min⁻¹; injector temperature 250°C; detector (FID) temperature 300°C; carrier gas helium; inlet pressure 5 psi; linear gas velocity 26 cm.s⁻¹; split ratio 56:1, injected volume 1 µl. One unit (U) is defined as the amount of enzyme that hydrolyses 1 µmol IPG ester per minute. Enantiomeric ratios, E, were calculated according to Chen et al. (1982; 1987) and were defined as the ability of the enzyme to distinguish between enantiomers. When E > 100, the enantiomeric excess, ee, was calculated.

All data were the results of three experiments. The hydrolysis of butyrate and caprylate esters by the blanks was always zero, the hydrolysis of acetate esters was negligible.

Results

Cloning of the ybfK gene

Using a BLAST search with the B. subtilis Thai I-8 carboxylesterase NP sequence, two open reading frames could be identified in the genome of B. subtilis 168. These open reading frames contained 900 and 888 base pairs and encoded the nap gene and ybfK gene respectively. The homology with the carboxylesterase NP of B. subtilis Thai I-8 was respectively 98% and 64% identity on protein level (figure 1), whereas the characteristic pentapeptide Gly – Xaa – Ser – Xaa – Gly was conserved in all three proteins.

![Comparison of the sequences of YbfK and carboxylesterase NP. Carboxylesterase NP B. subtilis Thai I-8 upper lane, YbfK B. subtilis 168 lower lane. The matching amino acids are in black. The Gly – Ala – Ser – Ala – Gly motif is underlined.](figure1.png)
TABLE 2: Purification of YbfK from the cytoplasm of *B. subtilis*. N.d.: not determined.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Specific activity (U.mg⁻¹)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonication</td>
<td>330</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>HiTrapQ</td>
<td>14</td>
<td>1.7</td>
<td>0.12</td>
<td>100</td>
</tr>
<tr>
<td>Phenylsepharose</td>
<td>6</td>
<td>1.02</td>
<td>0.17</td>
<td>60</td>
</tr>
</tbody>
</table>

YbfK could not be detected in lysed *B. subtilis* cells using SDS-PAGE and subsequent silver staining and therefore, the *ybfK* gene was cloned and fused to the strong *nap* promoter. The *ybfK* chromosomal sequence and the promoter sequence of the *nap* gene of *B. subtilis* 168 were amplified by the polymerase chain reaction. Both PCR fragments were cloned in pUC18, resulting in the plasmids pUCybfK and pUCpnap. The plasmids were digested with KpnI and SacI (pUCybfK) and SacI and HindIII (pUCpnap), respectively, and the digested fragments were cloned in pMA5, an *E. coli*/B. subtilis shuttle vector using the KpnI and HindIII restriction sites in a triple ligation. Thus, the *ybfK* gene was positioned downstream of the strong *nap* promoter. Digestion with BamHI and subsequent self-ligation of the resulting vector removed the *E. coli* replicon and positioned *ybfK* and the *nap* promoter sequence downstream of the strong Gram positive HpaII promoter (Brückner et al., 1984; Dartois et al., 1994; Zyprain & Matzura, 1986). Both promoters are expressed constitutively in *B. subtilis*. The shortened plasmid was used to transform the lipase A and esterase A negative *B. subtilis* strain 1050 (Dartois et al., 1993; 1994), resulting in strain Bs1050(pMAybfK). DNA sequencing confirmed that the DNA sequence of the construct was correct.

Isolation of the enzyme

A two-step purification protocol was established to purify YbfK from the cytoplasm of lysed Bs1050(pMAybfK) cells (table 2). Firstly, anion exchange chromatography at pH 8 was performed. YbfK bound to a HitrapQ column whereafter the protein could be eluted with 30% sodium chloride. Measurement of the (S)-naproxen methyl ester hydrolysis now revealed an activity of YbfK. The lack of enzymatic activity preceding this purification step could not be due to the detection limit of the HPLC system, since the total protein concentration decreased, whereas the volume of the protein solution remained almost unchanged. Therefore, the most likely explanation is the presence of an inhibitor of YbfK in the cytoplasm of Bs1050(pMAybfK). SDS-PAGE and Coomassie staining revealed that purification was incomplete (data not shown). Thus, a second chromatography step based on hydrophobic interaction was performed. YbfK bound strongly to a phenylsepharose column and eluted during the isocratic flow with a phosphate buffer without ammonium sulphate. SDS-PAGE and silver staining confirmed the purity of the sample (figure 2). After purification, 6 mg of purified protein was obtained from a 600 ml culture.
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Enzymatic activity of YbfK

Hydrolysis of the methyl ester of naproxen

The catalytic activity of YbfK towards the methyl ester of naproxen was determined. After 4 h incubation, 24.6% of the methyl ester of (S)-naproxen was converted to (S)-naproxen by 5 µg of the enzyme. The same amount of carboxylesterase NP (B. subtilis Thai I-8) induced 100% hydrolysis within 30 minutes. As a consequence, comparison of the specific activities of YbfK and carboxylesterase NP revealed a 25-fold lower specific activity of YbfK towards the naproxen ester (e.g. the specific activity was 4.6 and 0.17 U.mg⁻¹ for carboxylesterase NP and YbfK, respectively). However, the production of (S)-naproxen by YbfK is still far above the detection limit of the HPLC system and for this, it can be attributed to enzymatic activity of the potential esterase YbfK. Moreover, the hydrolysis of the substrate by the blanks was always below detection.

Hydrolysis of 1,2-O-isopropylidenglycerol esters

The substrate specificity and enantioselectivity of YbfK towards IPG esters with different aliphatic side chains were determined and compared with carboxylesterase NP of B. subtilis Thai I-8. Figure 3 summarises these specific activities. The highest activity of YbfK was measured when (+)-IPG-caprylate was used as a substrate (0.022 U.mg⁻¹). Surprisingly, YbfK was unable to hydrolyse (-)-IPG caprylate esters. The detection limit of the GC system used was 1 ng IPG, resulting in a selectivity of YbfK towards (+)-IPG-caprylate of at least 99.9% (E > 200). Additionally, YbfK was unable to hydrolyse (-)- and (+)-IPG butyrate, and (-)- and (+)-IPG acetate were hydrolysed but no selectivity was observed (E = 1.2). In contrast, carboxylesterase NP showed affinity towards both stereoisomers of the IPG esters, ranging from an enantioselectivity of E = 1.6 (IPG caprylate and acetate esters) to E = 1.2 (IPG butyrate esters).
Figure 3: Upper panel: enantioselective hydrolysis of racemic esters of 1,2-O-isopropylidenglycerol caprylate by YbfK. Lower panel: specific activity of *B. subtilis* YbfK towards IPG-acetate, butyrate and caprylate in comparison to carboxylesterase NP of *B. subtilis* Thai I-8. The assay conditions were described in material and methods. Carboxylesterase NP, *B. subtilis* Thai I-8 (○), YbfK, *B. subtilis* 168 (■).

**Discussion**

Since 1992, the US Food and Drugs Administration (FDA) and the European Committee for Proprietary Medicinal Products require to characterise each stereoisomer in a drug to be marketed as a racemic mixture. Therefore, the production and isolation of single enantiomers has become an important process in the pharmaceutical industry.

Enzymes, such as esterases, are capable of enantioselective hydrolysis and can be applied as catalysts in the resolution of racemates. For instance, carboxylesterase NP of *B. subtilis* Thai I-8 was found to be effective (> 99% ee) in the resolution of propionate esters with an aromatic ring containing a 2-substituent, such as 2-arylpropionates, 2-(aryloxy)propionates and N-arylalanine esters. Some enantioselectivity towards substrates with chirality in the alcohol part, such as IPG esters, was observed (Azzolina *et al.*, 1994; 1995; Mutsaers & Kooreman, 1991; Quax & Broekhuizen, 1994; Smeets & Kieboom, 1992).

The fact that *B. subtilis* contains many paralogous genes was obvious since the sequencing of the *B. subtilis* 168 genome was completed in 1997 (Kunst *et al.*, 1997). Therefore, it became possible to investigate carboxylesterase NP homologues. In the genome of *B. subtilis* 168, 8 genes have been functionally characterised as esterases (Bischoff & Ordal, 1991; Chen *et al.*, 1995; Eder *et al.*, 1996; Eggert *et al.*, 2000; Higerd & Spizizen, 1973; Kneusel *et al.*, 1994; Kunst *et al.*, 1997; Moore & Arnold, 1996; Nilsson *et al.*, 1994; Quax
An enantioselective esterase of Bacillus subtilis

& Broekhuizen, 1994; Riefler & Higerd, 1976; Zock et al., 1994). These esterases, carboxylesterase NP (Quax & Broekhuizen, 1994), lipase B (Eggert et al., 2000), p-nitrobenzylesterase (Chen et al., 1995; Moore & Arnold, 1996), esterase A (Higerd & Spizizen, 1973; Riefler & Higerd, 1976), brefeldin A esterase (Kneusel et al., 1994), two phosphodiesterases (encoded by the genes phoD and glpQ) (Eder et al., 1996; Nilsson et al., 1994) and a MCP glutamate methyl esterase (Bischoff & Ordal, 1991), are, with the exception of carboxylesterase NP, not homologous to carboxylesterase NP of B. subtilis Thai I-8. In addition, 9 genes can be classified as potential esterases (Kunst et al., 1997). Among these potential esterases, homology is observed towards carboxylesterases (ybfK and yvaK genes), an arylesterase (ydjP), an acetylesterase (yesY), an erythromycin esterase (ybfO) and four phosphodiesterases (yfKN, yhdW, yofJ, yqik). Surprisingly, ybfK shows similarity to the carboxylesterase of B. subtilis Thai I-8.

We have isolated and cloned this parologue of carboxylesterase NP, YbfK, to determine whether the 36% difference in amino acid sequence resulted in altered stereospecific characteristics towards IPG esters. It should be noted that a classical enzyme screening programme would probably never have identified this enzyme nor carboxylesterase NP, since wild type cells show no activity towards (S)-naproxen and IPG esters. In addition, these enzymes are not expressed under normal conditions as judged by the absence on 2-dimensional gels of the B. subtilis proteome. Overexpression of YbfK was achieved by cloning the gene in a multi-copy vector containing a combination of two strong promotors, the HpaII promotor and the promotor of the nap gene (Brückner et al., 1984; Dartois et al., 1994; Quax & Broekhuizen, 1994; Zyprian & Matzura, 1986). Using the HpaII promotor only, a slight overexpression of the protein encoded by the ybfK gene was observed.

Comparison of the specific activities and enantioselective properties of YbfK and carboxylesterase NP showed striking differences. Firstly, the catalytic activity of YbfK towards the methyl ester of (S)-naproxen was 25-fold lower. The obvious explanation for the observed difference in specific activity is found in the 36% difference in amino acid sequence. Secondly, YbfK combined a very narrow substrate specificity towards IPG esters with markedly enantioselective properties, whereas carboxylesterase NP could hydrolyse all IPG esters but no enantioselectivity was observed. YbfK hydrolysed only acetate esters of (-)- and (+)-IPG and caprylate esters of (+)-IPG. Since no activity towards (-)-IPG-caprylate was measured, the selectivity for (+)-IPG-caprylate is more than 99.9%. Due to this enantioselectivity, YbfK can be used for chiral resolution of IPG-caprylate (figure 3). Although the specific activity of YbfK towards (+)-IPG-caprylate is almost 200 times lower than the specific activity of carboxylesterase NP towards (S)-naproxen methyl ester, it should be more than sufficient for the development of a (+)-IPG production process. Compared to other studies, the amount of IPG produced is likely to be enough for large-scale production in a bioreactor using immobilised YbfK (Battistel et al., 1991).
In the past, a number of bacterial enzymes have been used in kinetic resolution experiments with IPG esters. Carboxylesterase A, an intracellular esterase (70-73 kDa) of *B. coagulans*, showed the highest enantioselectivity, yielding (+)-IPG from various racemic esters (e.g. an enantioselectivity of 94% for IPG-caprylate was observed). Although no sequence data are available for this esterase, it is very unlikely that this is a carboxylesterase NP or YbfK homologue, because its molecular weight is more than twice as high (Aragozzini et al, 1991; Maconi et al., 1990; Molinari et al., 1996).

In conclusion, we have isolated an enzyme, suitable for the enantioselective production of (+)-IPG, by analysis of paralogous genes. We propose to name this enzyme carboxylesterase B and the corresponding gene *cesB*. Similarly, we propose to rename the carboxylesterase NP (naproxen) of *B. subtilis* 168 into carboxylesterase A and the corresponding gene *cesA*.

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