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Functional expression of *Candida antarctica* lipase B in the *Escherichia coli* cytoplasm—a screening system for a frequently used biocatalyst

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Abstract In this paper, we report for the first time the functional expression of lipase B from the yeast *Candida antarctica* (CalB) in the *Escherichia coli* cytoplasm. The enzyme possessing three disulfide bonds was functionally expressed in the strain Origami B. Expression under the control of a *lac* promoter yielded 2 U mg^{-1}, whereas expression of a thioredoxin–CalB fusion protein yielded 17 U mg^{-1}. The native enzyme was most efficiently expressed under control of the *csp*A promoter (11 U mg^{-1}). Coexpression of different chaperones led to a strong increase in active protein formation (up to 61 U mg^{-1}). A codon-optimized synthetic variant of calb did not show significant effects on functional protein yield. Functional CalB expression was not only achieved in shake flasks but also in microtiter plate scale. Therefore, this CalB expression system is suitable for high-throughput applications, including the screening of large gene libraries as those derived from directed evolution experiments.

Introduction

Triacylglycerol lipases (EC 3.1.1.3) are popular and efficient catalysts of many industries, e.g., the detergent industry, oleochemistry, food industry, and fine chemical preparations (Schmid and Verger 1998), where they catalyze the hydrolysis as well as the synthesis of triglycerides and other mostly hydrophobic esters.

Candida antarctica lipase B (CalB) is an efficient catalyst for many reactions and is applied, for example, for stereoselective transformations and polyester synthesis (Anderson et al. 1998; Bornscheuer and Kazlauskas 1999). The enzyme has a solvent accessible active site (Uppenberg et al. 1994) and displays no interfacial activation (Martinelle et al. 1995). The fact that CalB activity in organic media is similar to that found in water, especially its high enantioselectivity toward secondary alcohols, makes CalB one of the most important lipases used in biotechnology nowadays.

Previously, CalB was expressed in *Aspergillus oryzae* for large industrial scale purposes (Hoegh et al. 1995). For research applications, the enzyme was successfully expressed in the yeasts *Pichia pastoris* (Rotticci-Mulder et al. 2001) and *Saccharomyces cerevisiae* (Zhang et al. 2003). The expression of CalB displaying a molecular weight of 33 kDa in the easy-to-handle prokaryotic expression system *Escherichia coli* has failed (Rotticci-Mulder 2003). This is regrettable because *E. coli* exhibits many great advantages in comparison with other expression systems and enables the fast and cheap high-throughput screening of large gene libraries. Recently, periplasmatic expression of a CalB-derived enzyme was reported, while no data concerning achieved activities were published in that work (Chodorge et al. 2005). Several attempts to improve CalB for special applications by rational enzyme design are reported in the literature. Although some yielded good results (Patkar et al. 1998; Rotticci 2000), the power of rational enzyme design is limited by the poor understanding of the enzyme's catalytic properties.

The reason for the lack of functional expression of CalB in the *E. coli* cytoplasm, however, is undisputed. The complex tertiary structure of the enzyme requires the formation of three disulfide bonds to reach its functional conformation. Producing such a protein in *E. coli* is challenging because the cellular environment, folding machinery, and conformational quality control checkpoints of prokaryotes are different from those of eukaryotes (Baneyx and Mujacic 2004). We expectedly observed inclusion body formation in the initial expression experiments of CalB in *E. coli* (unpublished results).

In principle spoken, expression problems can occur in three different stages of protein expression: the transcription of the protein encoding gene, the translation of the

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mRNA, and posttranslational events, e.g., the folding of newly synthesized peptide chains.

In this paper, we report the development of a suitable expression system for functional CalB expression in the *E. coli* cytoplasm that also offers the opportunity for high-throughput applications, such as screening of large gene libraries, including those derived from directed evolution experiments. The work also contains a comparison of some frequently used expression optimization strategies. The finally established expression system makes use of the cold shock inducible vector pColdIII and the coexpression of molecular chaperones.

Materials and methods

Chemicals

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (Taufkirchen, Germany) and Roth GmbH (Karlsruhe, Germany) unless otherwise specified.

Strains and plasmids

E. coli DH5α was purchased from Clontech (Heidelberg, Germany). *E. coli* Origami2(DE3), Origami B, and the plasmid pET-32b(+) were purchased from Novagen (Darmstadt, Germany). The plasmid pUC18 was purchased from MBI Fermentas (St. Leon-Rot, Germany), pColdIII and the Chaperone Plasmid Set, containing plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2, and pTf16, were purchased from Takara (Otsu, Japan). The construct pPCR/calB containing the codon-optimized *calB* gene was synthesized at GENEART (Regensburg, Germany) using the GeneOptimizer technology. In addition to codon bias optimization, regions of very high (>80%) or low (<30%) GC content were avoided whenever possible. Furthermore, *cis*-acting sequence motifs such as internal TATA-boxes, chi-sites, ribosomal entry sites, ARE, INS, and CRS sequence elements, as well as repetitive sequences and RNA secondary structures, were avoided. The construct pPICZαA/calB was available in our laboratory. Table 1 gives an overview about the used strains and plasmids.

Cloning of calB variants

CalB wt was amplified from the template construct pPICZαA/calB using primers containing restriction sites for *Eco*RI and *Nde*I, respectively, and subsequently cloned into the vector pUC18 leading to the construct pUC18/CalB wt. For subcloning into pET-32b(+), the lipase gene was amplified using primers containing the restriction sites for *Eco*RI and *Not*I, respectively, and subsequently cloned into the vector (pET-32b(+)/calB wt). For subcloning into pColdIII, calB was amplified and cloned using the restriction sites for *Nde*I and *Eco*RI (pColdIII/calB wt).

CalB syn was amplified from pPCR/calB according to the protocol given for calB wt and subsequently cloned into the plasmids pUC18, pET-32b(+) and pColdIII, respectively, leading to the constructs pUC18/calB syn, pET-32b(+)/calB syn, and pColdIII/calB syn (Table 1).

Table 1 Plasmids and strains used in this work

Plasmid	Gene of interest	Pro	Inducer	Ori	Resistance marker	Reference
PPICZαA/calB	calB_wt	AOX1	Methanol	pUC	Zeozin	Invitrogen (2002)
PPCR/calB	calB_syn	/	/	ColE1	Ampicillin	Geneart (Regensburg, Germany)
PUC18/calB	calB_wt/calB_syn	lac	IPTG	pBR322	Ampicillin	MBI Fermentas (St. Leon-Rot, Germany)
PColdIII/calB	calB_wt/calB_syn	cspA	Cold shock + IPTG	ColE1	Ampicillin	TaKaRa (2003a,b)
PET32-b(+)/calB	Trx-calB_wt/Trx-calB_syn	T7	IPTG	pBR322	Ampicillin	Novagen (1998)
PGro7	groES-groEL	araB	L-arabinose	pACYC	Chloramphenicol	TaKaRa (2003a,b)
PG-Tf2	groES-groEL-tig	Pzt1	Tetracyclin	pACYC	Chloramphenicol	TaKaRa (2003a,b)
PTf16	tig	araB	L-arabinose	pACYC	Chloramphenicol	TaKaRa (2003a,b)
PKJE7	dnaK-dnaJ-grpE	araB	L-arabinose	pACYC	Chloramphenicol	TaKaRa (2003a,b)
PG-KJE8	dnaK-dnaJ-grpE groES-groEL	araB Pzt1	L-arabinose Tetracyclin	pACYC	Chloramphenicol	TaKaRa (2003a,b)
Strains	Genotype					Reference
DH5α	supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 end A1 gyrA96 thi1relA1					Clontech (Heidelberg, Germany)
Origami B	Δara-leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL F'[lac^{+} (lacI^{q})pro] gor522::Tn10 (Tc^{R}) trxB::kan					Novagen (2004)
Origami 2(DE3)	Δ×ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F'[lac+ lacI q pro] (DE3) gor522::Tn10 trxB (StrR, TetR)					Novagen (2004)

Pro Promoter, *Ori* origin of replication

Lipase expression and coexpression of chaperones

All expression experiments were done four- to sixfold. The activities given in the "Results" part are mean values.

pUC18 expression

Origami B and DH5α cells were transformed with pUC18 constructs. Cells were grown in 100 ml Luria–Bertani media containing 100 μg ml^{-1} ampicillin (LB_{amp}) up to an optical density of 0.6 at 600 nm at 37 °C and 180 rpm. Subsequently, lipase expression was induced by addition of isopropylthiogalactoside (IPTG; final concentration 1 mM). Cells were grown for an additional 4 h at 30 °C and 180 rpm and were harvested by centrifugation.

pET-32b(+) expression

Origami 2(DE3) cells were transformed with pET-32b(+) constructs. Cells were grown in 100 ml LB_{amp} at 37 °C and 180 rpm up to an optical density of 0.6 at 600 nm and processed as described before. Alternatively, cells were cooled on ice before induction and expression was carried out at 15 °C for 24 h.

pColdIII expression

Origami B cells were transformed with pColdIII constructs. Cells were grown in 100 ml LB_{amp} at 37 °C and 180 rpm up to an optical density of 0.4–0.6 at 600 nm. Subsequently, cultures were chilled on ice for 30 min, and lipase expression was induced by adding IPTG (final concentration 1 mM). Cells were grown for an additional 24 h at 15 °C and 180 rpm and were harvested by centrifugation.

Co-expression of chaperone plasmids and pColdIII constructs

Origami B cells were transformed with chaperone plasmids. Cells were grown in 100 ml LB containing 34 μg ml^{-1} chloramphenicol (LB_{cm}) at 37 °C, and competent cells were prepared. The recombinant cells were transformed with the pColdIII constructs and selected on LB_{cm+amp}. For expression, cells were grown in LB_{cm+amp} containing 1 mg ml^{-1} L-arabinose (in case of pGro7, pKJE7, and pTf16), 5 ng ml^{-1} tetracycline (in case of pG-Tf2), or L-arabinose and tetracycline (in case of pG-KJE8) in concentrations given above at 37 °C and 180 rpm up to an optical density of 0.4–0.6 at 600 nm. Cultures were chilled on ice for 30 min. Lipase expression was subsequently induced by adding IPTG (final concentration 1 mM). Cells were grown for an additional 24 h at 15 °C and 180 rpm and were harvested by centrifugation.

Tributyrin agar plate assay

Cells were grown on LB agar plates containing 1% emulsified tributyrin and the appropriate antibiotics and, in case of coexpression of pGro7, pKJE7, and pTf16, 1 mg ml^{-1} L-arabinose, 5 ng ml^{-1} tetracycline in case of coexpression of pG-Tf2, or L-arabinose and tetracycline in case of pG-KJE8. After cell growth at 37 °C for 24 h, plates were covered with soft agar (0.6% agar in water) containing 1 mM IPTG and incubated at 30 °C, 15 °C, or room temperature for expression. Functional lipase expression was indicated by the formation of clear halos around the colonies.

Cell lysate activity assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and densitometric analysis

Cells were disrupted 30-sec sonication for three times in sodium phosphate buffer (50 mM, pH 7.5), and cell debris (insoluble fraction) was removed by centrifugation. Cell lysates (soluble fraction) were tested for activity using the pH stat device (Metrohm, Filderstadt, Germany). Hydrolysis of the substrate (5% tributyrin, emulsified in water with 2% gum arabic) was monitored by titration using 10 mM NaOH. Protein content of the cell lysate was measured using the Bradford assay (Bradford 1976). One unit of lipase activity was defined as release of 1 μmol fatty acid per minute.

Insoluble and soluble fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis following standard procedures (Laemmli 1970) using 50 μg of cleared cell lysate (corresponding to 0.2 to 0.4 ml cell culture) and insoluble cell debris from 0.5 ml cell culture, respectively. The gels were stained with Coomassie brilliant blue.

The percentage of soluble CalB on the total cell protein was measured densitometrically using the program Scion Image.

Expression in microtiter plates and activity assay

Origami B cells harboring pColdIII constructs and the chaperone plasmid pGro7 were grown in a 96-well microtiter plate (Greiner, Nürtingen, Germany), containing 150 μl LB_{cm+amp} plus 1 mg ml^{-1} L-arabinose up to an optical density of 0.4–0.6 at 37 °C and 400 rpm. Cells were chilled on ice for 30 min, and the lipase expression was induced by adding IPTG up to a final concentration of 1 mM. After 24 h expression at 15 °C and 200 rpm shaking, cells were harvested by centrifugation and were lysed by adding 50 μl lysis buffer (50 mM sodium phosphate, pH 7.5, 1 mg ml^{-1} lysozyme; DNAse 0.02 ml ml^{-1}). Lysates were incubated for 10 min at 37 °C and 300 rpm and chilled on ice for 30 min. After incubation for 1 h at −80 °C, lysates were thawed at RT, and cell debris was removed by 30 min centrifugation at 4,000 rpm and 4 °C.

To detect lipase activity, 20 µl of clear cell lysate was added to 180 µl of assay solution [162 µl solution B (1 g Triton X-100+0.2 g gum arabic in 200 ml 0.1 M Tris–HCl pH 7.5) + 18 µl solution A [60 mg p-nitophenyl phosphate (pNPP) in 20 ml *n*-propanol]. The formation of *p*-nitrophenolate was monitored after 5 min by measuring the absorbance at 410 nm. Alternatively, lipase activity was quantified using the pH stat assay as described above.

Results

Gene cloning

The *calB wt* gene dispensed from the N-terminal pre-propeptide sequence was subcloned from pPicZαA/calB (Rusnak 2004) into the *E. coli* expression vectors pUC18, pET-32b(+), and pColdIII, respectively (Table 1). The analysis of the *calB* gene, which was originally amplified from genomic DNA of *C. antarctica*, revealed two alterations from the published CalB sequence (CAA83122.1) on amino acid level (T57A, A89T).

The construct pPCR/calB contained a *calB* gene that was optimized for expression in *E. coli*. The gene differs at 253 nucleotides (26.5%) from the wt calB sequence (Fig. 1). On amino acid level, the synthetic gene encodes the published protein (CAA83122.1). Subsequently, the synthetic gene was subcloned into the mentioned expression vectors (Table 1).

Lipase expression in three different vector systems

CalB has not yet been actively expressed in *E. coli* (Rotticci-Mulder 2003). We therefore decided to use the *E. coli* Origami B and Origami2(DE3) strains that are characterized by their thioredoxin reductase and glutathione reductase deficiency. In fact, Origami B cells transformed with pUC18/calB wt showed halo formation on tributyrin agar plates, while DH5α cells transformed with the same plasmid did not. However, the CalB activity in the Origami B cells transformed with pUC18/calB wt or pUC18/calB syn was rather low, exhibiting hydrolysis of as little as ~2 U tributyrin per milligram total soluble protein for both constructs (Fig. 4). In an SDS-PAGE analysis, no protein band corresponding to the mass of CalB (33 kDa) was detected in the soluble fraction (data not shown), while the CalB content in the insoluble fraction was 10–12% (Table 2).

To enhance the yield of active enzyme, *calB wt* and *calB syn* genes were fused to a thioredoxin tag (Trx•Tag) using the vector pET-32b(+) and expressed in *E. coli* Origami2 (DE3). The transformed cells showed clear halo formation on tributyrin agar plates when grown at room temperatures, while cultivation at 37 °C did not lead to any detectable enzyme activity (data not shown). Whereas the shaking flask expression of Trx–CalB at 30 °C yielded in a strong increase of enzyme amount in the insoluble fraction (18–19%, Table 2) but not in activity and therefore in solubility of CalB, expression at 15 °C yielded in up to 17 U mg^{-1} soluble protein for the wt gene and 8 U mg^{-1} for the synthetic gene (Fig. 4). The usage of the temperature-

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wt    CTACCTTCCGGTTCGGACCCTGCCTTTTCGCAGCCCAAGTCGGTGCTCGATGCGGGTCTGACCTGCCAGGGTGCTTCGCCATCCTCGGTC
syn   CTGCCGAGCGGTAGCGATCCGGCGTTTAGCCAGCCGAAAAGCGTTCTGGATGCGGGTCTGACCTGTCAGGGTGCGAGCCCGAGCAGCGTT

wt    TCCAAACCCATCCTTCTCGTCCCCGGAACCGGCACCACAGGTCCACAGTCGTTCGACTCGAACTGGATCCCCCTCTCTGCGCAGCTGGGT
syn   AGCAAACCGATTCTGCTGGTTCCGGGCACCGGCACCACCGGTCCGCAGAGCTTTGATAGCAACTGGATTCCGCTGAGCACCCAGCTGGGT

wt    TACACACCCTGCTGGATCTCACCCCCGCCGTTCATGCTCAACGACACCCAGGTCAACACGGAGTACATGGTCAACGCCATCACCGCGCTC
syn   TATACCCCGTGTTGGATTAGCCCGCCGCCGTTTATGCTGAATGATACCCAGGTGAACACCGAATATATGGTGAACGCGATCACCGCGCTG

wt    TACGCTGGTTCGGGCAACAACAAGCTTCCCGTGCTTACCTGGTCCCAGGGTGGTCTGGTTGCACAGTGGGGTCTGACCTTCTTCCCCAGT
syn   TATGCGGGTAGCGGCAATAATAAACTGCCGGTGCTGACCTGGAGCCAGGGTGGTCTGGTTGCGCAGTGGGGTCTGACCTTTTTTCCGAGC

wt    ATCAGGTCCAAGGTCGATCGACTTATGGCCTTTGCGCCCGACTACAAGGGCACCGTCCTCGCCGGCCCTCTCGATGCACTCGCGGTTAGT
syn   ATTCGCAGCAAAGTGGATCGTCTGATGGCGTTTGCGCCGGATTATAAAGGCACCGTTCTGGCGGGTCCGCTGGATGCGCTGGCGGTTAGC

wt    GCACCCTCCGTATGGCAGCAAACCACCGGTTCGGCACTCACCACCGCACTCCGAAACGCAGGTGGTCTGACCCAGATCGTGCCCACCACC
syn   GCGCCGAGCGTTTGGCAGCAGACCACCGGTAGCGCGCTGACCACCGCGCTGCGTAATGCGGGTGGTCTGACCCAGATTGTTCCGACCACC

wt    AACCTCTACTCGGCGACCGACGAGATCGTTCAGCCTCAGGTGTCCAACTCGCCACTCGACTCATCCTACCTCTTCAACGGAAAGAACGTC
syn   AATCTGTATAGCGCGACCGATGAAATTGTTCAGCCGCAGGTTAGCAATAGCCCGCTGGATAGCAGCTACCTGTTCAACGGCAAAAATGTT

wt    CAGGCACAGGCCGTGTGTGGGCCGCTGTTCGTCATCGACCATGCAGGCTCGCTCACCTCGCAGTTCTCCTACGTCGTCGGTCGATCCGCC
syn   CAGGCGCAGGCGGTTTGTGGTCCGCTGTTTGTGATTGATCATGCGGGTAGCCTGACCAGCCAGTTTAGCTATGTGGTTGGTCGTAGCGCG

wt    CTGCGCTCCACCACGGGCCAGGCTCGTAGTGCAGACTATGGCATTACGGACTGCAACCCTCTTCCCGCCAATGATCTGACTCCCGAGCAA
syn   CTGCGTAGCACCACCGGCCAGGCGCGTAGCGCGGATTATGGTATCACCGATTGCAATCCGCTGCCGGCGAATGATCTGACCCCGGAACAG

wt    AAGGTCGCCGCGGCTGCGCTCCTGGCGCCGGCAGCTGCAGCCATCGTGGCGGGTCCAAAGCAGAACTGCGAGCCCGACCTCATGCCCTAC
syn   AAAGTTGCGGCGGCAGCGCTGCTGGCGCCGGCAGCGGCGGCCATTGTTGCGGGTCCGAAACAGAATTGTGAACCGGATCTGATGCCGTAT

wt    GCCCGCCCCTTTGCAGTAGGCAAAAGGACCTGCTCCGGCATCGTCACCCCCTGA
syn   GCGCGTCCGTTTGCGGTTGGTAAACGTACCTGCAGCGGTATTGTGACCCCGTAA
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Fig. 1 Alignment of calB wt amplified from *C. antarctica* and the synthetic gene *calB syn* derived from the vector pPCR/calB. Differences are highlighted

Table 2 Densitometric analysis of CalB content in cell extracts from different expression experiments

	CalB content in insoluble fraction (%)		CalB content in soluble fraction (%)		CalB content in whole cell protein (%)		Content of soluble CalB in whole cell protein (%)	
	Wt	Syn	Wt	Syn	Wt	Syn	Wt	Syn
pUC18	12	10	N. d.	N. d.	4	3	N. d.	N. d.
pET32-b(+) (30°C)	19	18	N. d.	N. d.	6	6	N. d.	N. d.
pET32-b(+) (15°C)	24	26	N. d.	N. d.	8	9	N. d.	N. d.
pColdIII	42	38	N. d.	N. d.	14	13	N. d.	N. d.
pColdIII + pGro7	49	28	11	10	23	16	7	7
pColdIII + pG-Tf2	42	29	9	7	20	14	6	5
pColdIII + pTf16	22	19	10	5	14	9	7	3
pColdIII + pKJE7	31	15	N. d.	N. d.	10	5	N. d.	N. d.
pColdIII + pG-KJE8	5	6	4	4	4	5	3	3

For evaluation of Coomassie brilliant blue-stained SDS-PAGEs the program Scion Image was used
N. d. Not detectable

inducible pColdIII vector system yielded in activities of 11 U mg^{-1} (wt) and 9 U mg^{-1} (syn), respectively (Fig. 4). Also in this system, the halo formation on tributyrin agar plates was only observed at low cultivation temperatures (data not shown.) Nevertheless, an SDS-PAGE analysis revealed a high amount of insoluble protein upon expression from pET-32b(+) (24 and 26%, respectively) as well as from pColdIII (42 and 38%, respectively), even for expression at 15 °C (Fig. 2, Table 2).

Lipase expression with coexpression of molecular chaperones

An increase in soluble protein yield, involving the coexpression of different chaperones in the *E. coli* cytoplasm, has been reported (Nishihara et al. 1998, 2000). Hence, the pColdIII constructs harboring the gene for the native CalB protein were coexpressed with several combinations of chaperones provided in the TaKaRa Chaperone Plasmid Set (Table 1). All recombinant Origami B cells possessing both CalB- and chaperone expression plasmids showed a clear halo formation on tributyrin agar plates and expression of CalB in the shaking flask scale (Fig. 3), while the amount of soluble lipase differed significantly (Fig. 4).

CalB wt was most efficiently expressed under the coexpression of pGro7 (61 U mg^{-1}) (Fig. 4). Expression of functional enzyme was also enhanced by coexpression of pG-Tf2 (33 U mg^{-1}), pTf16 (24 U mg^{-1}), and in smaller scale, also by coexpression of pG-KJE8 (18 U mg^{-1}). Coexpression of pKJE7 showed no significant effect on CalB expression. The results of the activity assay were confirmed densitometrically, where the highest amount of soluble CalB was found by coexpression of pGro7, followed by coexpression of pG-Tf2 and pTf16 (Table 2).

The results were similar with the synthetic *calB* gene, indicating a positive influence of coexpressed pGro7, pG-Tf2, pTf16, and pG-KJE8. As already shown for the pET-32b(+) and pColdIII expression, the achieved values for lipase activities and soluble enzyme content were lower for the synthetic gene compared to the wt gene (Fig. 4, Table 2).

Lipase expression in microtiter plate scale

As a prerequisite for the development of a high-throughput screening system for CalB derived enzyme variants, the coexpression of CalB wt or syn with pGro7 in Origami B cells was conducted in 96-well microtiter plate scale. The activities of the cleared cell lysates were analyzed by a

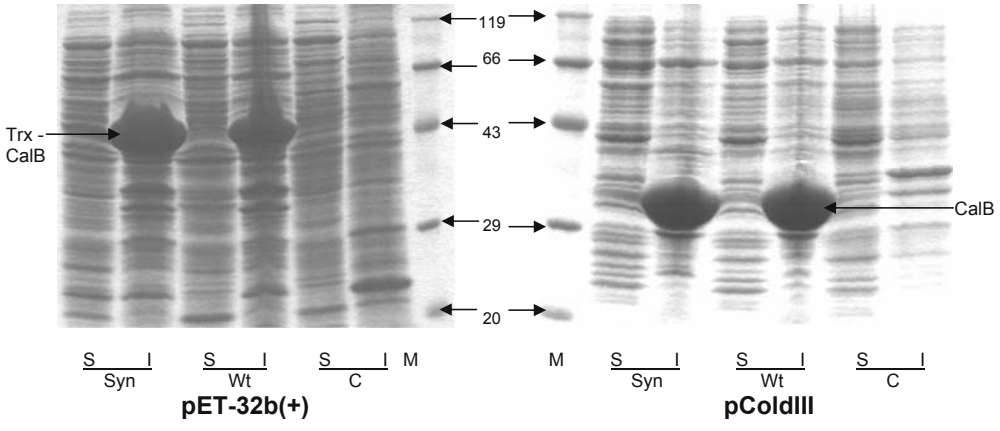

Fig. 2 SDS-PAGE separation of soluble (*S*) and insoluble (*I*) fractions obtained from CalB expression experiments at 15 °C using the expression vectors pET32-b(+) [in Origami 2 (DE3) cells] and pColdIII (in Origami B cells), respectively. CalB bands (33 kDa) and Trx-CalB fusion protein bands (45 kDa) are marked with *arrows*. *M* Molecular weight standard, *C* fractions from a control with empty vector

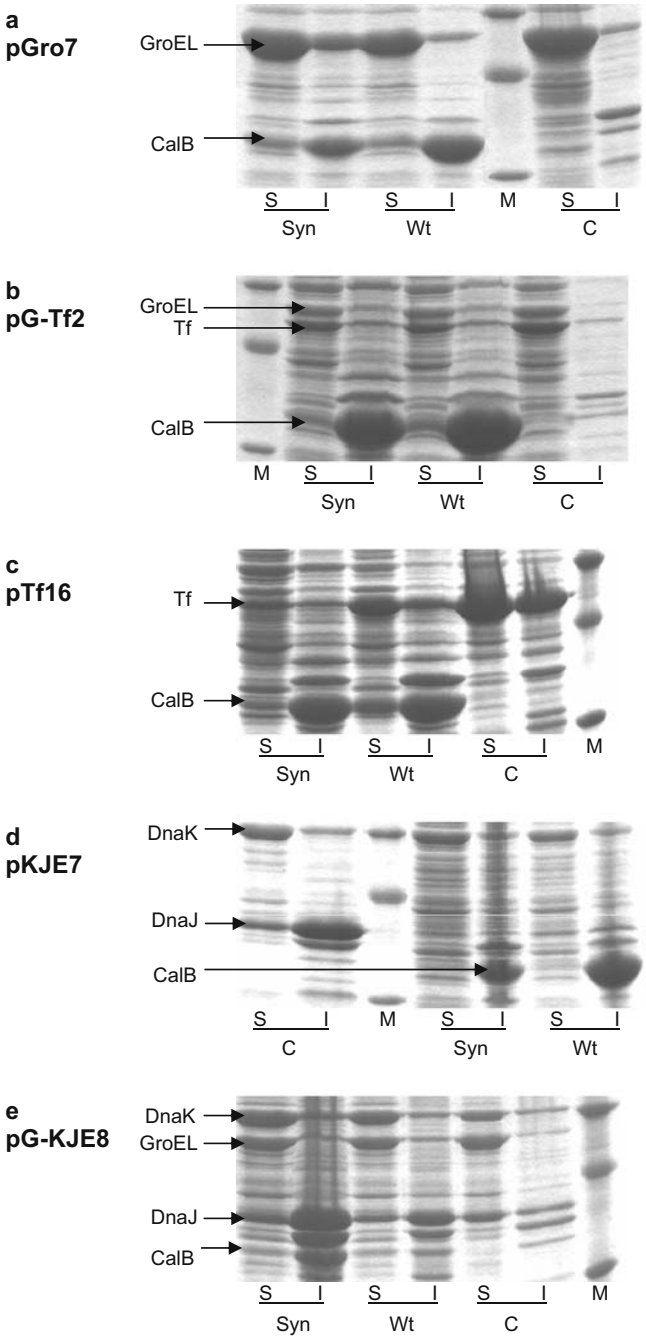

Fig. 3 SDS-PAGE separation of soluble (*S*) and insoluble (*I*) fractions obtained from coexpression experiments of CalB using pColdIII constructs with different chaperone plasmids (*a* pGro7, *b* pG-Tf2, *c* pTf16, *d* pKJE7, *e* pG-KJE8) in Origami B cells. CalB (33 kDa) and chaperones (GroEL 60 kDa, Tf 56 kDa, DnaK 70 kDa, DnaJ 40 kDa) are marked with *arrows*. *M* Molecular weight standard (29, 43 and 66 kDa), *C* Fractions from a control with empty vector

colorimetric assay using pNPP as a substrate. Both constructs were functionally expressed in comparable amounts, as indicated by the formation of yellow p-nitrophenolate in similar intensities (data not shown). Control cells lacking the lipase gene showed significantly reduced absorption values at 410 nm. To compare the activities from the microtiter plate expression with the values derived from the shaking flask coexpression of the constructs pColdIII/calB_wt and pGro7, the activities of five representative wells with the substrate tributyrin were analyzed using the pH stat assay. The growth conditions in the single wells were the same. They showed specific activities from 57 to 81 U mg^{-1} soluble protein in the cleared cell lysate and total activities of 10 to 15 U ml^{-1} cell culture, respectively. The total lipase expression level in the wells was quantified by densitometric analysis to 0.04±0.01 μg CalB per milliliter cell culture.

Discussion

The *calB wt* gene was amplified from the pPicZαA/calB construct that descended from our early projects, where CalB dispensed from the N-terminal pre-pro-peptide sequence was functionally expressed in *P. pastoris* (Rusnak 2004). In that project, the analysis of the *calB* gene amplified from genomic DNA of *C. antarctica* revealed two alterations from the published CalB sequence (CAA83122.1) on amino acid level (T57A, A89T). The two deviations appeared in two independent amplification approaches, where the gene was amplified from two different extracts of genomic DNA. Therefore, they are most likely natural variations of the lipase gene occurring in *C. antarctica* as a consequence of evolution. The lipase displayed comparable activity to the published values of the wild-type CalB when expressed in *P. pastoris*, and so we continued the work with the amplified gene (Rotticci-Mulder et al. 2001; Rusnak 2004). Nevertheless, an influence of the deviations on lipase activity is not completely out of the question.

The construct pPCR/calB contained a *calB* gene that was optimized for expression in *E. coli.* The gene differs at 253 nucleotides (26.5%) from the wt calB sequence (Fig. 1). On amino acid level, the synthetic gene encodes the published protein (CAA93122.1).

In this work, we compared activity data obtained from various expression strategies. Although the given values cannot be taken as absolute kinetic values because they derive from measurements of crude enzyme preparations, they mirror the conditions present during the high-throughput screening of libraries of enzyme variants more exactly than data derived from purified enzymes.

For expression of CalB in *E. coli*, we used the strains Origami B and Origami2(DE3) that are characterized by their thioredoxin reductase and glutathione reductase deficiency. These deficiencies reportedly enhance disulfide bond formation in the cytoplasm of *E. coli* (Prinz et al. 1997). The observation that Origami B cells transformed with pUC18 / calB wt showed halo formation on tributyrin agar plates while DH5α did not supports the assumption that the necessity of forming three correct disulfide bonds in the native CalB protein is the major bottleneck in the production of functional CalB in the *E. coli* cytoplasm.

The expression of the *calB wt* and *calB syn* genes fused to a thioredoxin tag using the vector pET-32b(+) at 30 °C

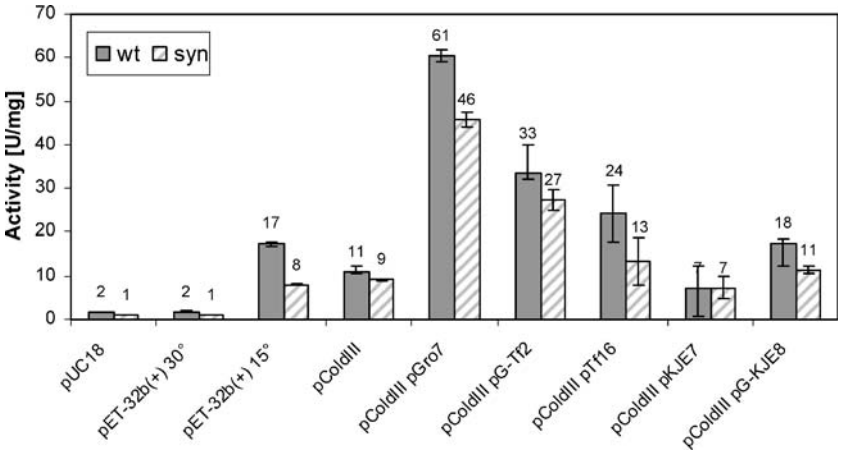

Fig. 4 Hydrolytic activity of cleared cell lysates from *E. coli* Origami 2(DE3) cells (in case of pET32-b(+) expression) and Origami B cells (all other constructs) harboring the specified constructs towards tributyrin. The mean value is given from 4–6 independent expression experiments, as well as the standard deviations

increased the yield of active enzyme only slightly, whereas the total protein amount was increased significantly. The observation fits to the known problem, that high expression levels of heterologously expressed enzymes often increase the risk to obtain incorrectly folded proteins. A high-yield production of functional lipases in *E. coli* cells by using the T7 *lac* promoter has been previously reported (Kim et al. 2000; Nthangeni et al. 2001). However, in the reported expression experiments, most of the target proteins were prokaryotic enzymes with no need for secondary modifications and folding parameters, as required by most eukaryotic enzymes. By fusion of the target protein to the extremely soluble *trxA* protein, several eukaryotic enzymes, e.g., mammalian cytokines and growth factors, were expressed in a soluble form in the *E. coli* cytoplasm (LaVallie et al. 1993). Using a T7 *lac* promoter and the *trxA* fusion Tag, as, e.g., provided by the pET-32b(+) vector system, the murine interleukin-2 (IL-2) was expressed in high yield in soluble form (Novy). In contrast to IL-2, CalB possesses three disulfide bonds, whose correct linkage may not be enhanced significantly by fusion to the *trxA* Tag under the given expression conditions.

A decrease of the expression temperature strongly increased the yield of functional enzyme in the pET-32b (+) system as well as in the pColdIII vector system encoding the native CalB protein. Such a positive influence of decreased expression temperatures on functional protein expression was reported previously (Liao 1991; de la Torre et al. 2002). The rationale behind the use of low temperatures for gene expression is based on the proposition that the rate of protein folding will be only slightly affected at about 15 to 20 °C, whereas the rates of transcription and translation, being biochemical reactions, will be substantially decreased. This, in turn, will provide sufficient time for protein refolding, yielding active proteins, and avoiding the formation of inactive protein aggregates, i.e., inclusion bodies (Makrides 1996). The expression temperature is obviously crucial for disulfide bond formation in CalB, for even in the suboptimal pUC18 expression system, active lipase was detected when cells were grown at 30 °C, and incubation at 37 °C led to no active CalB at all (data not shown).

CalB was subsequently coexpressed with several combinations of chaperones, as similar strategies led to an increase in soluble protein yield before (Nishihara et al. 1998, 2000; Ikura et al. 2002). For these experiments, the pColdIII constructs were chosen, because they express, in contrast to the pET-32b(+) constructs, the native CalB protein. The fusion of an enzyme to a protein tag, such as *trxA*, can lead to a change of the catalytic properties (Mutsuda et al. 2003). In case of CalB, we previously observed a strongly decreased activity of a variant with a C-terminal His-tag (Rusnak 2004). Therefore, screening of mutants derived from the fusion protein TrxA–CalB would not unconditionally lead to the detection of improved variants of native CalB. From this point of view, the expression using the vector pColdIII seems favorable, even in spite of the slightly higher activities achieved with the pET-32b(+) system. Moreover, expression using pColdIII led to higher amounts of total CalB in the cell indicating a positive effect of the cold-responsive promoter *csp*A on efficient gene expression at reduced temperatures as already reported (Goldstein et al. 1990).

CalB was most efficiently expressed under the coexpression of pGro7, indicating a strong impact of the GroES/GroEL chaperone system on the correct folding of CalB. It is known that these factors play an important role in folding α/β folds which are enriched in hydrophobic and basic residues as are also present in CalB. Also, the molecular mass of CalB with 33 kDa fits to the GroES/GroEL preferred proteins <60 kDa (Baneyx and Mujacic 2004). In combination with *trxB gor* suppressor cells (e.g., Origami), the yield of properly disulfide-bonded proteins was increased by the coexpression of these folding modulators (Bessette et al. 1999; Levy et al. 2001; Jurado et al. 2002). A positive influence of functional expression was also detected by coexpression of the trigger factor (encoded by pG-Tf2 and pTf16, whereas the coexpression of DnaK-DnaJ-GrpE showed no significant effect on functional CalB expression. These results fit to observations that a positive influence of the DnaK–DnaJ system is mostly restricted to target proteins >60 kDa (Baneyx and Mujacic 2004).

Very conspicuous are the lower activities obtained by using the synthetic gene in comparison to the wt gene.

These results appear astonishing because codon optimization may overcome the problem that the amount of tRNA species can vary widely in the different organisms. The expression levels of some eukaryotic proteins, e.g., a domain of the human type 1 neurofibromin protein was increased significantly (Hale and Thompson 1998) by codon optimization. Unfortunately, the amount of functional enzyme is not directly correlated to the expression level; therefore, it is conditionally correlated to codon usage. The negative effect in our work could be explained by a faster expression from the optimized gene leading—just as higher expression temperatures—to higher CalB concentrations, which in turn have negative effects on correct folding of the enzyme. All the more astonishing is the fact that the total amount of CalB in the cell was also lowered by the usage of this gene variant. It can be assumed that transcription of the synthetic variant leads, if not to increased concentrations, even to accelerated translation and therefore to folding problems.

The system allows for the high-throughput screening of lipase variants for hydrolytic activity toward hydrophobic substrates, as Origami B cells expressing GroES and GroEL (encoded by pGro7) and CalB wt or CalB syn, respectively, showed clear halo formation on agar plates supplemented with tributyrin. A preselection of active clones before complex screening assays with specific substrates that are often susceptible to faults is therefore possible. Both constructs were furthermore functionally expressed in a 96-well microtiter plate scale in comparable amounts, as indicated by the turnover rates for *p*-nitrophenylpalmitate. Control cells lacking the lipase gene showed significantly reduced absorption values at 410 nm, which is a prerequisite for a reliable assay system for the identification of active clones. The expression in microtiter plate led to similar activities than achieved by expression in shaking flasks. The relatively high deviations between single wells may be explained by substantial expression differences due to varying inoculation volumes, aeration differences across the plates, and pipetting inaccuracies, which could be optimized by the usage of robotic systems.

This is the first expression system suitable for high-throughput screening of CalB variants in a prokaryotic system. The *E. coli* system has some major advantages over the published high-throughput expression system in *S. cerevisiae* (Zhang et al. 2003; Suen et al. 2004). Besides lower costs and easier handling, the total amount of clones that can be screened daily is much higher in *E. coli.* The total expression period in microtiter plates is 48 h, while the *S. cerevisiae* system needs a 9-day expression period. Therefore, the described *E. coli* expression system is a very notable development for the mutagenesis of CalB, which is one of the most important lipases in biotechnology. The method reported here, applying subsequent gene-, vector-, host-, and cotransformant variation can furthermore serve as an example for an overall useful expression optimization strategy for hard-to-express proteins.

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