Heat-inducible autolytic vector for high-throughput screening

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In directed evolution, a high-throughput screening system is often a prerequisite for sampling the enzyme variants. When the target enzyme is expressed intracellularly, for example when Escherichia coli is used as the host, chemical or enzymatic disruption of cell membrane is often required in many cases, which can be tedious, time-consuming, and costly. In this study, a set of heat-inducible autolytic vectors were constructed to solve this problem, in which the SRRz lysis gene cassette from bacteriophage λ was placed downstream of heat-inducible promoters, λ cI857/p_R promoter and its mutant, cI857/p_R(M). The artificial autolytic units were inserted into the backbone of pUC18 (away from the multiple cloning sites). For the wild promoter, cI857/p_R, the SRRz lysis cassette was expressed by temperature up-shift from 28° to 38°C, and the lysis efficiency of transformed bacterial cells was found to be consistent and could reach 96.3% as measured by the reporter β-galactosidase assay. In order to obtain a higher cell growth rate, the mutant promoter cI857/p_R(M) was utilized to allow bacteria growth at 35°C and lysis at 42°C. However, this heat-inducible system showed significant inconsistency in terms of lysis efficiency. Bacillus subtilis 168 lipase A gene was further inserted into the multiple cloning sites of the autolytic vector containing cI857/p_R and 93.7% of the expressed lipase activity was found in the culture medium upon heat induction, demonstrating the utility of the vector for expression and rapid extracellular assay of heterologous enzymes.

INTRODUCTION

Aiming to search protein variants with improved or even novel activity by screening or selection from a large pool of protein variants (1–3), directed evolution has been widely used in engineering enzymes since it emerged in the past decade (4). Screening, where all the variants in a certain library are generally assayed individually, is commonly used in directed evolution, as selection in most cases is unattainable. A quick and high-throughput screening strategy is thus significant for many directed evolution efforts, especially when *Escherichia coli* is used as the expression host, since most heterologous proteins or enzymes are expressed intracellularly, while the cell membrane is not permeable to most substrates. Here we set out to develop a set of autolysis vectors for large-scale screening (as needed in, for example, directed evolution) that require only a physical signal, heat.

Many previous efforts in self-disruptive *E. coli* systems utilizing the autolysis mechanism were based on the modification of the *E. coli* genome (5), controlled expression by a chemical inducer (i.e., isopropyl-β-D-thiogalactopyranoside; IPTG) of bacterial phage lysis genes, such as the *SRRz* genes of phage λ, gene *E* of φX 174, gene *e* and gene *t* of phage T4 (6–9), or the joint action of additional osmotic, chemical, or enzymatic treatment (10), which may be time-consuming and costly for screening. We report here two heat-inducible autolytic vectors in which the lysis gene cassette, *SRRz* from bacteriophage λ was placed downstream of two heat-inducible promoters, λ cI857/p_R, and a mutant form of the promoter, cI857/p_R(M) (8,11,12). The cell lysis profiles for these two autolytic vectors were quantitatively analyzed. The wild cI857/p_R promoter is reported to be stringently repressed at temperatures lower than 30°C and inducible by a temperature shift to 38°C (13,14).

The mutant promoter cI857/p_R(M) (with a T→C mutation in the cI857/p_R promoter) was also chosen, since it has an extended heat stability allowing bacterial growth around 37°C and thus affords a faster cell growth profile (8,11,12). These two promoters have been used in preparing empty bacterial cell envelopes or “bacterial ghosts” in conjunction with the gene *E* of φX 174 (8,11,12), which provides the basis for the construction of heat-inducible autolytic vectors in our study. The mechanism for cell lysis induced by *SRRz* genes is known (6,8). The *S* gene product causes lesions in the cytoplasmic membrane through which the *R* and *Rz* gene products degrade the murein.

MATERIALS AND METHODS

Construction of Heat-Inducible Vectors

The autolytic genes used in our study was the *SRRz* cassette of bacteriophage λ (6,8). The *SRRz* gene cassette was placed under the transcriptional control of heat-inducible promoters and upstream of the strong *rrnB* terminator. A 1082-bp DNA fragment containing the promoter λ cI857/p_R was generated by PCR using DeepVent[®] polymerase (New England Biolabs, Ipswich, MA, USA) with λ phage DNA (cI857 Sam7; Promega, Madison, WI, USA) as the template and primers cI857/p_RFor and cI857/p_RRev (see Table 1, the underlined nucleotides indicate the *SapI*, *EcoRI*, and *XhoI* sites, respectively). For the mutant promoter, cI857/p_R(M), site-directed mutagenesis was performed by introducing a T→C base mutation into the cI857/p_R promoter with primers cI857/p_RFor and cI857/p_RRev(M) (Table 1, the underlined nucleotides in the latter indicates the *XhoI* site, and the boxed nucleotide represents the site mutation) and with λ phage DNA as template. The 246-bp *rrnB* terminator was amplified by PCR using ExTaq[™] polymerase (Takara, Dalian, China) with the genomic DNA of *E. coli* DH5-α (Dingguo, Beijing, China) as the template and with primers *rrnB*For and *rrnB*Rev (Table 1, the underlined nucle-

otides indicate the *Eco*RI, *Nco*I, and *Afl*III sites, respectively). The *cl857/p_R* promoter was first ligated with the *rrnB* terminator (doubly digested with *Eco*RI and *Afl*III) and then inserted between the *Sap*I and *Afl*III sites of pUC18 vector (Takara), yielding the plasmid pUC18-*cl857/p_R-rrnB*.

The *SRRz* cassette was amplified by PCR from the λ DNA (*cl857* Sam7). As the *cl857* Sam7 template is an *S* minus variant, which has a stop codon (TAG) in the *S* gene, site-directed mutagenesis was performed to replace the stop codon with the original one (TGG). Four primers were used, *SRRz*1For, *SRRz*1Rev, *SRRz*2For, and *SRRz*2Rev (Table 1, the underlined nucleotides indicate the *Xho*I and *Nco*I sites, respectively, and the boxed nucleotides indicate site mutations). Two PCR fragments (using *SRRz*1For/*SRRz*1Rev and *SRRz*2For/*SRRz*2Rev primer pairs, respectively) were obtained by PCR using DeepVent_R polymerase. These two fragments were combined at a 1:1 ratio to yield the full-length *SRRz*, via overlapping extension PCR and inserted into pUC18-*cl857/p_R-rrnB* to obtain pUC18-*cl857/p_R-SRRz-rrnB* (between the *Xho*I and *Nco*I sites).

The vector pUC18-*cl857/p_R(M)-SRRz-rrnB* was constructed by inserting the *cl857/p_R(M)* promoter between the *Sap*I-*Xho*I sites of the plasmid pUC18-*cl857/p_R-SRRz-rrnB*, replacing the *cl857/p_R* promoter (Figure 1). Restriction enzymes were either from New England Biolabs or Promega. Oligonucleotides were either from Sangon (Shanghai, China) or Takara. Sequence analysis was performed either by Takara or by Sanboyuanzhi (Beijing, China). The kits for DNA purification, gel recovery, and plasmid mini-prep were all from Qiagen (Valencia, CA, USA).

Cloning of Lipase A Gene Into pUC18-*cl857/p_R-SRRz-rrnB*

A 546-bp PCR fragment of the lipase A gene from *Bacillus subtilis* 168 was amplified using *Pfu* DNA polymerase (Tiangen, Beijing, China) and with primers BSLAFor and BSLARev (Table 1, the underlined nucleotides indicate the *Sac*I and *Xba*I sites, respectively). Then this PCR fragment

Figure 1. Construction of heat-inducible vectors pUC18-*cl857/p_R(M)-SRRz-rrnB*. The *SRRz* gene cassette was under the control of heat-inducible promoters and upstream of the *rrnB* terminator. The *cl857/p_R* promoter was first ligated with the *rrnB* terminator through the *Eco*RI site, then inserted between the *Sap*I and *Afl*III sites of pUC18 (in the backbone), yielding pUC18-*cl857/p_R-rrnB*. The *SRRz* cassette was inserted between the *Xho*I and *Nco*I sites of pUC18-*cl857/p_R-rrnB* to obtain pUC18-*cl857/p_R-SRRz-rrnB*. pUC18-*cl857/p_R(M)-SRRz-rrnB* was obtained by replacing the *cl857/p_R* promoter with the *cl857/p_R(M)* promoter between the *Sap*I and *Xho*I sites of pUC18-*cl857/p_R-SRRz-rrnB*.

was inserted into the *Sac*I-*Xba*I sites of pUC18-*cl857/p_R-SRRz-rrnB*, yielding pUC18-*cl857/p_R-SRRz-rrnB-lipase*.

Sequential Expression of *LacZ* or Lipase A and *SRRz* Lysis Genes

Saturated overnight cultures, transformed *E. coli* BL21 (Novagen, Madison, WI, USA) with pUC18-*cl857/p_R-SRRz-rrnB* were diluted 100-fold into LB media containing ampicillin and grown at 28°C, 250 rpm, for about 3.5 h to reach an OD₆₀₀ of 0.4–0.5. Then the expression of the *LacZ* reporter gene (located in the genome of *E. coli* BL21) for β -galactosidase was initiated with 1 mM IPTG (Takara) for 1.5 h, when the OD₆₀₀ was about 0.8–1.0. Cell cultures were divided into several 7-mL aliquots, induced by a temperature shift from 28°C to 38°C for 2 h. After the temperature induction, the temperature was down-shifted to 28°C for the expression of the lysis gene and cell lysis. At indicated time intervals, cells were harvested for the lysis efficiency assay. All the experiments were done in triplicate.

The recombinant cells BL21 containing pUC18-*cl857/p_R(M)-SRRz-rrnB* were grown at 35°C. After 1 h of IPTG induction of β -galactosidase, *SRRz* expression and cell lysis were induced at 42°C for 0.5 h, and then the temperature was down-shifted to 35°C for further growth and cell lysis.

For lipase A lysis experiments, the BL21 cells containing pUC18-*cl857/p_R-SRRz-rrnB-lipase* were also grown at 28°C to reach an OD₆₀₀ of 0.4–0.5. Then the expression of lipase was initiated with 0.5 mM IPTG for 3 h, followed by induction of cell lysis at 38°C for 2 h. All lysis experiments were done in triplicate.

Enzyme Extraction and Lysis Efficiency Calculation

The efficiency of cell disruption was evaluated by the indigenous β -galactosidase. Cell cultures were separated by centrifugation at 15,700× *g* for 4 min. The supernatant was collected for extracellular β -galactosidase activity assay. The cell pellets were resuspended in 0.5 mL lysis buffer (50 mM Tris-Cl, pH 7.2, 5% glycerol, 50 mM NaCl), then frozen in liquid N₂ for 1 min, and thawed in a 25°C water bath (repeated three times). Then 30 pulses of sonication (3 s each with a 3 s interval) in an ice-water bath were applied, and the sonicates were then centrifuged at 15,700× *g* for 4 min for intracellular reporter enzyme activity. The lysis efficiency was calculated as the ratio between extracellular β -galactosidase and the total activity (sum of the extracellular and the intracellular activity). β -Galactosidase activity was assayed essentially as described (15) using *o*-nitrophenyl- β -D-galactopyranoside (ONPG; Sigma, St. Louis, MO, USA). The reaction mixture consisted of 125 μ L enzyme solution (diluted in 0.1 M sodium phosphate buffer, pH 7.6) and 50 μ L 4 g/L ONPG solution in the same buffer. The alkaline phosphatase assay was carried out with the substrate *p*-nitrophenyl phosphate (Amresco, Parkway, OH, USA) as described (16). The reaction system contained 150 μ L enzyme solution and 50 μ L 4 g/L *p*-nitrophenyl phosphate (all in the buffer containing 1 M Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 μ M ZnCl₂). Lipase A activity was measured according to a published protocol with *p*-nitrophenyl palmitate (pNPP; Sigma) as substrate (17). The reaction mixture consisted of 10 μ L enzyme solution (diluted in 50 mM sodium phosphate buffer, pH 8.0) and 190 μ L 0.4 mM pNPP substrate (50 mM sodium phosphate buffer, pH 8.0,

Table 1. Primers Used in This Study

Primer Designation	Sequence
cl857/ <i>p_R</i> For	5'-AGACGCTAGCTCTTCCGCTTGATGATTATCAGCCAGCAGAG-3'
cl857/ <i>p_R</i> Rev	5'-CTAGAATTCAAGTCTCGAGATGCAACCATTATCACCGCC-3'
cl857/ <i>p_R</i> Rev(M)	5'-AGTACAGACTCGAGATGCAACCATTATCACGCCAGAGGTAAAATAGTCAACACGC [□] CGGT-3'
<i>rrnB</i> For	5'-CTAGAATTCAAGTCCATGGAGGCATCAAATAAACGAAAG-3'
<i>rrnB</i> Rev	5'-CGAACATGTTAGATATGACGACAGGAAG-3'
<i>SRRz1</i> For	5'-GGACCTGACTCGAGGCCACTGTCTGTCTGAATTC-3'
<i>SRRz1</i> Rev	5'-AAGGTCACGAATGAAC [□] AGGCGA-3'
<i>SRRz2</i> For	5'-CCT [□] GTTTCATTCTGACCTTCT-3'
<i>SRRz2</i> Rev	5'-CGGTTTTACCATGGTAGGCATTATATACTCCGCTGGA-3'
BSLAFor	5'-TCTCCGAGCTCCATGGCTGAACACAATCCAG-3'
BSLAREv	5'-CTACTCTAGATTAATTCGTATTCTGGCCCC-3'

The underlined nucleotides indicate restriction sites, the bolded nucleotides are complementary with the templates, and the boxed nucleotides represent site mutations.

0.2% sodium deoxyxholate, 0.1% gum aerobic). All assays were performed with a SpectroMax[®] 190 microtiter reader (Molecular Devices, Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

Construction of Heat-Inducible Autolytic Vectors

As shown in Figure 1, the *SRRz* gene cassette from phage λ was placed under the transcriptional control of the two heat-inducible promoters and upstream of the strong *rrnB* terminator.

Heat Induction, *SRRz* Expression, and Lysis Profiles

For *E. coli* cells transformed with pUC18-cl857/*p_R*-*SRRz*-*rrnB*, cell lysis was induced by a temperature shift from 28° to 38°C for 2 h, and the extent of lysis was assayed every 2 h after the heat induction. As shown in Figure 2, at 2 h post-heat induction, the cells were observed to be almost completely disrupted with a lysis efficiency of 90.2% $\pm$ 9.0%, according to the β -galactosidase activity assay. At 4 and 6 h post-induction, the lysis efficiency was 95.2% $\pm$ 1.0% and 96.3% $\pm$ 0.4%, respectively. Then as time went on, this number decreased as the survived cells continued to grow and the intra-

cellular β -galactosidase enzyme activity increased, while the extracellular β -galactosidase remained nearly unchanged (Figure 2A). After overnight cultivation, the nominal lysis efficiency decreased to 42.6% $\pm$ 1.7%. In addition, at 4 h post-induction, 92.2% $\pm$ 2.1% of the periplasmic enzyme alkaline phosphatase was found in the extracellular fraction, which was comparable to what was seen for β -galactosidase (95.2% $\pm$ 1.0%). Microscopic observations confirmed that the cells were nearly completely disrupted (data not shown). The cl857/*p_R* promoter was efficiently repressed in the absence of heat induction, which was apparent from the lysis profile of the uninduced cells (Figure 2B).

Transformed *E. coli* cells with pUC18-cl857/*p_R*(M)-*SRRz*-*rrnB* were grown at 35°C to the early exponential phase. Although the reported temperature for the growth is 37°C (11), 35°C was chosen to prevent leaky expression of the autolytic genes. The recombinant cells were sequentially induced by IPTG and a temperature shift from 35° to 42°C for expression of β -galactosidase and the *SRRz* gene products. Surprisingly, erratic lysis profiles were observed. The lysis efficiency at 2 h post-induction ranged from 98.1% $\pm$ 0.9% to 30.1% $\pm$ 3.3% in several repeated experiments. Additionally, significant clonal differences were observed. For example, for a set of experiments carried out for three

Figure 2. Expression of lysis gene cassette *SRRz* in *Escherichia coli* BL21 cells under the control of the cl857/*p_R* promoter. (A) Effects of *SRRz* gene expression on growth of BL21 cells. Cells were grown for 2 h at 38°C for the induction of the lysis genes, following the expression of β -galactosidase at 28°C. The temperature was then down-shifted to 28°C. (B) Lysis efficiency profiles, as calculated as the ratio between the extracellular β -galactosidase activity and the total activity (sum of the extracellular and the intracellular activity). The maximal lysis efficiency, 96.3% $\pm$ 0.4% was observed at 6 h post-heat induction, and the number dropped after overnight growth. The control showed that the promoter cl857/*p_R* was sufficiently repressed at 28°C.

single colonies descending from a single clone, lysis efficiencies of $94.6\% \pm 1.2\%$, $53.6\% \pm 2.4\%$, and $30.1\% \pm 3.3\%$, respectively, were obtained. This pattern of inconsistency was not reported previously (8,11).

Lipase A Expression and Release in pUC18-cl857/p_R-SRRz-rrnB-lipase

The *B. subtilis* 168 lipase A gene was inserted into the cloning region of pUC18-cl857/p_R-SRRz-rrnB to test the utility of this vector for expression and release of heterologous genes. After heat induction (at 38°C for 2 h), $93.7\% \pm 1.8\%$ of lipase A was found in the culture medium, which was consistent with that of β -galactosidase under similar conditions. This result indicates that the heat-inducible autolytic activity functions well for enzymes cloned in the vector.

Conclusion

In summary, in our study, a heat-inducible cell autolytic system was constructed for automatically releasing intracellular enzymes of interest from *E. coli* cells, which should be useful for applications in high-throughput screening in directed evolution and other screening-intensive technologies, such as metagenome. Cell autolysis was efficiently achieved by expression of the cloned SRRz gene from phage λ under the control of a heat-inducible promoter, cl857/p_R. The lysis efficiency was up to 96.3% according to the β -galactosidase assay. The control of the cl857/p_R promoter was stringent, with a basal level lysis of <1.4%. This heat-inducible vector was further utilized to express *B. subtilis* 168 lipase A. The release efficiency of lipase A reached 93.7%, comparable to that of β -galactosidase. Lastly, this artificial SRRz regulation and expression unit should be transferable to the backbones of other vectors.

Interestingly, using a mutant form of the promoter, cl857/p_R(M), and when cell lysis was induced at 42°C, the autolytic system appeared to be unstable as the cell lysis profiles showed significant inconsistency and clonal difference, rendering this mutant promoter not useful for the purpose of

consistent cell lysis. The reason is not clear.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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