In vivo selection for the enhancement of Thermotoga maritima exopolygalacturonase activity at neutral pH and low temperature

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The aim of this study was to develop an Escherichia coli-based metabolic selection system for the uncovering of new oligogalacturonate-active enzymes. Based on the expression of the specific permease TogMNAB, this system enabled the entry of oligogalacturonates into the cytoplasm of E. coli thus providing a modified strain usable for this purpose. This tool was used for the metabolic selection of Thermotoga maritima exopolygalacturonase (TmGalU) mutants enabling the uptake of sodium trigalacturonate as the sole carbon source by the bacterium. In only one round of error-prone PCR and selection, mutants of TmGalU with a 4-fold increased turnover at pH 7.0 and 2-fold more active at 37°C than wild-type enzyme were isolated. These results show the versatility of this strain for the evolution of oligogalacturonate-active enzymes.

Keywords: directed evolution/exopolygalacturonase/metabolic selection/oligogalacturonates/Thermotoga maritima

Introduction

Pectic polymers are composed of an α-(1→4) linked D-galacturonic acid backbone and are the major components of the plant cell wall. They are depolymerized by specialized enzymes found in bacteria and fungi. For example, Erwinia chrysanthemi, an enterobacterium that is responsible for various plant diseases by destroying the integrity of the cell wall (Hugouvieux-Cotte-Pattat et al., 1996), secretes a powerful toolbox of pectin-degrading enzymes, including the endopECTIC lyases PeI, PeB, PeC, PeD, PeE, PeF, PeL and PeZ, the pectin acetyltransferases PaEX and PaEY, the pectin methylesterase PemA and the pectin esterase PehX (Kazemi-Pour et al., 2004). The resulting products of lyases are oligogalacturonates harboring an unsaturated moiety at their non-reducing ends. Polygalacturonases are involved in the hydrolysis of the α-(1→4) glycosidic bond. All these enzymes are of great industrial importance and are exploited today in several commercial enzymatic mixtures (Hoondal et al., 2002) and in industrial processes, such as fruit juice production or degumming and retting of fiber crops (Kashyap et al., 2001).

As part of our program for the uncovering of new enzymatic activities, we recently cloned, overexpressed and characterized a hyperthermostable exopolygalacturonase (exo-PG) from Thermotoga maritima (TmGalU, locus gene TM0437) (Parisot et al., 2003). Several characterized bacterial polygalacturonases have an exo-selectivity, usually releasing dimers. However, this oligogalacturonate-active enzyme exhibited an original exo-selectivity, releasing one unit of monogalacturonate. A half-life of several hours was observed at 90°C (pH 6.0), and the optimal activity was reached at 85°C (pH 6.0). However, the residual activity on sodium polygalacturonate at 37°C was <1% of the optimal activity. These properties reflect the ability of T. maritima to grow in an extreme environment (Nelson et al., 1999) and allow the prediction of a poor hydrolytic activity of TmGalU when expressed in a mesophilic host such as Escherichia coli.

The directed evolution of enzymes, which are the laboratory counterpart of Darwinian evolution, is a two-step process to explore and modify biocatalyst properties. The first step is the generation of variant gene libraries from a wild-type (WT) gene and the second one is the evaluation of the resulting libraries by a screening or selection system. Numerous methods for the creation of the genetic diversity have been developed (Neylon, 2004), such as error-prone PCR (EPP) (Leung et al., 1989) or DNA shuffling (Stemmer, 1994a, 1994b), and have proven to be very efficient. The second step is the recognized bottleneck of a directed evolution experiment. Indeed, the throughput of the screening should be as high as possible but it is often decreased by technical limitations, despite the use of robots. In vivo or metabolic selection is usually considered more efficient for screening a large library (10^5–10^10 screenable variants), since only improved mutants survive the selection leading to the successful evolution of diverse enzymes (Taylor et al., 2001).

We report here the design of a metabolic selection system and demonstrate its functionality by the evolution of TmGalU in order to enhance its hydrolytic activity when expressed in E. coli. Using this metabolic selection, we were able, after only one cycle of EPP and selection, to isolate and characterize active mutants having thermal and pH characteristics different from the WT enzyme.

Materials and methods

The bacterial strains and plasmids used in this study are listed in Table I. Previously described plasmid pETpgTm [pET21d(+) derivative] (Parisot et al., 2003) was used to subclone the tngalu gene (1.3 kb) into the expression plasmid pACYCDuet-1 (Novagen) under the control of the T7-promoter and between the restriction sites NdeI and XhoI. This plasmid (pGalWT) contained chloramphenicol gene resistance. The plasmid pO604 (pTog, 10 kb) encoding for TogMNAB was a generous gift from Hugouvieux-Cotte-Pattat (Hugouvieux-Cotte-Pattat et al., 2001). This is a pBR322 derivative that confers resistance to ampicillin.
**Table I.** Plasmids and strains used in this study

<table>
<thead>
<tr>
<th>Plasmid/strain</th>
<th>Genotype/phenotype</th>
<th>Reference/origin</th>
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<tbody>
<tr>
<td>PET21(Δ+)</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, replication origin ColE1</td>
<td>Novagen</td>
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<tr>
<td>PACYCDuet-1</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, replication origin P15A</td>
<td>Novagen</td>
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<tr>
<td>PETpG7Tm</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, PET21Δ(+) derivative, (tmgalu)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Parisot et al. (2003)</td>
</tr>
<tr>
<td>PgalWT</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, PACYCDuet-1 derivative, (tmgalu)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>PBT7, pBT9, pB12</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, PACYCDuet-1 derivatives with mutant TmGalU genes</td>
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<tr>
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<td>Ap&lt;sup&gt;R&lt;/sup&gt;, PET21Δ(+) derivatives with mutant TmGalU genes</td>
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<tr>
<td>PO604 or pTog</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, pBR322 derivative, (togMNB)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Hugouvieux-Cotte-Pattat et al. (2001)</td>
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<tr>
<td>DHR5a</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; g80lacZΔM15 Δ((lacZYA-argF)U169 recA1 endA1 hisD17(r&lt;sub&gt;K&lt;/sub&gt;, m&lt;sub&gt;6&lt;/sub&gt;) phoA supE4 thy-1 gyrA96 relA1 λ&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Invitrogen</td>
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<td>Bl21(DE3)</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; dcm ompT hsdR&lt;sub&gt;1&lt;/sub&gt;(m&lt;sub&gt;6&lt;/sub&gt;, m&lt;sub&gt;15&lt;/sub&gt;) gal (DE3)</td>
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<td>HMS174(DE3)</td>
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<td>Novablue(DE3)</td>
<td>endA1 hisD17(r&lt;sub&gt;K&lt;/sub&gt;, m&lt;sub&gt;6&lt;/mub&gt;) supE4 thy-1 recA1 gyrA96 relA1 lac F&lt;sup&gt;+&lt;/sup&gt; proA&lt;sup&gt;B&lt;/sup&gt; B&lt;sup&gt;+&lt;/sup&gt; lacPΔAM15::Tn10 (C&lt;sup&gt;60&lt;/sup&gt;) (DE3)</td>
<td>Novagen</td>
</tr>
<tr>
<td>NM522</td>
<td>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM5) Δ(r&lt;sub&gt;K&lt;/sub&gt;, m&lt;sub&gt;6&lt;/mub&gt;) [F&lt;sup&gt;+&lt;/sup&gt; proAB lacFΔZ8M15]</td>
<td>Hugouvieux-Cotte-Pattat et al. (2001)</td>
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The E. coli strains used [Bl21(DE3), Novablue(DE3), Tuner(DE3), HMS174(DE3)] were purchased from Novagen.

**Media and growth conditions**

Cells were grown in Luria–Bertani (LB), SOB, SOC, M9 or Davis medium. Agar plates of these media were obtained by adding agar to a final concentration of 1.5 g l<sup>−1</sup>. M9 minimal medium (MM) was composed of Na<sub>2</sub>HPO<sub>4</sub> (6 g l<sup>−1</sup>), KH<sub>2</sub>PO<sub>4</sub> (3 g l<sup>−1</sup>), NH<sub>4</sub>Cl (1 g l<sup>−1</sup>) and NaCl (0.5 g l<sup>−1</sup>), and pH was adjusted to 7.0 with KOH 1 M. Davis MM was composed of K<sub>2</sub>HPO<sub>4</sub> (5.34 g l<sup>−1</sup>), KH<sub>2</sub>PO<sub>4</sub> (2 g l<sup>−1</sup>), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1 g l<sup>−1</sup>) and sodium citrate trihydrate (0.5 g l<sup>−1</sup>). Then, pH was adjusted to 7.0 with KOH 1 M. MgSO<sub>4</sub> (10<sup>−3</sup> M) and thiamine (30 µg ml<sup>−1</sup>) were added to both MM after sterilization. CaCl<sub>2</sub> (10<sup>−4</sup> M) was added to M9 after sterilization when specified.

Antibiotics were added at the following concentrations: ampicillin (Ap), 100 µg ml<sup>−1</sup>; chloramphenicol (Cm), 20 µg ml<sup>−1</sup>. Carbon sources (D-glucose, sodium D-galacturonate and sodium trigalacturonate) were added to a final concentration of 5 g l<sup>−1</sup>. All chemical compounds were purchased from Sigma–Aldrich and sterilized on a 0.2 µm filter (Sartorius) when needed. Unsaturated sodium trigalacturonate was prepared as previously described (Parisot et al., 2002).

**Random mutagenesis**

Random mutations were introduced by mutagenic PCR. Universal primers T7P and T7T flanking the gene tmgalu of pGalWT were used and 20 pmol of each primer was mixed with 50 ng of plasmid pGalWT in a 50 µl PCR. The reaction conditions were as follows: 1 × Dynazyme buffer, 1 mM of dTTP and dCTP, 0.2 mM of dATP and dGTP, 7 mM MgCl<sub>2</sub> and 2.5 U of polymerase Dynazyme Ext (Finnzymes). The reaction was thermocycled as follows: 1 cycle at 92°C, 3 min, then 40 cycles at 92°C, 30 s; 50°C, 30 s; 72°C, 2 min. Mutagenized PCR products were extensively digested overnight at 37°C by the NdeI and XhoI restriction enzymes and cloned back into the pACYCDuet-1 vector digested by the same enzymes.

**Transformation of electrocompetent BL21(DE3)/pTog cells**

The electroporator used was an Eppendorf 2510. Typical transformation at 1700 V of BL21(DE3)/pTog cells with 1 ng of pGalWT yielded more than 10<sup>8</sup> colonies, corresponding to a transformation efficiency of 10<sup>9</sup> cfu/µg of pGalWT.

**Generation of randomized library of TmGalU variants**

Random-mutagenized tmgalu genes ligated to the pACYCDuet-1 vector were transformed into Library Efficiency<sup>®</sup> DH5α<sup>TM</sup> Competent Cells (Invitrogen) and plated onto LB/Cm medium. Overnight incubation at 37°C furnished typically 20 000 colonies. An average of 25% of recombinant clones was estimated by PCR screening on randomly picked colonies. Primers GALU1 (5’-GGATC AGAGCTCATGATCATGGAAGAATGGC) and GALU2 (5’-TGACGATCTGATATTTTCAGCAGAGCAGGTTACC) flanking the gene tmgalu were used and 20 pmol of each primer was mixed in a 50 µl PCR. The reaction conditions were as follows: 1 × Dynazyme buffer, 0.5 mM of dNTP and 2.5 U of polymerase Dynazyme Ext. The thermocyclization of the reaction was achieved according to: 1 cycle at 94°C, 5 min, then 30 cycles at 92°C, 30 s; 65°C, 30 s; 72°C, 1 min and 45 s.

Plated bacteria were pooled and the batch of plasmids was extracted using the QIAprep Spin Miniprep Kit (Qiagen). An adequate volume of 1/50 water-diluted plasmid preparation was used to transform electrocompetent BL21(DE3)/pTog cells, providing roughly 10 000 recombinant clones.

**Selection and purification of evolved variants**

Electrotransformed cells were vigorously shaken in 1 ml of SOC medium for 1 h at 37°C and washed twice with liquid Davis MM in order to remove all traces of rich nutrients. Rinased cells were plated onto agar Davis containing Ap, Cm and IPTG (80 µM) supplemented with GA<sub>3</sub> and incubated at 37°C for 60 h. Emerged colonies were picked into 1.5 ml of liquid Davis MM supplemented with GA<sub>3</sub> (5 g l<sup>−1</sup>) and cultured at 37°C for 24 h with vigorous shaking. Streaking of three arbitrarily chosen positive strains was carried out on Davis agar + GA<sub>3</sub> and incubated at 37°C. Isolated colonies were obtained after 24 h of incubation. Single colonies of B7, B9 and B12 strains were then cultivated in liquid LB.
containing Ap and Cm and kept in glycerol at −80°C. The 1.3-kb DNA fragments encoding for mutant TmGalU enzymes were sequenced in both forward and reverse directions.

**Assay of uGA3 uptake in BL21 (DE3)/pTog**

We used the procedure described in Hugouvieux-Cotte-Pattat et al. (2001) with slight modifications. E. coli BL21(DE3), Novabue(DE3), NMS22/pTog, BL21(DE3)/pTog and Novabue(DE3)/pTog cells were grown in LB or LB/Ap medium (20 ml) until optical density (OD) at 600 nm reached 1 U. Cells were concentrated 10-fold and 500 µl of the cell suspension was incubated at 37°C with uGA3 (250 µM). Samples (200 µl) were removed after 30 min then added to 800 µl of ice-cold 0.1 M LiCl solution. Next, the suspension was centrifuged, resuspended in 200 µl of water and, finally, the cells were lysed by ultrasound Raytheon 250 W apparatus. uGA3 concentration was measured using the thiobarbituric acid (TBA) method (Nedjma et al., 2001) by comparison with standard calibration curves obtained in the presence of known concentrations of galacturonic acid. In standard conditions, NaOH (1 M, 20 mM) was added to 800 µl of the sample and the mixture was shaken briefly. The solution was heated at 80°C for 5 min and cooled. HCl (1 N, 240 mM) was added, the solution was shaken and then 200 µl of a solution (0.04 M) of TBA was added. The sample was incubated once again at 80°C for 5 min then briefly cooled before measurement of the OD at 540 nm (200 µl) on a 96-well microplate with an iEMS Labsystems plate reader.

**Purification of WT and mutant enzymes**

B7, B9 and B12 genes were amplified using primers GALJP-R (5′-ATATCTCGAGTTTCAGCAGAGCTTAC GAGTTCAGGCTTTTACGAGCTT TAC) flanking the gene tmgalu and 20 pmol of each primer were mixed in a 50 µl PCR. The reaction conditions were as follows: 1 × Phusion HF buffer, 0.5 mM of dNTP and 1 U of high-fidelity Phusion polymerase (Finnzymes). The reaction was thermocycled according to: 1 cycle at 98°C, 30 s, then 30 cycles at 98°C, 5 s; 50°C, 20 s; 72°C, 40 s and finally at 72°C for 10 min. PCR products were digested overnight at 37°C by the Ncol and Xhol restriction enzymes and cloned back into the pET21d(+) vector digested by the same enzymes. The E. coli BL21(DE3) strains expressing the WT and mutant tmgalu genes were grown in 50 ml of LB/Ap medium at 37°C to an OD at 600 nm of 0.8 U and incubated further with isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM) for 4 h. The cell culture was centrifuged (2500 g, 15 min) and resuspended in 2.5 ml of lysis buffer (Qiagen) containing NaCl (300 mM) and imidazole (10 mM). The cells were lysed by ultrasound Raytheon 250 W apparatus, centrifuged (18 000 g, 15 min) and the clear lysate was incubated at 70°C for 0.5 h in order to denature E. coli proteins. After centrifugation at 18 000 g for 15 min at 4°C, the debris was discarded and the supernatant was loaded on a column containing 1 ml Ni²⁺/NTA resin (Qiagen). The column was first washed twice with 10 ml of the washing buffer (concentration of imidazole was 50 mM for this buffer). The protein attached to the resin was eluted with the elution buffer (containing 250 mM imidazole). The purified WT and mutant proteins were analyzed by SDS–PAGE (12% polyacrylamide gel, 0.1% SDS, 375 mM Tris–HCl buffer pH 8.8) at a constant voltage (120 V). The gel was colored with Coomassie blue G-250 (Bio-Rad). A sample of purified protein analyzed by capillary electrophoresis (Agilent 2100 Bioanalyzer) displayed a single peak thus indicating the homogeneity of the preparation. Protein concentrations were also determined by this method.

**Measurement of the exo-PG activity**

Enzymatic activities were calculated from the increase in reducing saccharides using the 3,5-dinitrosalicylic acid (DNS) method by comparison with standard calibration curves obtained in the presence of known concentrations of galacturonic acid. In standard conditions, reaction mixtures containing 50 µl sodium trigalacturonate (10 mM in 100 mM sodium acetate buffer pH 6.0) were incubated for 30 min with 2 µl (appropriate concentration) of the WT and mutant exo-PG preparations at 70°C. Enzymatic reactions were quenched by addition of NaOH 1 M (12.5 µl), then DNS reagent (50 µl) was added. The resulting yellow solution was incubated for 10 min at 90°C and diluted with water (100 µl). OD at 540 nm of the samples (200 µl) was measured on a 96-well microplate with an iEMS Labsystems plate reader.

**Thermal inactivation**

The standard conditions described for the measurement of the enzyme activity were used to determine the residual enzymatic activity after partial thermal denaturation. Thus, 200 µl aliquots of the WT and mutant exo-PG (100 µg ml⁻¹) were incubated at 90°C for 4 h. The residual activity was measured every hour at 70°C.

**Effect of temperature and pH**

The activity of the WT and mutant exo-PG was measured in the standard conditions given above at temperatures from 30 to 100°C using sodium trigalacturonate (10 mM) as a substrate. Similarly, the optimal pH was determined in the standard conditions by varying pH from 5.0 to 8.0.

**Enzyme kinetics**

Kinetic studies with purified enzymes were performed with sodium trigalacturonate as a substrate. The enzymes (appropriate amounts for initial rate time scale) were incubated in preheated 100 mM sodium acetate buffer (pH 6.0 or 7.0) containing 10 mM GA3 at 37 or 90°C for 40 min. The residual activity was measured as described above.

**Results**

The metabolic selection presented herein was designed for the search for new oligogalacturonate-active enzymes. For this purpose, we assayed T. maritima exo-PG variants enabling the growth of E. coli at 37°C on an agar MM supplemented with sodium trigalacturonate (GA3) as the sole carbon source. According to the principle of in vivo selection and to the poor activity of WT TmGalU in these conditions, only improved mutants should enable the growth on MM + GA3 at 37°C. Indeed, growth would be mediated by the uptake of GA3 into the bacterium cytoplasm and subsequent hydrolysis of GA3 by an efficient mutant of TmGalU would yield monogalacturonate (GA1) metabolized via the well-known Entner–Doudoroff pathway (Peekhaus and Conway, 1998).
growth could be observed when the amount of liberated GA$_1$ reached a sufficient level (Scheme 1).

**Determination of the optimal combination of strain and MM**

The first step in the development of this selection system was to find a medium that would support the growth of *E. coli* on GA$_1$ as the sole carbon source. While using a T7 RNA polymerase-based expression system, different DE3 strains of *E. coli* (BL21, Tuner, Novablue, HMS174) were tested on different agar MM in order to determine the best combination of medium and strain allowing the fastest growth at 37°C. The classical M9 agar medium was first used but the growth on glucose (Glc, 5 g l$^{-1}$) was very slow even after 48 h at 37°C. When CaCl$_2$ (10$^{-2}$ M) was added, M9 supported a faster and significant growth of the tested strains on Glc but this medium did not support growth on GA$_1$ (5 g l$^{-1}$) for any tested strains even after 3 days of incubation. Conversely, when using Davis agar MM, BL21(DE3) and Tuner(DE3) grew after only 12 h at 37°C on both Glc and GA$_1$ (data not shown). This MM contained sodium citrate (0.5 g l$^{-1}$) known to be unusable by *E. coli* (Lara and Stokes, 1952) and, consequently, no growth occurred using Davis agar without GA$_1$ even after 14 days of incubation at 37°C. Finally, addition of IPTG (80 μM) did not affect cell growth and could be used to allow a weak induction of the enzymes. Thus, the combination Davis agar/BL21(DE3) was chosen as the starting point of this study.

**Cytoplasmic uptake of oligogalacturonates by expression of a specific permease**

*Erwinia chrysanthemi* expresses an impressive number of pectinolytic enzymes and uses oligogalacturonates as carbon sources. Hugouvieux-Cotte-Pattat et al. (2001) demonstrated that two transporters, TogT and TogMNAB, were responsible for the oligogalacturonate uptake in *E. chrysanthemi* 3937. In this study, the *E. coli* NM522 strain was shown to be unable to mediate naturally the uptake of unsaturated or saturated oligogalacturonates. However, when transformed with the plasmid pO604 (hereafter named pTog) encoding for the transporter TogMNAB, the uptake of both saturated and unsaturated oligogalacturonates into the cytoplasm became possible. We have successfully applied this strategy to the BL21(DE3) strain. The intracellular concentration of unsaturated sodium trigalacturonate (uGA$_3$) was measured after a classical transport reaction and an unambiguous increase in intracellular uGA$_3$ concentration with BL21(DE3) and Novablue(DE3) carrying pTog was observed (Fig. 1). On the basis of previous results (Hugouvieux-Cotte-Pattat et al., 2001), we assumed that the transporter behavior was the same for GA$_3$ and uGA$_3$. This experiment showed the functionality of TogMNAB in BL21(DE3)/pTog thus allowing the uptake
of oligogalacturonates into the cytoplasm. Growth of the latter on Davis + GA₃ was not affected by the encoded TogMNAB and almost the same growth rate was measured. Electrocompetent BL21(DE3)/pTog cells were prepared and tested for a transformation efficiency of >10⁸ cfu/µg of pGalWT. BL21(DE3)/pTog, when transformed with pGalWT, did not grow on Davis agar supplemented by GA₃ even after 20 days at 37°C. The adaptation of TmGalU to its mesophilic host using EPP and metabolic selection with BL21(DE3)/pTog was then possible.

**Generation of a random library of TmGalU variants**

Random mutations were introduced into the *mgalu* WT gene by EPP (0.4% average error rate). The resulting genes were cloned into the plasmid pACYCDuet-1 under the control of a T7 promoter and the resulting plasmids were transformed into *E. coli* DH5α competent cells and plated onto LB medium. This step allowed us to assess the transformation efficiency and the number of recombinant clones after a PCR control on randomly picked colonies. Plasmids were extracted from pooled clones and transformed into BL21(DE3)/pTog by electroporation. The amount of transformed DNA was adjusted to obtain about 10,000 recombinant mutants of TmGalU, which were plated onto Davis agar containing GA₃ (5 g l⁻¹) and incubated at 37°C.

**Selection and isolation of enhanced TmGalU mutants**

After 24 h of incubation at 37°C, 12 small colonies (named B1 to B12) emerged and 36 h later they reached a maximal diameter of 2 mm. These colonies were picked into liquid Davis MM supplemented with GA₃ (5 g l⁻¹) as the sole carbon source and cultivated at 37°C for 24 h. Two cultures (B3 and B8) were rejected because of their slow growth. Three (B7, B9 and B12) of the resulting 10 positive cultures were arbitrarily chosen, their plasmids (pB7, pB9 and pB12) were extracted and the corresponding *mgalu* genes were sequenced (Fig. 2). Plasmids pB7, pB9 and pB12 were separately used to transform BL21(DE3)/pTog giving rise to colonies on Davis agar containing GA₃. This result confirms that the mutant genes were responsible for the altered growth phenotype of the recombinant strain.

**Effects of temperature and pH on B7, B9 and B12 activities**

Because activity enhancement could be due to an increased expression of the mutants in the bacterium, the expression profiles of the WT and mutant proteins were first checked by SDS–PAGE analysis. Although the expression level of WT and mutants was quite low with pACYCDuet-1, no significant variation was noticed, ruling out this hypothesis (data not shown). In order to improve the expression level, mutant genes were subcloned into the high-copy vector pET21d(+) also allowing the introduction of a C-terminal His₆-Tag. Overexpression was obtained after IPTG induction, and WT and mutant enzymes were purified to homogeneity (>95%) by heat treatment followed by nickel affinity chromatography yielding 30–40 mg of tagged protein per liter of culture.

At pH 6.0, the optimal hydrolytic activity of WT TmGalU on sodium polygalacturonate was reached at 85°C. At 37°C, the activity was ~1% of the optimal (Parisot et al., 2003) for this substrate. The quantification of activity enhancement for the mutant enzymes was made using GA₃ as a substrate. Hydrolysis of this trisaccharide yielded GA₂ and GA₁ followed by subsequent hydrolysis of GA₂ leading to the liberation of two GA₁. The overall increase in reducing power due to the liberation of GA₁ reducing units was measured. However, as two simultaneous hydrolytic reactions occurred, the parameters for GA₃ represent only apparent constants in these conditions.

A preliminary kinetic experiment gave an apparent *Kₘ* value for the WT enzyme of 0.5 mM for GA₃ at 90°C, pH 6.0. Substrate saturation was reached before 10 mM. Then, *kₐ₅* values were estimated for a GA₃ concentration of 10 mM at different temperatures and pH (Table II) assuming that the *Kₘ* would not change significantly between pH 6.0 and 7.0. For B9 and B12 mutants, turnovers at 90°C were higher than for the WT enzyme. Interestingly, turnovers of B9 and B12 mutants were 4- and 1.5-fold higher at pH 7.0 and 6.0, respectively, as compared to that of the WT.

Analysis of the plots of relative activity vs. temperature (pH fixed at 6.0) showed that the hydrolytic activity of the mutants was doubled at 37°C (Fig. 2A) and, at least for B9 and B12, preserved at 90°C (Fig. 2B). Moreover, analysis of the plots of relative activity vs. pH revealed that the hydrolytic activities of B9 and B12 were preserved at pH 6.0 (Fig. 2C). Finally, increases of 35 and 100% were measured at pH 7.0 for B9 and B12 mutant enzymes, respectively (Fig. 2D).

In addition, thermostability was measured at 90°C and we noticed an exponential decay of hydrolytic activities on GA₃ (10 mM, pH 6.0). Mutant enzymes showed a lower stability at 90°C over time than the native enzyme (*t½*/₅ = 500 h). Indeed, it was dramatically decreased for B7 and B12 (*t½*/₅ = 21 and 50 min, respectively) while still significant for B9 (*t½*/₅ = 568 min) (Fig. 3).

**TmGalU 3D-model and amino acid substitutions in B7, B9 and B12**

Crystal structures have been resolved for the endopolygalacturonase II from *Aspergillus niger* (PGN) (van Santen et al., 1999; Armand et al., 2000), *Erwinia carotovora* ssp.
carotovora (PGE) (Pickersgill et al., 1998) and Aspergillus aculeatus (PGA) (Cho et al., 2001) endopolygalacturonases. Because of the lack of an available 3D-structure for TmGalU, a 3D-model for the WT protein was built based on sequence homology with the glycoside hydrolase family 28 (www.cbs.dtu.dk). Sequence alignments (Fig. 4) and three-dimensional superpositions of TmGalU and the latter polygalacturonases furnished a good assumption for Asp 239, Asp260 and Asp 261 to be the catalytic residues. The distances found between acid/base and activating residues were 5.2 and 6.3 Å (data not shown), values in accordance with previous findings (Cho et al., 2001). The 3-D model was thus used to locate the different mutations in B7, B9 and B12. They were all far from the catalytic residues at an average distance of 15–20 Å, located at the surface of the protein and clustered in the C-terminal region (Fig. 4).

**Table II.** Turnover values ($s^{-1}$) of WT and mutant TmGalU on GA$_3$

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>37°C</th>
<th>90°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
<td>pH 6.0</td>
</tr>
<tr>
<td>WT</td>
<td>1.7 ± 0.2</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>B12</td>
<td>6.2 ± 0.3</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>B9</td>
<td>5.0 ± 0.8</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>B7</td>
<td>3.8 ± 0.4</td>
<td>nd</td>
</tr>
</tbody>
</table>

Results are the mean values of three independent experiments. $k_{cat}$ values were estimated at 10 mM GA$_3$ concentration at different temperatures and pH.

Fig. 2. Relative activities of WT and mutant TmGalU (A) at 37°C, (B) at 90°C (both assayed at pH 6.0 for 30 min), (C) at pH 6.0 and (D) at pH 7.0 (both assayed at 70°C for 30 min). Sodium trigalacturonate (10 mM) was incubated with WT and mutant enzyme preparations at the appropriate temperatures and pHs. Relative enzymatic activities were calculated from the increase in reducing saccharides using the DNS method by comparison with standard calibration curves obtained in the presence of known concentrations of sodium galacturonate and expressed by comparison with the most active enzyme in each case.

Fig. 3. Thermal stability of WT and mutant TmGalU. WT and mutant enzyme preparations were incubated at 90°C and residual relative (compared with WT) hydrolytic activities on sodium trigalacturonate (10 mM, pH 6.0) were calculated as described in Fig. 2. WT (filled diamond); B7 (open circle); B9 (filled triangle); B12 (filled square).
Discussion

This study constitutes an original example of the creation of an artificial metabolic pathway in E. coli. An engineered BL21(DE3) strain was developed in several steps. The first step was the development of the selection assay, including the choice of an appropriate combination of MM and DE3 strain. We showed that Davis agar MM was very suitable for the growth of BL21 and Tuner (both DE3) on GA 1 as the sole carbon source. The second challenging step was the uptake of the substrate into the cytoplasm of BL21(DE3). In Gram-negative bacteria, carbohydrates can enter the periplasm by passing through aspecific porins, such as OmpC and OmpF, allowing the diffusion of molecules below about 600 Da (Nikaido, 1994; Koebnik et al., 2000).

Cytoplasm uptake of the carbon source (GA 3) was enabled by the transformation of BL21(DE3) by the previously described plasmid pO604 (named pTog here, see Table I) encoding for TogMNAB (Hugouvieux-Cotte-Pattat et al., 2001). This result was the transposition of the observations made with NM522/pTog. Differences between cytoplasmic uGA3 concentrations were noticed depending on the strain (Fig. 1). These differences could arise from different expression levels of the transporter in the strains. The engineered strain BL21(DE3)/pTog on one hand and the Davis agar medium on the other hand constituted the two key elements for the adaptation of TmGalU to E. coli.

Evaluation of a library of 10 000 recombinant clones led to the emergence of 10 growing colonies and 3 clones were analyzed.

Turnovers of analyzed mutants increased by 4-fold at pH 7.0 but only 1.5-fold at pH 6.0. This clearly reflects modifications of the pH range of activity that became less stringent for the mutants than for the WT enzyme. This has been previously observed for the directed evolution of Thermotoga neapolitana xylose isomerase, where the authors showed a significantly increased specific activity on glucose over the entire active pH range for isolated mutants (Sripradundh et al., 2003). Independent of pH, turnovers remained higher at 90°C than at 37°C, showing that these mutant enzymes retained a significant thermostable character.

Two parameters important for WT activity (pH and temperature) were modified during the selection assay. We measured 35 and 100% increases in hydrolytic activity at pH 7.0 for B9 and B12, whereas the hydrolytic activity of the three isolated mutants was doubled at 37°C. This suggests that, for these mutants, both neutral pH and low temperature adaptations were obtained under these conditions.

Thermostability was not preserved for the isolated mutant proteins. However, residual hydrolytic activities at 90°C for B9 and B12 were at the level of the WT enzyme on the time scale of the experiments. Furthermore, the apparently reduced activity of the B7 mutant reflected its dramatically reduced stability at high temperatures (t1/2 = 21 min, at 90°C).

The 3-D model showed a right-handed parallel β-helix fold characteristic of pectinolytic enzymes and first observed in pectate lyase C (Yoder et al., 1993). Eight amino acids were shown to be strictly conserved among polygalacturonases but only six were present in TmGalU (Fig. 4): Asn 237 (226, 196, 178), Asp 239 (228, 198, 180), Asp 260 (249, 219, 201), Asp 261 (250, 220, 202), Arg 327 (306, 274, 256) and Lys329 (308, 276, 258), numbers in parentheses refer to Erwinia carotovora (PGE), to Aspergillus aculeatus (PGA) and to Aspergillus niger (PGN) endopolygalacturonases, respectively. Residues Asp 287 and Asn 288 did not find any equivalent among these endopolygalacturonases (Fig. 4). This fact could be related to the specific processing properties of TmGalU, which is an exo-enzyme releasing only monomers.

DNA sequencing and exploitation of the 3-D model for TmGalU indicated that long-range interactions were responsible for the improvement in activity of TmGalU mutants. Although distant from the active site, all the mutations observed (excepted E53A) were located in the C-terminal region of the enzyme, suggesting the great importance of this
part of the protein for the neutral pH adaptation. This is in accordance with previously published results indicating that, for catalytic activity and thermal stability, both close and distant mutations appear ‘similarly’ effective in improving biocatalysts (Morley and Kazlauskas, 2005). However, the presence of the C-terminal His 6-Tag on subcloned mutant proteins might have an influence on their in vitro behavior as previously observed for the *Bacillus stearothermophilus* α-galactosidase (Dion et al., 2001) and suggests that the recorded parameters did not perfectly reflect the in vivo adaptation.

This study enabled the development of a metabolic selection, which was used for the evolution of TmGalU. This system is a valuable platform for the further directed evolution of other oligogalacturonate-active enzymes such as pectate lyases. Indeed, it renders possible experiments using saturated or unsaturated oligogalacturonates as substrates in the cytoplasm of *E. coli*. Further improvement of TmGalU by DNA shuffling and selection is currently in progress in our laboratory.

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References