DNA family shuffling of hyperthermostable β-glycosidases

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The structural compatibility of two hyperthermostable family 1 glycoside hydrolases, *Pyrococcus furiosus* CelB and *Sulfolobus solfataricus* LacS, as well as their kinetic potential were studied by construction of a library of 2048 hybrid β-glycosidases using DNA family shuffling. The hybrids were tested for their thermostability, ability to hydrolyse lactose and sensitivity towards inhibition by glucose. Three screening rounds at 70 °C led to the isolation of three high-performance hybrid enzymes (hybrid 11, 18 and 20) that had 1.5–3.5-fold and 3.5–8.6-fold increased lactose hydrolysis rates compared with parental CelB and LacS respectively. The three variants were the result of a single crossover event, which gave rise to hybrids with a LacS N-terminus and a main CelB sequence. Constructed three-dimensional models of the hybrid enzymes revealed that the catalytic $(\beta\alpha)_8$-barrel was composed of both LacS and CelB elements. In addition, an extra intersubunit hydrogen bond in hybrids 18 and 20 might explain their superior stability over hybrid 11. This study demonstrates that extremely thermostable enzymes with limited homology and different mechanisms of stabilization can be efficiently shuffled to form stable hybrids with improved catalytic features.

Key words: directed evolution, hybrid enzyme, lactose hydrolysis, protein thermostability.

INTRODUCTION

Enzyme optimization has taken off with the recent developments in laboratory evolution of proteins. Random mutagenesis combined with DNA shuffling [1], DNA family shuffling [2], iterative truncation for creation of hybrid enzymes [3] and sequence homology independent recombination [4] are several techniques that have been applied to modify or improve the performance of a wide array of biologically active proteins. A higher operational stability is one of the most desired enzyme improvements and has been found hard to achieve by rational protein design. By directed evolution, the half-life of thermal inactivation of several mesophilic proteins has been extended dramatically, often without compromising low-temperature activity [5,6]. Subtle structural changes were observed in the stabilized enzyme variants, which resulted in immobilization of flexible loops or optimization of long-range interactions [6,7].

Relatively few studies report on the directed evolution of extreme thermostable proteins or thermozymes. These proteins already have an extreme resistance against thermal inactivation, but often have relatively low activities at moderate temperatures. In the few known cases, single genes that encode hyperthermophilic proteins were subjected to random mutagenesis *in vitro* or *in vivo* and enzyme variants were screened for increased activities at ambient temperatures [8–10]. An increase in low-temperature activity resulted from a small number of mutations, which often lead to a severe reduction in thermostability. To our knowledge, there have been no reports yet on the creation of enzyme hybrids by DNA family shuffling of genes from hyperthermophilic origin. This could be due to the fact that studies on the thermostability of proteins from hyperthermophiles have indicated that no general rules exist for protein thermostabilization and that, even within a protein family, members may have evolved distinct mechanisms to resist extreme conditions [11]. Thus each protein seems to have been evolved individually to withstand the high temperatures of hyperthermophilic habitats [12]. In this respect, it is not unlikely that family shuffling of proteins from hyperthermophiles does yield hybrid enzymes with affected stability.

Despite these uncertainties, we have attempted to generate stable functional hybrids by DNA shuffling of two genes coding for hyperthermostable β-glycosidases. The 'parental' enzymes are the β-glucosidase CelB from *Pyrococcus furiosus* {optimum temperature (T_{opt}) for growth [$T_{opt(growth)}$] = 100 °C} and β-glycosidase LacS from *Sulfolobus solfataricus* [$T_{opt(growth)}$ = 85 °C], which are among the most thermo-active members of family 1 glycoside hydrolases, with optimal temperatures for hydrolysis of 105 and 95 °C respectively [13,14]. Thermostable family 1 glycoside hydrolases are being recognized as promising candidates for carbohydrate engineering [15–18] and several site-directed mutagenesis studies on their substrate specificity and activity engineering have been reported [19–21]. CelB and LacS are 53 % and 56 % identical at the protein and DNA level respectively, and are similar with respect to catalytic mechanism and substrate specificity [11]. However, the molecular basis of the high thermostability appears to be different in CelB and LacS. A biochemical comparison suggested that CelB is mainly stabilized by hydrophobic interactions, whereas salt-bridge interactions are crucial for the stability of LacS [11]. Indeed, analysis of the crystal structures of CelB [20] and LacS [22] revealed a higher number of ion-pairs in the less stable LacS protein [23]. Besides being model enzymes for the study of enzyme stability and high-temperature catalysis, CelB and LacS have been recognized as potentially interesting biocatalysts for the hydrolysis of the milk sugar lactose (galactose β-1,4-D-glucose) to monomeric glucose and galactose [16]. However, for CelB, a significant glucose inhibition was observed, whereas LacS displayed limited stability.

In the present study, we report the generation of functional glycoside hydrolase hybrids by shuffling of the genes coding for *P. furiosus* CelB and *S. solfataricus* LacS, and it is the first study

Abbreviations used: CFE, cell-free extract; 3D, three-dimensional; GOD-PAP, glucose oxidase-phenol 4-aminophenazone peroxidase; IPTG, isopropyl β-D-thiogalactoside; PheImGlc, (5*R*,6*R*,7*S*,8*S*)-5-(hydroxymethyl)-2-phenyl-5,6,7,8-tetrahydroimidazol[1,2-a]pyridine-6,7,8-triol; pNp-Gal, *p*-nitrophenyl-β-D-galactopyranoside; pNp-Glc, *p*-nitrophenyl-β-D-glucopyranoside; S14A, $Ser^{14} \rightarrow$ Ala mutant; T_m, melting temperature; T_{opt}, optimum temperature; X-Gal, 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside.

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on DNA family shuffling of extreme thermostable enzymes. In order to study catalysis at suboptimal temperatures, a library of 2048 active variants has been screened for optimized activity at 70 °C, for which hydrolysis of lactose in combination with a reduced inhibition by glucose has been taken as a model system. After three screening rounds, three high-performance variants and one variant with reduced stability were isolated, purified and characterized in detail at the biochemical and molecular level. Using crystal structures of CelB [20] and LacS [22], three-dimensional (3D) models of the hybrids were constructed by homology modelling. The results show that distantly related thermoenzymes can be shuffled into hybrids with improved catalytic features. Moreover, we found that these enzymes can tolerate a significant number of non-wild-type residues while remaining extremely thermostable.

MATERIALS AND METHODS

Library construction

For the production of *P. furiosus* β-glucosidase CelB in *Escherichia coli* an efficient expression system has been developed by cloning the *celB* gene in the pET9d vector, resulting in pLUW511 [24]. Similarly, the *lacS* gene [25] was cloned in the pET9d vector (kanamycin resistant and containing the T7 promoter; Novagen, Madison, WI, U.S.A.). The *lacS* gene was PCR-amplified using *Pfu* turbo polymerase (Stratagene, Amsterdam, The Netherlands) and homologous primers BG745 (5′-GCGC-GCCC**ATG***GCA*TACCATTTCCAGATAGCTTT-3′, where the introduced *Nco*I restriction site is underlined, *lacS* start codon is in bold type and the introduced codon for alanine at position 1a is in italics) and BG746 (5′-GCGCGCGGATCC**TTA**GTG-CTTTAATGGCTTTACTG-3′, where the introduced *Bam*HI restriction site is underlined and *lacS* stop anticodon in bold type). The PCR product was digested with *Nco*I and *Bam*HI and cloned in *Nco*I/*Bam*HI-digested pET9d vector to form pWUR6.

Subsequently, the plasmids pLUW511 and pWUR6 were used as starting material for the DNA shuffling procedure in which *Pfu* turbo DNA polymerase (Stratagene) was used in all assembly and DNA amplification steps. The *celB* and *lacS* genes were amplified by PCR using the homologous primer sets BG238/BG239 [24] and BG745/BG746 respectively. Using DNase I, the genes were randomly digested as described previously [26]. The digestion mixture contained 4 μg of DNA and 1 μg of DNase I in 50 μl of DNase I buffer [5 mM Tris/HCl (pH 7.4) and 1 mM $MnCl_2$] and was performed in triplicate at 20 °C. Reactions were stopped at intervals by addition of 5 μl of 0.5 M EDTA and stored on ice. DNA fragments were analysed on a 1.5 % (w/v) agarose gel and fragments in the range of 50–300 bp (derived from a 13–15 min incubation) were excised and purified using the QiaexII gel extraction kit (Qiagen, Hilden, Germany). The fragmented genes were assembled by performing a PCR without primers. The reaction mix contained 0.5 μg of each fragmented gene, 0.2 mM of each dNTP, 2.5 units of *Pfu* turbo DNA polymerase (Stratagene) in the supplied buffer in a total volume of 50 μl. Assembly reactions were carried out with annealing temperatures of 40 °C as reported previously [27]. Due to the limited homology of the *celB* and *lacS* genes, however, the use of lower annealing temperatures for re-assembly was explored. Attempts with annealing temperatures of 10 °C were unsuccessful. Hybrid genes were obtained by using a Tgradient thermocycler (Biometra, Göttingen, Germany) when the mixtures were subjected to 5 min at 95 °C, followed by two temperature programmes of 21 cycles. The first 21 cycles consisted of 1 min at 95 °C, 1 min at 20 °C with 1 °C increase per cycle and 1 min at 72 °C with 5 s increase per cycle. The second 21 cycles were 1 min at 95 °C, 1 min at 40 °C and 2 min 40 s at 72 °C with 2 s increase per cycle. To prevent amplification of wild-type genes, the shuffled constructs were enriched in PCR reactions using hybrid primer sets. The amplification mixtures contained 100 ng of recombined DNA fragments, 5 pmol of each primer, 0.2 mM of each dNTP and 2.5 units of *Pfu* Turbo polymerase in the supplied buffer in a total volume of 50 μl. The temperature programme was 2 min at 95 °C, followed by 25 repeats of 30 s at 95 °C, 1 min at 58 °C and 45 s at 72 °C with a 20 s increase per cycle, with a final step of 5 min at 72 °C. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen) before digestion with *Nco*I and *Bam*HI. Using T4 DNA ligase, hybrid genes were ligated in the *Nco*I/*Bam*HI sites of pET9d vector. *E. coli* JM109(DE3) (*lacZ*⁻ strain) electro-competent cells were transformed with the ligation mixture using a Electroporator 2510 (Bio-Rad Laboratories, Veenendaal, The Netherlands) and plated on to selective TY agar (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 30 μg/ml kanamycin and 1.5 % granulated agar) containing 1.6 μg/ml 5-bromo-4-chloroindol-3-yl β-D-galacto-pyranoside (X-Gal). Colonies with complemented β-glycosidase activity showed a blue phenotype and were transferred to sterile flat-bottomed 96-well microplates that contained selective TY medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 30 μg/ml kanamycin) with 10 % (w/v) glycerol. Each plate contained negative [*E. coli* JM109(DE3)/pET9d] and positive [*E. coli* JM109(DE3)/pLUW511 or pWUR6] controls. Colonies (2048 in total) were isolated and grown overnight with shaking at 37 °C. These glycerol stocks were stored at −80 °C.

Screening for lactose hydrolysis at 70 °C

For the preparation of cell-free extract (CFE), the glycerol stock plates were replicated in 96-well plates containing selective TY medium and grown for 72 h with shaking at 37 °C. Induction of the expression system was not necessary, because leakage of the lacUV5 promoter results in a low level of constitutive T7 polymerase expression, as described previously [24]. A freezing-thawing step lysed the cells, and the CFE was subjected to a heat incubation of 60 min at 70 °C, which denatured most of the *E. coli* host proteins. After overnight incubation at 4 °C, the majority of the denatured proteins were precipitated, leaving a clear CFE. Microplates, containing either 200 μl of L buffer [100 mM lactose in 150 mM sodium citrate (pH 5.0)] or 200 μl of LG buffer (L buffer containing 10 mM glucose) per well, were covered to prevent evaporation and preheated in a 70 °C water-bath for 15 min. In an insulated microplate holder, 5 μl of CFE was transferred to the plates containing L buffer and LG buffer. The plates were covered, manually inverted for mixing and placed in a 70 °C waterbath. Hydrolysis was stopped after 60 min by placing the microplates in a water/ice mixture. The liberated glucose was measured by transferring 5 μl of the hydrolysis reaction to 200 μl of glucose oxidase-phenol 4-aminophenazone peroxidase (GOD-PAP; Roche, Basel, Switzerland) glucose detection mixture in 96-well microplates. After 30 min incubation at 20 °C, the developed colour was measured at 492 nm in a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The amount of glucose liberated was calculated from a calibration curve. The accuracy of the GOD-PAP kit allowed for a reliable calculation of the liberated glucose in the presence of 10 mM glucose.

Initial characterization of high-performance hybrids

For a more detailed analysis, hybrids were grown overnight in 15 ml of selective TY medium with shaking at 37 °C. At D_{600} = 1.0, isopropyl β-D-thiogalactoside (IPTG) was added to a final

concentration of 0.5 mM. Cells were harvested and resuspended in 0.75 ml of 150 mM sodium citrate (pH 5.0). Cells were lysed by sonication and CFEs were incubated at 70 °C for 60 min. This yielded at least 90 % pure hybrid β-glycosidase after centrifugation at 13000 ***g*** for 30 min, as determined by SDS/PAGE. The hybrids were tested for their lactose-hydrolysing activity and thermostability. For the lactose-hydrolysing activity, samples (0.25 μg of enzyme) were added to 0.5 ml of either preheated 100 mM or 300 mM lactose in 150 mM sodium citrate (pH 5.0) and incubated at 70 °C for 15 min. Glucose was detected with the GOD-PAP kit. Thermostability of the hybrids was tested by incubating 50 μl of 50 μg/ml enzyme solution at 70 °C for 1.5 h and measuring the residual activity, which was divided by the initial activity to give the inactivation rate.

Purification high-performance hybrids

E. coli JM109(DE3) clones harbouring pLUW511, pWUR6 or pET9d derivatives with hybrid genes were grown overnight in 125 ml of selective TY medium with shaking at 37 °C. Prior to harvesting, the cultures were induced for 4 h by the addition of IPTG to a final concentration of 0.5 mM. Cells were collected by centrifugation at 5400 ***g*** for 10 min, resuspended in 150 mM sodium citrate buffer (pH 5.0) and lysed by sonication (Sonifier B12; Branson, Danbury, CT, U.S.A.). *E. coli* proteins were denatured by a heat incubation at 70 °C for 1.5 h and pelleted by centrifugation at 45000 ***g*** for 30 min. $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 1.0 M. After passage through a 0.2 μm filter (Schleicher & Schuell, Dassel, Germany), the supernatant was loaded on to a phenyl-Superose column coupled to an Äkta FPLC (Amersham Biosciences, Uppsala, Sweden), equilibrated with 20 mM Tris/HCl (pH 8.0) containing 1.0 M $(NH_4)_2SO_4$. Proteins were eluted by a linearly decreasing $(NH_4)_2SO_4$ gradient (1.0–0 M), and were eluted from the column at approx. 0.2 M $(NH_4)_2SO_4$. Active fractions were pooled and dialysed against 20 mM sodium phosphate buffer (pH 7.5). Proteins were pure as judged by SDS/PAGE. Absence of contaminating DNA was verified in wavelength scans in the range of 250–300 nm. Protein concentrations were determined by measuring the absorption at 280 nm, in which the calculated specific ϵ_{280} values of 128280 $M^{-1} \cdot cm^{-1}$ and 140370 $M^{-1} \cdot cm^{-1}$ were used for CelB and LacS respectively ([28], but see [28a]).

Enzyme activity assays

Enzymes were tested for the hydrolysis of lactose, cellobiose, *p*-nitrophenyl-β-galactopyranoside (pNp-Gal) and *p*-nitrophenyl-β-glucopyranoside (pNp-Glc) at 70 °C by discontinuous assays, as described previously [29]. Under the used conditions, the ϵ_{405} of pNp was determined as 15.8 $mM^{-1} \cdot cm^{-1}$. Kinetic data were obtained by fitting the data to the Michaelis–Menten equation using the non-linear regression program Tablecurve 2D (Jandel Scientific Software, San Rafael, CA, U.S.A.). Inhibition constants were determined for glucose and the thermostable transition-state analogue (5*R*,6*R*,7*S*,8*S*)-5-(hydroxymethyl)-2-phenyl-5,6,7,8-tetrahydroimidazol[1,2-a]pyridine-6,7,8-triol (PheImGlc; generously supplied by Dr Andrea Vasella, Eidgenossische Technische Hochschule, Zurich, Switzerland) [30] with pNp-Gal as a substrate, as described previously [31]. However, after the apparent K_m values were determined, K_i values were determined using a Dixon plot.

Stability studies

Half-life values of thermal inactivation of parent and hybrid enzymes were determined at 92 °C in 20 mM sodium phosphate buffer (pH 7.5) using 50 μg/ml enzyme, as described previously [29]. Optimal temperatures for catalysis were determined by measuring specific activities at 60, 70, 75, 80, 85, 90, 95, 100, 105 and 107 °C for the hydrolysis of 20 mM pNp-Gal in 150 mM sodium citrate buffer (pH 5.0). Below 90 °C, aliquots of 0.5 ml of buffer with substrate were preheated for 5 min in a water bath before addition of 5 μl of enzyme solution. After 5 min, the incubations were stopped by addition of 1 ml of 0.5 M Na_2CO_3. At 90 °C and above, 250 μl of buffer containing substrate was preheated in 1 ml glass-stoppered HPLC vials in a silicon oil bath for 5 min. Reactions were started by addition of 5 μl of enzyme solution and terminated by addition of 0.5 ml of 0.5 M Na_2CO_3. Liberated nitrophenol was measured at 405 nm using a spectrophotometer (Hitachi, Amsterdam, The Netherlands). The melting temperature (T_m) of wild-type and hybrid enzymes was determined by differential scanning calorimetry in a VP-DCS micro calorimeter (MicroCal, Northampton, MA, U.S.A.), as described previously [31].

Molecular analysis and homology modelling

The DNA sequence of hybrid genes was determined using the Thermo Sequenase kit (Amersham Biosciences) with IR-labelled primers (MWG, Ebersberg, Germany) and subsequent analysis on an automated sequencer (LiCor, Lincoln, NE, U.S.A.). 3D tetrameric models of the hybrid enzymes were obtained by molecular replacement using the crystal structures of CelB [20] and LacS (Protein Database entry 1GOW) as search models by SWISS-MODEL [32].

Construction of CelB Ser^{14} → Ala (S14A) mutant

Using pLUW511 as a template, a S14A substitution was introduced in the *celB* gene via the QuickChange Site-Directed Mutagenesis Kit (Stratagene) using primers BG996 (sense: 5′-TATTCTTGGgCTGGTTTCCAG-3′) and BG997 (antisense: 5′-CTGGAAACCAGcCCAAGAATA-3′; where the introduced mutation in the primers is in lower case). The sequence with the introduced mutation was verified by DNA sequence analysis (see the subsection above). CelB S14A protein was produced and purified as described for the wild-type CelB in the subsection above.

RESULTS

Isolation of high-performance hybrids

After the DNA shuffling procedure, hybrid PCR fragments were ligated into pET9d and transformed into *E. coli* JM109(DE3). Approx. 10000 colonies were screened for X-Gal-hydrolysing activity on TY agar plates at 37 °C. Approx. 20 % of the colonies had a blue phenotype, indicating functional β-galactosidase expression. At this level, the variety in functional hybrid enzymes was visible by the differences in the intensity of the blue coloration between individual colonies. The enzymes were then tested for (i) thermostability at 70 °C; (ii) lactose-hydrolysing capacity; and (iii) inhibition by glucose. The heat incubation inactivated a significant number of the variants, indicating the presence of functional but thermolabile hybrids. One of these instable variants, hybrid 1, was studied in more detail with respect to its stability and, after sequencing, a 3D model was constructed by homology modelling (see below). In each of the two subsequent screening rounds, 10 % of the hybrids were selected for re-screening, which finally yielded 20 thermostable hybrid enzymes with improved lactose-hydrolysing activity and/or a reduced inhibition by glucose. These variants were partially purified and tested at 70 °C for their activity on lactose, glucose inhibition

Table 1 Characterization of high-performance hybrids: lactose hydrolysis at 70 °C and thermostability

Residual activity is expressed as a percentage of the initial activity before incubation at 70 °C. ND, not determined.

Hybrid	Lactose-hydrolysing activity (units/mg)		Residual activity after 1.5 h at 70 °C (%)
	100 mM	300 mM	
2	333	540	128
3	157	396	3
4	261	562	89
5	308	456	140
6	384	553	138
7	166	310	2
8	378	540	134
9	385	549	116
10	782	1119	107
11	554	926	117
12	604	959	128
13	545	960	130
14	804	941	139
15	588	949	125
16	619	996	114
17	624	948	121
18	728	1146	139
19	32	73	3
20	727	1089	110
21	637	902	132
CelB	400	581	ND
LacS	223	263	ND

and stability. This more detailed characterization revealed that 11 hybrids showed significantly higher lactose-hydrolysing activity at 70 °C compared with the parental enzymes (Table 1). Remarkably, all of these hybrids were specifically selected with primers for an N-terminal LacS and a C-terminal CelB sequence. Hybrid 18 was the most active variant, whereas hybrids 11 and 20 represented high-performance hybrids with a relatively low (hybrid 11) or average (hybrid 20) activity. These three hybrids were selected for further analysis.

Kinetic parameters

To obtain more insight into their altered biochemical properties, the hybrid enzymes 11, 18 and 20 were purified to homogeneity together with the CelB and LacS parental enzymes and characterized. The kinetic parameters of the purified enzymes were determined at 70 °C for the hydrolysis of lactose, cellobiose and their respective chromogenic analogues pNp-Gal and pNp-Glc (Tables 2 and 3). At 70 °C, CelB was a little more active on the galactosides lactose and pNp-Gal than on pNp-Glc, whereas the efficiency of cellobiose hydrolysis was relatively low. With doubled activities on galactosides compared with glucosides, LacS displayed a more pronounced β-galactosidase activity than CelB, as reported previously [11]. LacS was less active than the other enzymes on all tested substrates, but had the highest affinity for all substrates, except cellobiose. Compared with LacS, CelB was approx. twice as active on all substrates, however, with equally augmented Michaelis–Menten constants. The sub-

Table 2 Kinetic parameters of wild-type (CelB and LacS) and hybrid enzymes for the hydrolysis of lactose and cellobiose at 70 °C

In the assays, 0.1 μg of enzyme was used in 150 mM sodium citrate (pH 5.0).

Enzyme	Substrate: Lactose			Substrate: Cellobiose*			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	$K_{m(lactose)}/K_{m(cellobiose)}$
CelB	59.8 ± 12.1	286 ± 15	4.78	3.2 ± 2.6	37.6 ± 3.2	11.8	18.7
LacS	37.9 ± 17.3	121 ± 13	3.19	13.5 ± 8.4	23.2 ± 2.9	1.72	2.81
Hybrid 11	71.8 ± 10.2	442 ± 18	6.16	11.8 ± 3.9	42.5 ± 2.7	3.60	6.08
Hybrid 18	88.6 ± 8.4	1022 ± 32	11.5	4.6 ± 1.8	264 ± 13	57.4	19.3
Hybrid 20	59.5 ± 5.5	447 ± 11	7.51	12.1 ± 2.4	60.1 ± 2.3	4.97	4.92

* CelB showed typical substrate inhibition on cellobiose.

Table 3 Kinetic parameters of wild-type (CelB and LacS) and hybrid enzymes for the hydrolysis of pNp-Gal and pNp-Glc at 70 °C

In the assays, 0.1 μg of enzyme was used in 150 mM sodium citrate (pH 5.0).

Enzyme	Substrate: pNp-Gal			Substrate: pNp-Glc*			Glucose†	PheImGlc†
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	K_i (mM)	K_i (nM)
CelB	2.60 ± 0.26	298 ± 9	115	0.80 ± 0.11	206 ± 11	258	43	9.3
LacS	1.57 ± 0.21	165 ± 6	105	0.12 ± 0.02	45 ± 2	375	46	8.4
Hybrid 11	3.99 ± 0.35	327 ± 9	82.0	0.71 ± 0.29	235 ± 28	331	110	5.9
Hybrid 18	5.63 ± 0.51	743 ± 13	132	0.58 ± 0.09	440 ± 21	759	88	12
Hybrid 20	6.58 ± 0.85	412 ± 20	62.6	0.75 ± 0.13	276 ± 17	368	80	13

* CelB and hybrids 18 and 20 showed typical substrate inhibition on pNp-Glc at concentrations over 3 mM (CelB) and 4 mM (hybrids 18 and 20). Parameters have been determined using activities obtained at lower substrate concentrations.

† Determined with pNp-Gal as a substrate.

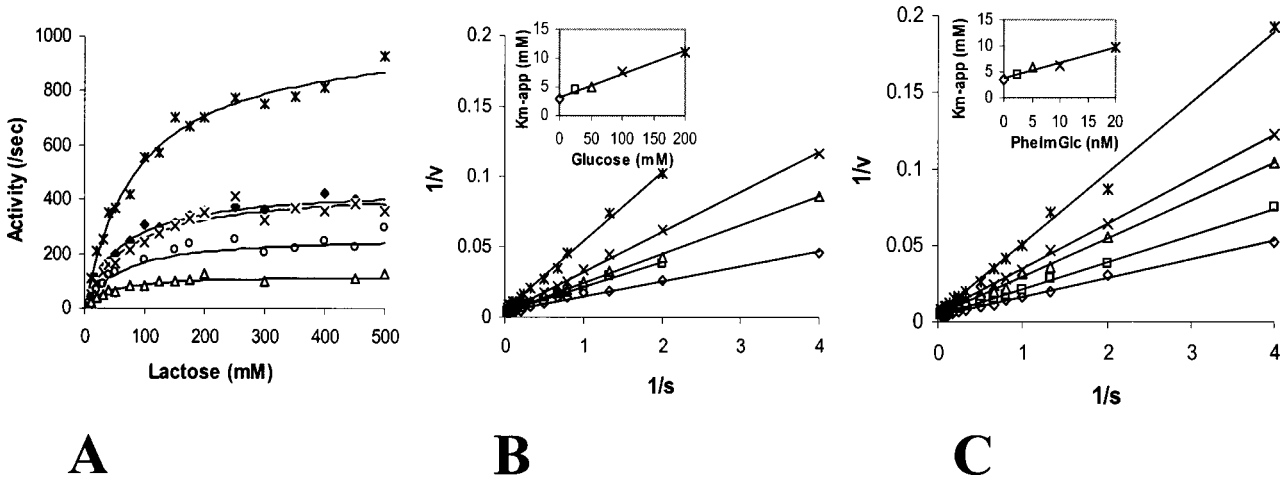

Figure 1 Michaelis–Menten and Dixon plots of hybrid enzymes

(**A**) Michaelis–Menten kinetics of CelB (○), LacS (△), hybrids 11 (×), 18 (✳) and 20 (◆) for the hydrolysis of lactose. (**B**) Inhibition of hybrid 20 by various glucose concentrations [0 (◇), 25 mM (□), 50 mM (△), 100 mM (×) and 200 mM (✳) glucose]. (**C**) Inhibition of hybrid 20 by various concentrations [0 (◇), 2.5 nM (□), 5 nM (Δ), 10 nM (×) and 20 nM (✳)] of the transition-state analogue PheImGlc. The reciprocal substrate (s) concentration is plotted against the activity (v). Insets in (**B**) and (**C**) show the dependence of the K_m for glucose and PheImGlc respectively.

strate affinities of the hybrids were similar to those of CelB. Hybrids 11, 18 and 20 had highest activity for lactose hydrolysis, which was the substrate used in the screening. The efficiency of lactose hydrolysis was approx. 1.5–3.5 and 3.6–8.6-fold higher than that of CelB and LacS respectively (Figure 1A). All hybrids showed increased turnover rates (k_{cat}) on cellobiose and chromogenic substrates, but only hybrid 18 had a higher efficiency for their hydrolysis (k_{cat}/K_m) compared with CelB and LacS. CelB was inhibited at higher concentrations of the substrates cellobiose and pNp-Glc. Hybrid 20 showed similar inhibition, but only with pNp-Glc. All other substrates were hydrolysed according to Michaelis–Menten kinetics.

Inhibition by glucose and PheImGlc

The inhibition constants of glucose and the thermostable transition-state analogue PheImGlc on the hydrolytic activity of CelB, LacS and hybrids 11, 18 and 20 were determined (Table 3). pNp-Gal was chosen as a substrate, since no substrate inhibition was observed for the hydrolysis of this compound with any of the tested enzymes. The inhibiting effect of glucose on the activity of CelB and LacS was similar; the inhibition constants for glucose were comparable, whereas the three high-performance hybrids were approx. 2-fold less sensitive for glucose inhibition (Figure 1B). This indicates that the affinity for glucose in the ground-state conformation has been diminished similarly in all hybrids with respect to LacS and CelB. PheImGlc is a glucose derivative that has a flat geometry at the anomeric C1 saccharide atom and, as such, is believed to resemble the transition-state in the glycoside hydrolysis reaction in retaining β-glycosidases [30]. For all enzymes, the strong inhibition by PheImGlc followed kinetics for competitive inhibition (Figure 1C). The inhibition constants for CelB and LacS were similar. The K_i value determined for hybrid 11 was slightly lower than that for CelB and LacS, whereas the K_i value for hybrids 18 and 20 was approx. 2-fold higher than the value of hybrid 11. This means that the active sites of the enzymes have subtle differences in structure, which affect the stabilization of the transition state of the reaction.

Table 4 Half-lives at 92 °C, $T_{opt(catalysis)}$ and T_m of wild-type (CelB and LacS) and hybrid enzymes

ND, not determined.

Enzyme	Half life at 92 °C (min)	$T_{opt(catalysis)}$ (°C)	T_m (°C)
CelB	⩾ 100	104	106.0
LacS	< 3	95	ND
Hybrid 1	30*	80	80.7
Hybrid 11	7	85	94.0
Hybrid 18	8	96	94.5
Hybrid 20	100	93	101.8

* Determined at 80 °C.

Stability

Hybrids 11, 18 and 20 were compared with CelB and LacS with respect to their thermal-inactivation rate, T_{opt} for catalysis [$T_{opt(catalysis)}$] and T_m (Table 4). The different analysis methods display approximately similar trends for the enzyme variants. Hybrid 1 was the least stable variant, whereas the stability of hybrids 11, 18 and 20 were intermediate to that of CelB and LacS. Hybrid 20 was the most stable hybrid with respect to the inactivation rate and T_m, whereas hybrid 18 had a slightly higher $T_{opt(catalysis)}$ (Figure 2). Remarkably, LacS was readily inactivated at 92 °C, whereas it displayed a broad $T_{opt(catalysis)}$, with a peak at approx. 95 °C. For CelB and hybrids 1 and 18, the determined optimal temperature for catalysis was very close to the determined T_m. For hybrids 11 and 20, however, the $T_{opt(catalysis)}$ value was almost 10 °C lower than the determined T_m value. This might suggest different inactivation patterns between the hybrids. It was not possible to determine a T_m value for LacS. This is most probably due to the fact that the unfolding of LacS does not involve two states, but rather occurs via a stable intermediate, which has been identified during guanidine hydrochloride-induced unfolding of LacS ([33], and T. Kaper and J. Van der Oost, unpublished work). In previous differential scanning calorimetric experiments with LacS [34], a two-state unfolding was observed, but these experiments were performed in 10 mM

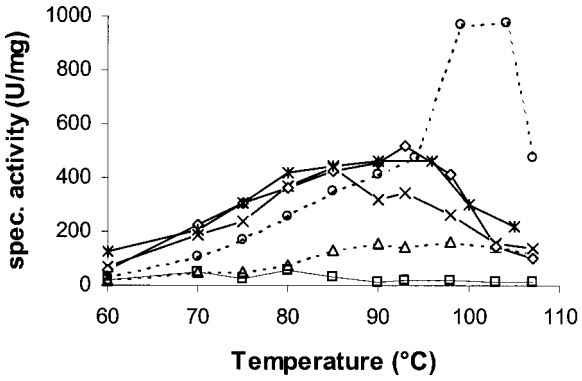

Figure 2 Typical influence of temperature on the activity of CelB, LacS and hybrids 1, 11, 18 and 20

Reactions were performed in 250 μl of 20 mM pNp-Gal in 150 mM sodium citrate (pH 5.0) with 0.05 μg of enzyme [CelB (○), LacS (△), hybrids 1 (□), 11 (×), 18 (✳) and 20 (◇)]. Reactions were incubated for 10 min at 60 and 70 °C, 5 min at 75–85 °C and 3 min at 90–107 °C. One unit equals the hydrolysis of 1 μmol of pNp-Gal/min.

3-(cyclohexylamino)propanesulphonic acid ('CAPS') buffer at pH 10. Under those conditions, the T_m of LacS was 86.3 °C.

Structural analysis of hybrids

The primary structure of several hybrids was determined by DNA sequence analysis. All hybrids were the result of a single crossover event in different regions with 5–22 bases with identical DNA sequence (Figure 3). All sequenced hybrids contained mainly the CelB amino acid sequence in which the C- or N-terminus had been substituted with the LacS sequence (Figures 3 and 4A). Starting at the N-terminus, hybrids 1 and 7 have 419 and 413 amino acids derived from CelB, followed by 55 and 61 residues of LacS until the C-terminus respectively (Figure 3A). Hybrid 9 was identical with wild-type CelB. Hybrids 11 and 20 were very similar. Both proteins start with a LacS sequence of approx. 40 residues, followed by the CelB sequence until the C-terminus. The only differences between hybrids 11 and 20 are the residues at position 43 and 45 (Figure 3B). Hybrid 11 has LacS residues at these positions, a methionine and an alanine residue respectively, whereas hybrid 20, like CelB, has an isoleucine and a serine residue at these positions. Hybrid 14 was found to be identical with hybrid 20, whereas hybrid 18 was identical with LacS until residue 33, except for a point mutation that resulted in $Thr^{23} \rightarrow Pro$, followed by 441 residues of the CelB sequence (Figure 3B). The *hybrid 19* gene consisted of 68 nucleotides of LacS sequence followed by the complete wild-type *celB* gene sequence.

To obtain insight into the structural characteristics of the hybrids, 3D models were constructed of hybrids 1, 11, 18 and 20 by homology modelling using the available crystal structures of CelB [20] and LacS [22] (Figure 4). Although the resolutions of the CelB and LacS structures (3.3 and 2.6 Å resolution respectively, where 1 Å = 0.1 nm) do not allow for a detailed analysis of the exact positions of the amino acid side chains, they do provide a basis for the interpretation of the structural rearrangements in the chimaeric enzymes. In hybrid 1, the LacS sequence replaced the CelB–CelB intersubunit contacts almost completely at the small subunit interface of the tetramer (Figure 4B). The 55 amino acid residues of the LacS C-terminus are 55% identical with the C-terminus of CelB. The LacS sequence is two residues longer than in CelB and fills up the cavity that is present in the centre of CelB at the tetrameric subunit interface. The C-terminus is involved in an elaborate ion-pair network in LacS, where the penultimate arginine residue reaches across and participates in a 16-residue ion-pair network

A

```
CelB   387 VSHLKAVYNAMKEGADVRGYLHWSLTDNYEWAQGFRMRFGLVYVD 432
LacS   402 VSHVYQVHRAINSGADVRGYLHWSLADNYEWASGFSMRFGLLKVD 447
1      387 VSHLKAVYNAMKEGADVRGYLHWSLTDNYEWAQGFRMRFGLVYVD 432
7      387 VSHLKAVYNAMKEGADVRGYLHWSLTDNYEWAQGFRMRFGLVYVD 432
           ***   *  *   ************ ****** ** *****  **

CelB   433 FETKKRYLRPSALVFREIATQKEIPEELAHLADLKFVT--RK    472
LacS   448 YNTKRLYWRPSALVYREIATNGAITDEIEHLNSVPPVKPLRH    489
1      433 YNTKRLYWRPSALVYREIATNGAITDEIEHLNSVPPVKPLRH    474
7      433 YNTKRLYWRPSALVYREIATNGAITDEIEHLNSVPPVKPLRH    474
             **  * ****** *****   *  *  **     *   *
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B

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CelB   M-KFPKNFMFGYSWSGFQFEMGLPGSE-VESDWWVWVHDKENIASGLVSGDLPENGPA 56
LacS   MYSFPNSFRFGWSQAGFQSEMGTPGSEDPNTDWYKWVHDPENMAAGLVSGDLPENGPG 58
11     MYSFPNSFRFGWSQAGFQSEMGTPGSEDPNTDWYKWVHDPENMAAGLVSGDLPENGPA 58
18     MYSFPNSFRFGWSQAGFQSEMGPPGSEDPNTDWWVWVHDKENIASGLVSGDLPENGPA 58
14/20  MYSFPNSFRFGWSQAGFQSEMGTPGSEDPNTDWYKWVHDPENIASGLVSGDLPENGPA 58
       *  **  * ** *  *** *** ****    **  ****  * * ************
                                                  ⇑ ⇑
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Figure 3 N- and C-terminal amino acid sequences of CelB, LacS and hybrids

(**A**) C-terminal amino acid sequence of CelB, LacS and hybrids 1 and 7. (**B**) N-terminal sequences of CelB, LacS and hybrids 11, 18 and 14/20. CelB sequence is underlined, whereas the corresponding regions of nucleotide sequence identity, where crossovers took place, are indicated in bold and italics. The two residues in which hybrids 11 and 20 differ are indicated by arrows. * indicates sequence identity. For reasons of clarity, the alanine residue at position 1a in the sequences is not shown.

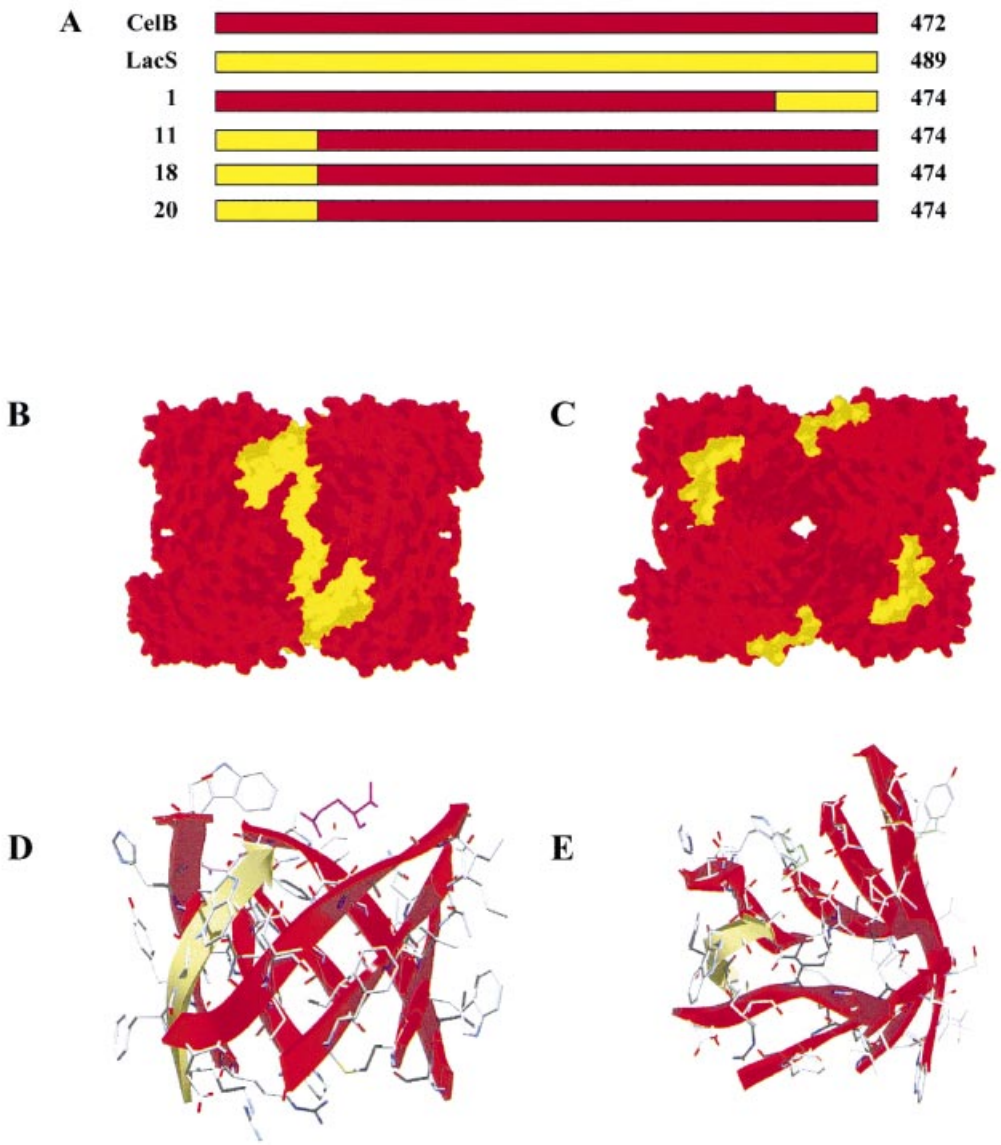

Figure 4 Structural representation of hybrids

(**A**) Schematic representation of primary structures of CelB, LacS, hybrids 1, 11, 18 and 20. Surface plot of tetrameric hybrid 1 (**B**) and tetrameric hybrid 20 (**C**). Side view (**D**) and top view (**E**) of hybrid 20 $(\beta\alpha)_8$-barrel. Images were generated using Swiss-PDB viewer [48] and visualized using POV-Ray for Windows (Persistence of Vision Development Team, Indianapolis, IN, U.S.A.).

that bridges the four subunits [22]. In general, ion-pairs have been shown to be involved in stabilization of proteins against high temperatures [35]. In the C-terminus of CelB, several charged residues are located so that they are possibly involved in an ion-pair, but align with non-charged residues in LacS and *vice versa*. Therefore, in hybrid 1, these possible ion-pairs can not be formed. Interestingly, the ion partner of the penultimate arginine residue in LacS, Glu^{345}, aligns with Glu^{330} in CelB, which could indicate a LacS-like C-terminal organization in hybrid 1. However, the changes in hydrophobic interactions and hydrogen bridges cannot be evaluated with these models. Overall, the loss in thermostability can be interpreted as an incompatibility of LacS and CelB in the structural stabilization of the C-terminus and the subunit interface. The active site in hybrid 1 is equal to that of CelB. This could explain the catalytic profile of hybrid 1, which is similar to that of CelB (results not shown).

In contrast with hybrid 1, hybrids 11, 18 and 20 contain LacS sequences that completely traverse the subunit of the protein (Figure 4C). A close-up of the catalytic $(\beta\alpha)_8$-barrel visualizes that in both variants the first β-strand of the CelB barrel has been replaced by its LacS counterpart (Figures 4D and 4E). CelB and LacS are 51 % identical in the first 43 and 45 amino acid residues of the protein (Figure 3B). In LacS, the N-terminus is tightly bound to the protein body by an ion–ion interaction of the α-amino group with the side chain of Asp^{473} [22]. Such an interaction is not possible in the hybrids, where an alanine (Ala^{1a}) has been introduced after the N-terminal methionine residue to enable a translational fusion of the *lacS* gene to the T7 promoter in a *Nco*I restriction site. In CelB, however, the

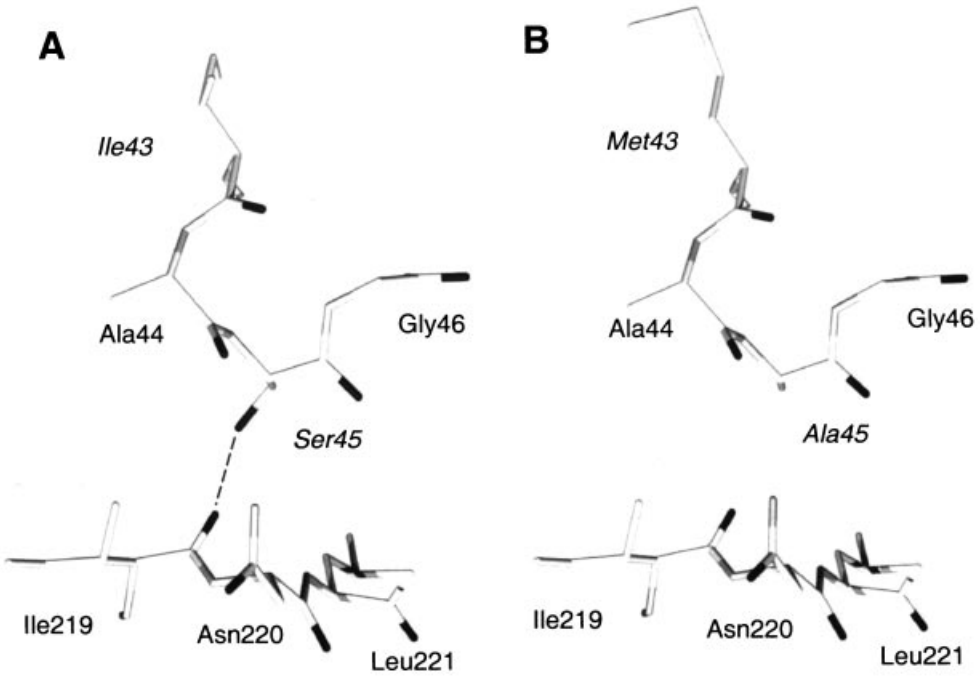

Figure 5 Modelled subunit interface interactions in hybrids 20 (A) and 11 (B) around residue 45

Images were generated using Swiss-PDB viewer [48] and visualized using POV-Ray for Windows (Persistence of Vision Development Team, Indianapolis, IN, U.S.A.).

α-amino group does not appear to make any ionic interactions. The side chain of Lys^{2} could form an ion pair with Glu^{455} in CelB. The interactions of the charged residues in the LacS stretch in hybrids 11, 18 and 20 appear to be similar to those in wild-type LacS, which could explain the relatively high stability of the hybrids.

Hybrids 11 and 20 differ only at two positions, Met^{43} and Ala^{45} in hybrid 11 and Ile^{43} and Ser^{45} in hybrid 20. Since we measured a significant higher stability for hybrid 20, this must solely result from the effects of the two different residues. Interestingly, both residues were located at the larger subunit interface of the glycosidase tetramer. Earlier studies [12] have shown that quartenary interactions contribute largely to the stability of proteins. These intersubunit interactions can be hydrophobic interactions, salt-bridges or hydrogen bonds. In modelling studies, the interactions of Met^{43} and Ile^{43} in hybrids 11 and 20 respectively, appeared to be very similar. However, an extra possible hydrogen bond was identified in hybrid 20, which involved the side chain of Ser^{45} and the backbone amide atom of Leu^{221}, located at the adjacent subunit (Figure 5A). Since hybrid 11 has an alanine at position 45, a similar interaction was absent in this variant (Figure 5B). In hybrid 18, the residues at positions 43 and 45 were the same as in hybrid 20. Surprisingly, it was significantly less stable than hybrid 20. The point mutation in hybrid 18, which resulted in $Thr^{23} \rightarrow Pro$, caused a small shift in the position of the N-terminal peptide chain in the 3D model compared with that in hybrid 20. This shift could have a destabilizing effect on the interactions that 23 N-terminal residues have with the rest of the protein and this can explain the lower stability of hybrid 18.

Throughout family 1 glycoside hydrolases, the residues that line the active site have been highly conserved [20]. Therefore CelB and LacS share a nearly identical active-site topology. However, Ser^{14} in CelB is a serine in half of the known family 1 sequences, whereas an alanine is present in the other sequences, including LacS (Ala^{15}). The hydroxy group of this serine side chain could possibly form a hydrogen bond with the residue at the non-reducing end of a hexose substrate in the active site. Overlay studies with the structure of the 6-phospho-β-galactosidase LacG from *Lactococcus lactis* with a bound galactose-6-phosphate in the active site [36] showed that Ser^{14} might interact with the 3-hydroxy of galactose or glucose, which have an identical orientation at this position. To investigate whether this residue was involved in substrate inhibition, Ser^{14} was substituted by an alanine by site-directed mutagenesis, resulting in mutant CelB S14A. However, this mutant was inhibited similarly by glucose as CelB and LacS and resembled CelB in substrate specificity and activity (results not shown).

DISCUSSION

In the present study, we have succeeded in generating thermostable hybrid β-glycosidases and isolating high-performance variants. A library was constructed by shuffling of the genes encoding the hyperthermostable family 1 glycoside hydrolases CelB of *P. furiosus* and LacS from *S. solfataricus*. This is the first example of improving extremely thermostable enzymes by a DNA family shuffling approach. The β-glycosidase-encoding genes used in this study are 56 % identical, which is relatively low compared with other shuffling studies in which 60–90 % identical genes were used [2,37–39]. However, the combination of hybrid primer sets with extremely low annealing temperatures in the reassembly procedure did lead to the formation of a variety of hybrid genes (Figure 3).

Previously [10], one of the parental enzymes in this study, the pyrococcal CelB, has been optimized for catalysis at 20 °C by random mutagenesis in combination with DNA shuffling. It was demonstrated that variants, which were selected for increased

activity with pNp-Glc at room temperature, were not equally more active on the β-(1,4)-linked glucose disaccharide cellobiose [10]. In contrast, the three high-performance hybrids that were selected in the present study for an improved hydrolysis of lactose also had increased turnover rates for hydrolysis of cellobiose, pNp-Glc and pNp-Gal (Tables 2 and 3). However, only hybrid 18 was more efficient than the wild-type enzymes in hydrolysis of all four tested substrates. This demonstrates the selectivity of the screening procedure: 'You get what you screen for' [40]. In the hydrolysis of pNp-Gal, the three selected hybrids were inhibited less by glucose compared with the parental enzymes (Table 3), which suggests a correlation with the increased turnover rates. Judging from the increased K_m values for the hydrolysis of pNp-Gal, the affinity for the Michaelis–Menten complex has been reduced in the hybrids with respect to the parental enzymes, which correlates with the reduced glucose inhibition. Therefore the increase in k_{cat} might result from a less favourable binding of the ground state of the substrate, which would lower the activation energy to reach the transition state of the reaction [41]. On the other hand, the relation between inhibition by the transition-state analogue PheImGlc and the activity of the enzymes seems less clear, since individual hybrids were inhibited either slightly more (hybrid 11) or less (hybrids 18 and 20) compared with the parental enzymes (Table 3). When the hybrids are compared, the higher affinity of hybrid 11 for PheImGlc correlated with the observed affinity for hydrolysis of pNp-Gal.

The three high-performance hybrids resulted from single crossover events near the N-terminus of the protein. On the basis of the determined sequences of hybrids 1, 11, 18 and 20, 3D structural models were constructed using the available structures of CelB [20] and LacS [22]. The hybrids are near-identical in sequence and consequently in structure, which means that structural explanations for substrate specificity have to result from differences between residues at positions 34–45 in the hybrids (Figure 3). CelB has a more pronounced β-glucosidase character than LacS for the hydrolysis of disaccharides (Table 2). Interestingly, hybrid 18 has the shortest LacS sequence (residues 1–33) and resembles CelB more when the ratio of the affinity for lactose and cellobiose (K_m) is considered, whereas hybrids 11 and 20 have more LacS-like specificities (Table 2). The peptide stretch from residue 33–45 is the determining factor in the substrate specificity for the hydroxy group at C4 of the sugar residue at the non-reducing end. However, such specificity correlation cannot be extended to the hydrolysis of chromogenic substrates, since these substrates have a different leaving group. For hydrolysis of disaccharides, the initial breaking of the glycosidic bond is most critical for the reaction rate, due to the high pK_a value of the leaving glucose (i.e. 12.4 [42]) [43]. This requires considerable distortion of the non-reducing sugar residue [44]. Since nitrophenol has a good leaving-group ability (pK_a = 7.2) [43], the rate-limiting step could change from glycosylation to deglycosylation of the enzyme. However, this depends on the substrate and makes it impossible to compare the kinetic data of the hydrolysis of pNp substrates in a similar fashion as for lactose and cellobiose. Interestingly, the residues at position 33–45 could be best described as third or fourth shell residues and are located distantly from the active site, but nevertheless appear to affect its structure.

The three hybrids have a stability that is between that of LacS and CelB, with hybrid 20 being the most stable variant (Table 4 and Figure 2). The loss in stability compared with CelB and increase in activity could be the result of increased flexibility, although this is not an absolute requirement for improved turnover numbers at lower temperatures, as has been found in other directed evolution studies [5,6]. The structural models were analysed with respect to the stability of the hybrids. The inferior stability of hybrid 1 results most probably from the loss of a relatively large number of stabilizing interactions at the C-terminus. The difference in stability between hybrids 11 and 20 is intriguing, since the two hybrids differ only in two residues. The 3D model shows four extra intersubunit hydrogen bridges in the hybrid 20 tetramer, which could explain the 7 °C difference in T_m between the two hybrids (Figure 5). The forces that counteract conformational entropy loss and result in protein folding have a net energy gain for folded proteins that equals only a few weak intermolecular interactions [45]. Therefore the introduction of four hydrogen bridges might result in such a large stability increase. Mutational studies of subunit interface interactions have dealt mainly with ion-pair interactions, but similar differences in T_m have been reported upon altering single residues at subunit interfaces [46,47].

The present study has demonstrated that DNA family shuffling is also applicable for directed evolution of highly thermostable enzymes with different mechanisms of stabilization. We have successfully shuffled the genes coding for the extremely thermostable β-glycosidases CelB from *P. furiosus* and LacS from *S. solfataricus*. Hybrid enzymes were selected for thermostability and improved catalytic properties for the hydrolysis of lactose at 70 °C. Composition of the hybrids showed that β-glycosidases from hyperthermophiles can tolerate significant foreign peptide stretches, while remaining very thermostable. These results have encouraged us to continue directed evolution of enzymes from extreme thermophilic origin. Laboratory evolution of enzymes remains the most promising strategy for improvement of enzymes, since the necessary structural changes for altered catalytic or stability features are too subtle to be predicted by rational design. Analysis of high-performance hybrids is likely to increase our understanding of protein stability and catalysis at elevated temperatures.

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