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Construction of Heat-Inducible Expression Vector of *Corynebacterium glutamicum* and *C. ammoniagenes*: Fusion of λ Operator with Promoters Isolated from *C. ammoniagenes*

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The heat-inducible expression vectors for *Corynebacterium glutamicum* and *C. ammoniagenes* were constructed by using the λO_L1 and the cryptic promoters, CJ1 and CJ4 that express genes constitutively in *C. ammoniagenes*. Although the promoters were isolated from *C. ammoniagenes*, CJ1 and CJ4 were also active in *C. glutamicum*. To construct vectors, the O_L1 from the λP_L promoter was isolated and fused to the CJ1 and CJ4 promoters by recombinant PCR. The resulting artificial promoters, CJ1O and CJ4O, which have one λO_L1, and CJ1OX2, which has two successive λO_L1, were fused to the green fluorescent protein (GFP) gene followed by subcloning into pCES208. The expression of GFP in the corynebacteria harboring the vectors was regulated successfully by the temperature-sensitive *cI857* repressor. Among them, *C. ammoniagenes* harboring plasmid pCJ1OX2G containing GFP fused to CJ1OX2 showed more GFP than the other ones and the expression was tightly regulated by the repressor. To construct the generally applicable expression vector using the plasmid pCJ1OX2G, the His-tag, enterokinase (EK) moiety, and the MCS were inserted in front of the GFP gene. Using the vector, the expression of *pyrR* from *C. glutamicum* was tried by temperature shift-up. The results indicated that the constructed vectors (pCeHEMG) can be successfully used in the expression and regulation of foreign genes in corynebacteria.

Keywords: Corynebacteria, heat-inducible expression vector, fusion, λ operator, promoter, recombinant PCR

Corynebacteria, Gram-positive bacteria with high GC content, are well-known microorganisms that are frequently used for the industrial production of food-grade amino acids and nucleotides [19]. These organisms can also be used as potential hosts for the production of recombinant proteins. Much progress in the manipulation of genes of the strains has been achieved through the development of effective transformation and cloning vectors. The developed vectors were made by using the endogenous cryptic plasmids with fusion to the *E. coli* vectors for the shuttling between the strains [2, 4–6, 10, 21]. Almost all the developed vectors adopted *E. coli* promoters such as *lac*UV5, *tac*, *trp*, and P_L promoter to express foreign proteins in corynebacteria [1, 15, 18]. The regulation/deregulation was achieved by addition of carbohydrates such as arabinose [7], lactose and its analog IPTG, acetic acid [3], or zinc [9] to the culture medium of the cells harboring the vector. Although the *E. coli* promoters were active in the corynebacteria, the activities were quite low and the regulation/deregulation of the gene expression was not efficient when compared with those of *E. coli*. These facts led researchers to screen the endogenous strong promoters and the regulation/deregulation system for the control of gene expression useful in corynebacteria [11–14]. The major obstacle in the construction of such vectors is the efficiency of expression and regulation/deregulation. One of the possible regulation/deregulation systems isolated from corynebacteria is the promoter and operator of the isocitrate lyase gene [20], but the addition of a large amount of acetate to regulate the gene expression can affect the physiology of the target microorganism. These facts prompted us to develop the heat-inducible expression vectors by fusion of λO_L to the strong cryptic promoters (CJ1 and CJ4) isolated by using the proteome of the cell extracts from *Corynebacterium ammoniagenes*. The λO_L was fused to the two strong promoters. The vectors also contain the temperature-sensitive (ts) *cI857* under the control of the CJ1 promoter. To monitor the activities of the artificial promoters, the GFP gene was used as a reporter. As a result, the regulation/deregulation of the artificial promoters was highly responsive

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upon the shift of temperature from 30°C to 42°C. Using the artificial vectors, the *pyrR* from *C. glutamicum* was expressed in *C. glutamicum* and *C. ammoniagenes* to test the regulation/deregulation. Here, we report the results.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

E. coli DH5αF' [16], Top10F' and GI724 (Invitrogen Inc.), and POP2136 (New England Biolabs Inc.) were used as hosts for transformation by plasmids. *C. glutamicum* ATCC 13032 and *C. ammoniagenes* ATCC 6872 were used as the transformation hosts for the *E. coli*/corynebacteria shuttle vectors and artificial vectors developed in this experiment. The strains and plasmids used are listed in Table 1. Both *E. coli* and corynebacteria were routinely grown and maintained in LB media with appropriate antibiotic when necessary. For the expression of foreign gene, *C. glutamicum* was cultivated in CgIII medium, which consisted of 1% trypton, 1% yeast extract, 0.25% NaCl, and 2% glucose (pH 7.4). *C. ammoniagenes* was grown in WM media, which consisted of 1% peptone, 1% yeast extract, and 0.25% NaCl (pH 7.4). The corynebacterial cells harboring recombinant plasmids were grown at 30°C and the temperature shifted up to 42 °C for the induction of the expression of the foreign genes. All the transformants were grown in the medium supplemented with appropriate antibiotics such as ampicillin (50 μg/ml) or kanamycin (25 μg/ml).

Gene Manipulation and Transformation

All the general recombinant DNA techniques were carried out according to Sambrook *et al.* [16]. Restriction enzymes were purchased from New England Biolabs Inc. *Taq* polymerase was from Promega Co. Purification of plasmid DNA was carried out by using the Qiagen gel extraction kit (Qiagen, U.S.A.). The other enzymes were molecular biology grade and chemicals were reagent grade. The transformation of *E. coli* has been described elsewhere [16]. The corynebacteria were transformed by electroporation as described by Liebel *et al.* [8].

PCR

The fusion of corynebacterial promoter and λO_L was carried out by recombinant PCR with the primer set as described in Table 2. For recombinant PCR, overlapping primers were used as described in Fig. 1. The PCR was performed in a thermal cycler (MJ Research)

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Genotype or description	Reference
Strains		
E. coli		
G1724	F$^-$λ$^-$ *lacI^q ampC::PtrpcI mcrA merB* INV(*rrnD-rnnE*)	Invitrogen, Inc
DH5αF'	F'*endA1 hsdR17*(rk$^-$mk$^+$) *supE44 thi$^-$ recA1 gyrA relA1 Δ(lacIZYA$^-$ argF) U169 deoR(80dlacΔ(lacZ)M15)*	
Top10F'	F'*[(lacI^q Tn10(Tet^r)] mcrA Δ(mrr-hsdRMS-mcrBC) (80 lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU* galK rpsL(Strr) endA1 nupG	Invitrogen, Inc
POP2136	F- *glnV44 hsdR17 endA1 thi-1 aroB mal- cI857 lambdaPR tet^R*	New England Biolabs, Ltd
Corynebacterium		
glutamicum	ATCC13032	
ammoniagenes	ATCC6872	
Plasmids		
pCES208	*E. coli-Corynebacterium* shuttle vector, *Kan^r*	Personal communication
pGFP	GFP under the control of *lac* promoter in pUC19, *Amp^r*	Clontech, Inc
pCJ1OG	CJ1 promoter+λO_L1+GFP fusion in pCES208	This study
pCJ4OG	CJ4 promoter+λO_L1+GFP fusion in pCES208	This study
pCJ1OX2G	CJ1 promoter+2 λO_L1+GFP fusion in pCES208	This study
pScI857	*cI857* under the control of SDF5 promoter in pCES208	This study
pCJ1cI857	*cI857* under the control of CJ1 promoter in pCES208	This study
pCJ1OG+ScI857	DNA fragment containing *cI857* under the control of SDF5 subcloned in CJ1OG	This study
pCJ4OG+ ScI857	DNA fragment containing *cI857* under the control of SDF5 subcloned in CJ4OG	This study
pCJ1OX2G+ScI857	DNA fragment containing *cI857* under the control of SDF5 subcloned in CJ1OX2G	This study
pCJ1OX2G+CJ1cI857	DNA fragment containing *cI857* under the control of CJ1 promoter subcloned in CJ1OX2G	This study
pCeHEMG857	CJ1OX2G+CJ1cI857+MCS+$(His)_6$-EK, *Kan^r*	This study
pCeHEMGR	pCeHEMG857 having *pyrR*	This study
pSDF5	Deleted *pyrH* promoter in pCES208, *Kan^r*	This study

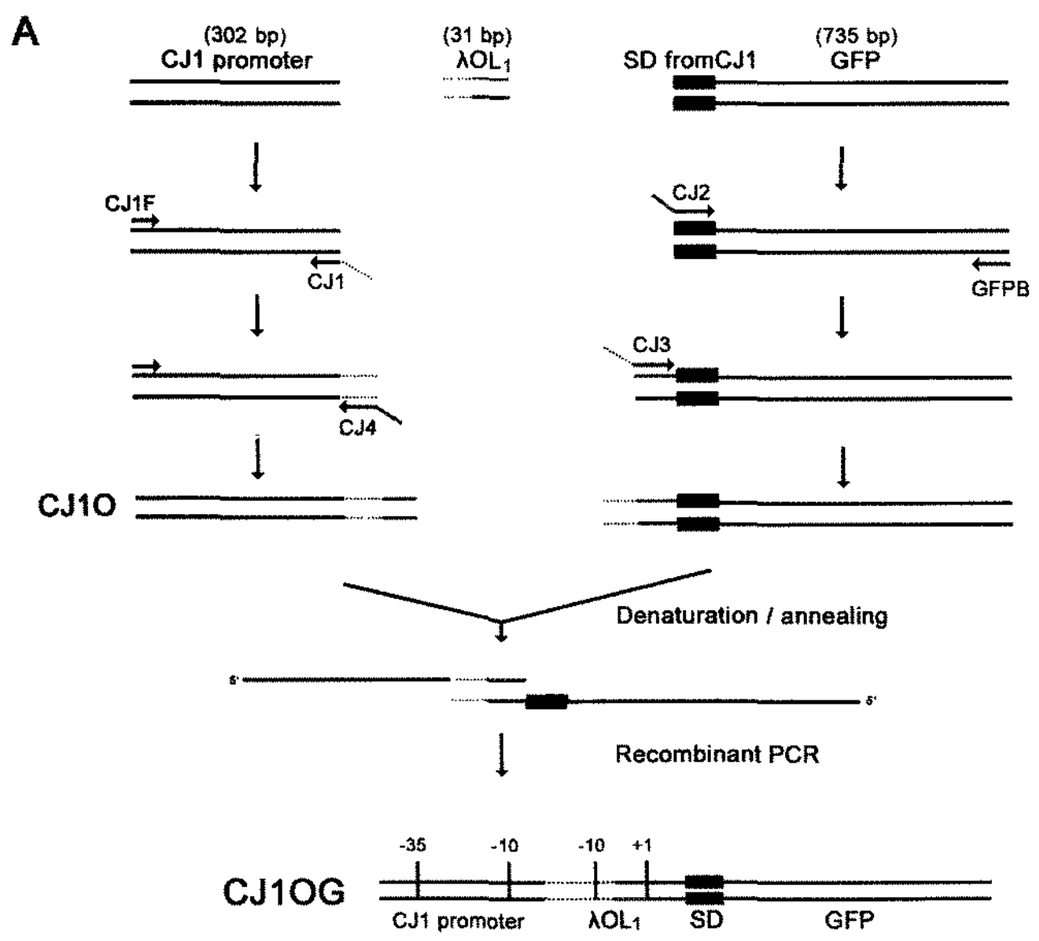

Fig. 1. A. Construction of CJ1OG containing λO_L1 fused to the CJ1 promoter by recombinant PCR.
The CJ1 promoter and λO_L1 were amplified by PCR with the primers, which have overlapping sequences between the two fragments. On the other hand, the GFP gene containing SD from CJ1 was also amplified with the primers overlapped to the λO_L1 fragment. After then, the three separate fragments were combined sequentially with overlapping sequence into one longer fragment. The constructed fragment CJ1OG contains −35 and −10 from the CJ1 promoter, +1 from λO_L1, SD from the CJ1 promoter, and GFP reporter gene. Numbers above the fragments show the size of each DNA. **B.** Nucleotide sequence of the junction region of CJ1OG and CJ4OG. Each −35 and −10 is underlined. The λO_L1 sequence is indicated by rectangular arrows and the repressor-binding site is underlined. +1 means transcription start of each promoter.

with *Taq* polymerase (Promega Co.). The reaction was started with predenaturation at 95°C for 1 min. Then, 30 cycles of denaturation (92°C, 30 sec), annealing (56°C, 30 sec), and polymerization (72°C, 30 sec) were carried out. Final completion of elongation was done by amplification at 72°C for 5 min.

RNA Extraction, RT-PCR, and 5'RACE

RNA was isolated from corynebacteria grown to OD_{600}=0.6. The cells were rapidly chilled on ice. Then, total RNA was extracted using the Qiagen RNAeasy Mini Kit according to the manufacturer's protocol. The isolated total RNA was used as template for the synthesis of cDNA. The synthesis was conducted using the ImProm-II Reverse Transcription System (Promega Co.) with the manuals provided by the manufacturer. 5' RACE was carried out according to the manufacturer's guide (Roche Applied Science) with the oligo d(A)-homopolymer tailing of the 3' end of the 1st cDNA of pCJ1OG, pCJ4OG, and pCJ1OX2G. The 1st cDNA was synthesized by RT-PCR with the primer GFPB. Then, the oligo d(A)-tailed 1st cDNA was amplified by PCR with an oligo d(T)-anchor primer, 5'GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV3' (V=G, A, or C), and gene-specific GFPmB primer, 5'CAAACTTG-ACTTCAGCACGT3'. The final amplified product was cloned by using a T & A cloning kit (Real Biotech Corp., Taiwan) followed by sequence analysis for the identification of transcription start site.

Expression of GFP by Heat-inducible Expression Vector

GFP was expressed under the control of recombinant synthetic promoter consisting of CJI or CJ4 promoter and λO_L. Briefly, cells harboring the plasmid were grown at 30°C overnight. Then, the cells were transferred to fresh medium followed by growth until A_{600}=0.6. The temperature was then shifted to 42°C for the indicated time to induce the expression. The cells were harvested by centrifugation and diluted to A_{600}=3.0 with 1 ml of PBS for the analysis of GFP expression.

Measurement of Fluorescence of GFP

The fluorescence was viewed by using an Axioskop epifluorescent microscope (Carl Zeiss Co. Ltd, Germany) equipped with a 50 W mercury arc lamp and filter block fitted with a 470–490 nm excitation filter and a 515–565 nm emission filter. The pictures were observed with ×100 magnification, captured with a CCD camera (PXL system, photometric), and analyzed with an imaging program RSImage (Roper Scientific, Inc.). To measure the fluorescence of the disrupted cells, the cells diluted with PBS (pH 7.4) to approximately A_{600}=1.0 were harvested and washed once with PBS and then resuspended in 1 ml of PBS, followed by disruption with a Bead-beater (Biospec Product, Inc.). The fluorescence of the supernatant was measured by using a Fusion fluorometer (VersaFluor Fluorometer; Bio-Rad Laboratories Inc.) equipped with a 485–420 nm excitation filter and 535–525 nm emission filter.

SDS-PAGE and Western Blotting

Proteins were separated by 12% SDS-PAGE. For Western blotting, the separated proteins were transferred to a PVDF membrane. The membrane was blocked by soaking with 1% BSA in TBST buffer (20 mM Tris-HCl, pH 8.3, 150 mM NaCl, and 0.05% Tween 20) and incubated with primary polyclonal antibody ($1:10^4$ dilution). After incubation for 30 min at room temperature, the membrane was washed and developed with Western Blue (Promega Co.). All steps were performed according to the manufacturer's guide.

Ni^{++}-NTA Column Chromatography

Bacterial cells were harvested and disrupted by sonication (with six 10 s bursts at 200–300 W and a 10 s cooling period between each burst) in buffer containing 1 mg/ml of lysozyme. After removal of the cell debris by centrifugation (10,000 ×g, 20 min, 4°C), 50% Ni^{++}-NTA slurry was added to the cleared supernatant followed by shaking (200 rpm on a rotary shaker) at 4°C for 60 min. The mixture was then loaded onto a column. After washing twice with the buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazol, pH 8.0), the protein was eluted out with the elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazol (pH 8.0).

Results and Discussion

Construction of Constitutive Expression Vectors by Fusion of λO_L to the Corynebacterial Promoters

For the expression of genes in corynebacteria, the strong constitutive expression promoters CJ1 (GenBank Accession No.: EU249515) and CJ4 (EU249518) isolated from *C. ammoniagenes* (unpublished, kindly provided by Dr. J.-H., Lee, CJ Corp, Korea) were used. Although the two promoters were isolated from *C. ammoniagenes*, they were also active in *C. glutamicum*. Furthermore, the CJ1 was shown to be active in *E. coli* (data not shown). For the construction of heat-inducible expression vectors, $\lambda O_L 1$ was fused to the downstream of −10 of CJ1 and CJ4, respectively. The schematic diagram for the construction of CJ1OG containing the CJ1 promoter by successive recombinant PCR is shown in Fig. 1A. The resulting promoters were designated CJ1O and CJ4O, respectively. The $\lambda O_L 1$ fragment contained a transcription start site (+1) and −10 derived from the λP_L promoter. The GFP gene was positioned to the downstream of the promoters to obtain the plasmids pCJ1OG and pCJ4OG. The nucleotide sequences of the fusion region of the vectors are shown in Fig. 1B. The results of the expression of GFP by the cells harboring pCJ1OG and pCJ4OG are shown in Fig. 2. Both artificial promoters constitutively expressed the GFP in *C. glutamicum* and *C. ammoniagenes*, by showing the fluorescence in contrast to the control that shows no fluorescence. These facts confirmed that the fusion of

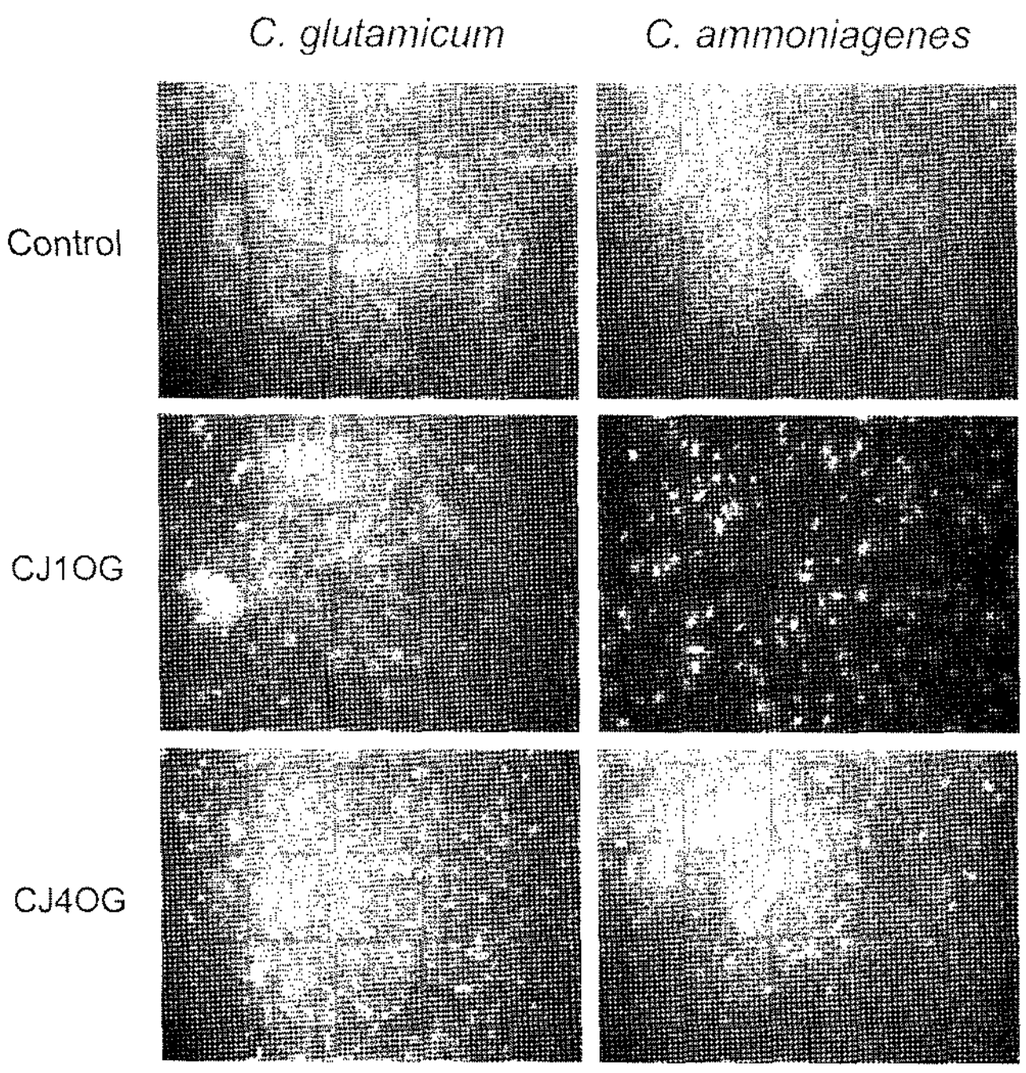

Fig. 2. Expression of GFP under the control of artificial promoters. The fluorescence of GFP was measured as described in Materials and Methods. Control is the host cells having no plasmids.

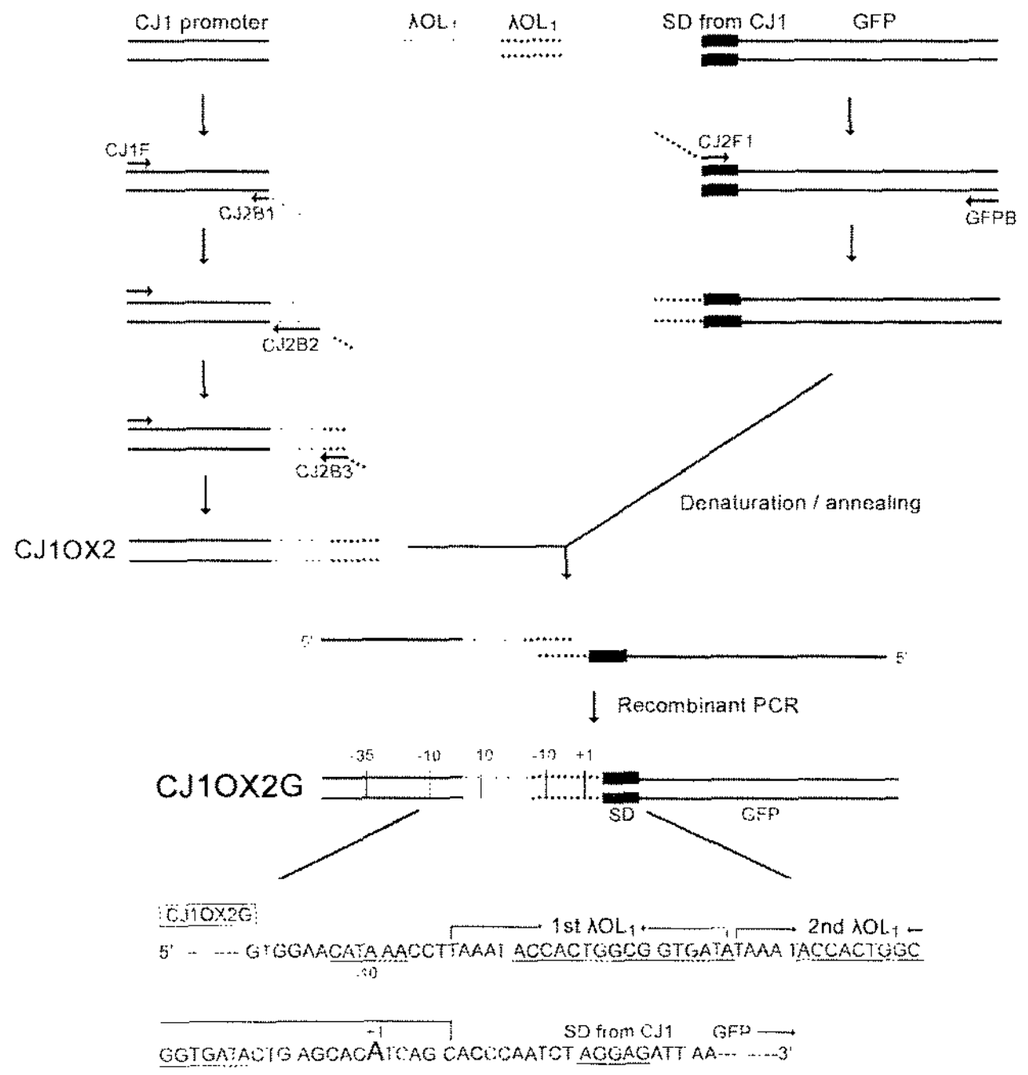

Fig. 3. Recombinant PCR for the construction of CJ1OX2G containing two sequential $\lambda O_L 1$'s (marked by rectangular arrows). The sequence of the junction region is shown. Repressor-binding sites are underlined. The transcription start site is marked as +1. −10 in the sequence (underlined) is derived from the CJ1 promoter. The detailed methods are the same as described in Fig. 1.

$\lambda O_L 1$ did not disturb the expression of the GFP by the CJ1 and CJ4 promoters. For the tight regulation of gene expression, fusion of the two successive $\lambda O_L 1$'s to the CJ1 promoter for the construction of CJ1OX2 was attempted. Fig. 3 shows the strategies for the construction and nucleotide sequence of the regulatory region of the CJ1OX2. The transcription start site, which is adenine as identified by 5'RACE, of the CJ1OG, CJ4OG, and CJ1OX2G was positioned at 14 bp, 65 bp, and 14 bp upstream of the SD sequence, respectively.

Cloning of Temperature-sensitive *cI857* (ts *cI857*)

To regulate the gene expression under the control of the CJ1O, CJ4O, and CJ1OX2 by temperature shift-up, the ts *cI*857 gene from *E. coli* GI724 was cloned and expressed in corynebacteria. The PCR was carried out using the primers ScIF and cIB (Table 2) to amplify the *cI857* followed by fusion using the recombinant PCR (Fig. 4A) to the promoter SDF5 (unpublished promoter, which has been isolated in this lavoratory from *C. glutamicum*. It was made by deletion of the natural *pyrH* promoter by Bal31 digestion and is a constitutive promoter). The resulting DNA fragment was named *ScI857*. Another construction was carried out for the expression of ts *cI*857 under the control of the CJ1 promoter in *C. ammoniagenes*. The primers CJ1F and CCB (1st PCR) and CCF and cIB (2nd

Table 2. Primer sequences used in this study.

Primer	Sequence (5'→3')
CCB	TGG TTT CTT TTT TGT GCT CAT GAT ATC TTA ATC TCC TAG
CCF	GGA GAT TAA GAT ATC ATG AGC ACA AAA AAG AAA CCA TTA AC
cIB	TCA GCC AAA CGT CTC TTC AGG CCA CTG AC
CJ1	TAT CAC CGC CAG TGG TAG GTT TAT GTT CCA CG
CJ1F	CAC CGC GGG CTT ATT CCA TTA CAT GGA
CJ2	CTG AGC ACA TCA GCA CCC AAT CTA GGA GAT TAA
CJ2B1	CCG CCA GTG GTA TTT AAG GTT TAT GTT CCA CGC
CJ2B2	CCA GTG GTA TTT ATA TCA CCG CCA GTG GTA TTT AAG
CJ2B3	ATG TGC TCA GTA TCA CCG CCA GTG GTA TTT ATA TCA CCG
CJ2F1	CGG TGA TAC TGA GCA CAT CAG CAC CCA ATC TAG GAG ATT
CJ4	TGC TGA TGT GCT CAG TAT CAC CGC CAG TGG TAG
CJ3	ACC ACT GGC GGT GAT ACT GAG CAC ATC AGC ACC C
GFPB	CTA TTT GAT TAG TTCATC CAT GCC ATG TG
GFPmB	CAA ACT TGA CTT CAG CAC GT
Oligo d(T) anchor	GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTT TTV
ScIF	CTG AAG GAG AAC ACC ACT ATG AGC ACA AAA AAG AAA CCA TTA

(V=C, A, or G)

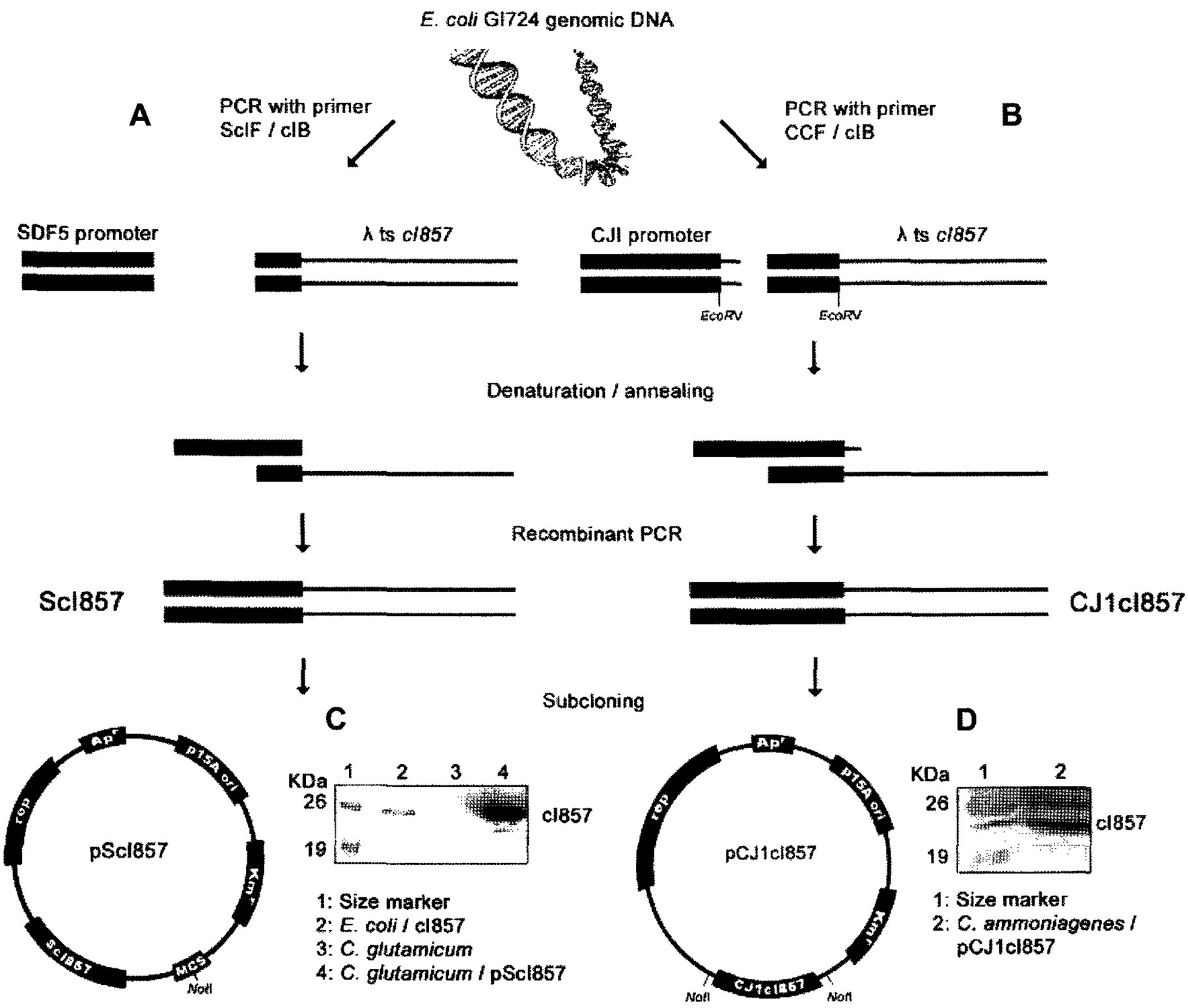

Fig. 4. Construction of vectors containing ts *cI857* under the control of the SDF5 or CJ1 promoter. ts *cI857* was isolated from *E. coli* G1724 by PCR amplification with the primers ScIF/cIB and CCF/cIB for the ScI857 and CJ1cI857 fragment, respectively.
Each fragment was subcloned to pCES208 for the construction of pScI857 (**A**) and pCJ1cI857 (**B**). The vector pScI857 was then introduced to *C. glutamicum*, and pCJ1cI857 to *C. ammoniagenes*, to confirm the expression of the cI857. The repressor cI857 expressed from pScI857 and pCJ1cI857 was identified by Western blot with the anti-cI857 antibody (**C** and **D**).

PCR) (Table 2) were used for the amplification of *cI857* and fusion to the CJ1 promoter by recombinant PCR, respectively (Fig. 4B). The resulting fused DNA fragment was designated *CJ1cI857*. The *ScI857* and *CJ1cI857* were cloned in the vector pCES208 to construct pScI857 and pCJ1cI857, respectively. Both the *C. glutamicum* harboring pScI857 and *C. ammoniagenes* harboring pCJ1cI857 constitutively expressed the ts *cI857* successfully, which was confirmed by Western blotting (Figs. 4C and 4D).

Construction of Heat-inducible Vectors and Regulation of Foreign Gene Expression in Corynebacteria

The heat-inducible vectors for the expression of GFP in corynebacteria were constructed by subcloning of DNA fragments (CJ1OG, CJ4OG, and CJ1OX2G) to pScI857 or pCJ1cI857. The constructed vectors (pCJ1OG+ScI857, pCJ4OG+ScI857, pCJ1OX2G+ScI857, and pCJ1OX2G+CJ1cI857) are shown in Fig. 5A. After transformation of the vectors to *C. glutamicum* or *C. ammoniagenes*, the regulation of expression of GFP by the repressor cI857 was investigated. As shown in Fig. 5B and 5C, *C. glutamicum* harboring plasmid pCJ1OG+ScI857 or pCJ4OG+ScI857 showed an increase in GFP expression when induced by temperature shift-up in a time-dependent manner. The level of GFP expression in the cells harboring pCJ1OG+ScI857 was recovered to the level of cells having only pCJ1OG after 4 h induction. However, in the case of cells harboring pCJ4OG+ScI857, the level of GFP expression was increased according to the induction but not recovered to the level of cells harboring pCJ4OG only. These results indicated that the expression of GFP by CJ1OG+ScI857 was not influenced by the λO_L1 inserted to the downstream of the CJ1 promoter, in contrast to the CJ4OG+ScI857 in which the expression of GFP was hindered by the λO_L1 inserted to the downstream of the CJ4 promoter. However, all of these results also suggested that the constructed vectors for the expression of a foreign gene in corynebacteria could be used successfully in the regulation of gene expression by *cI857*

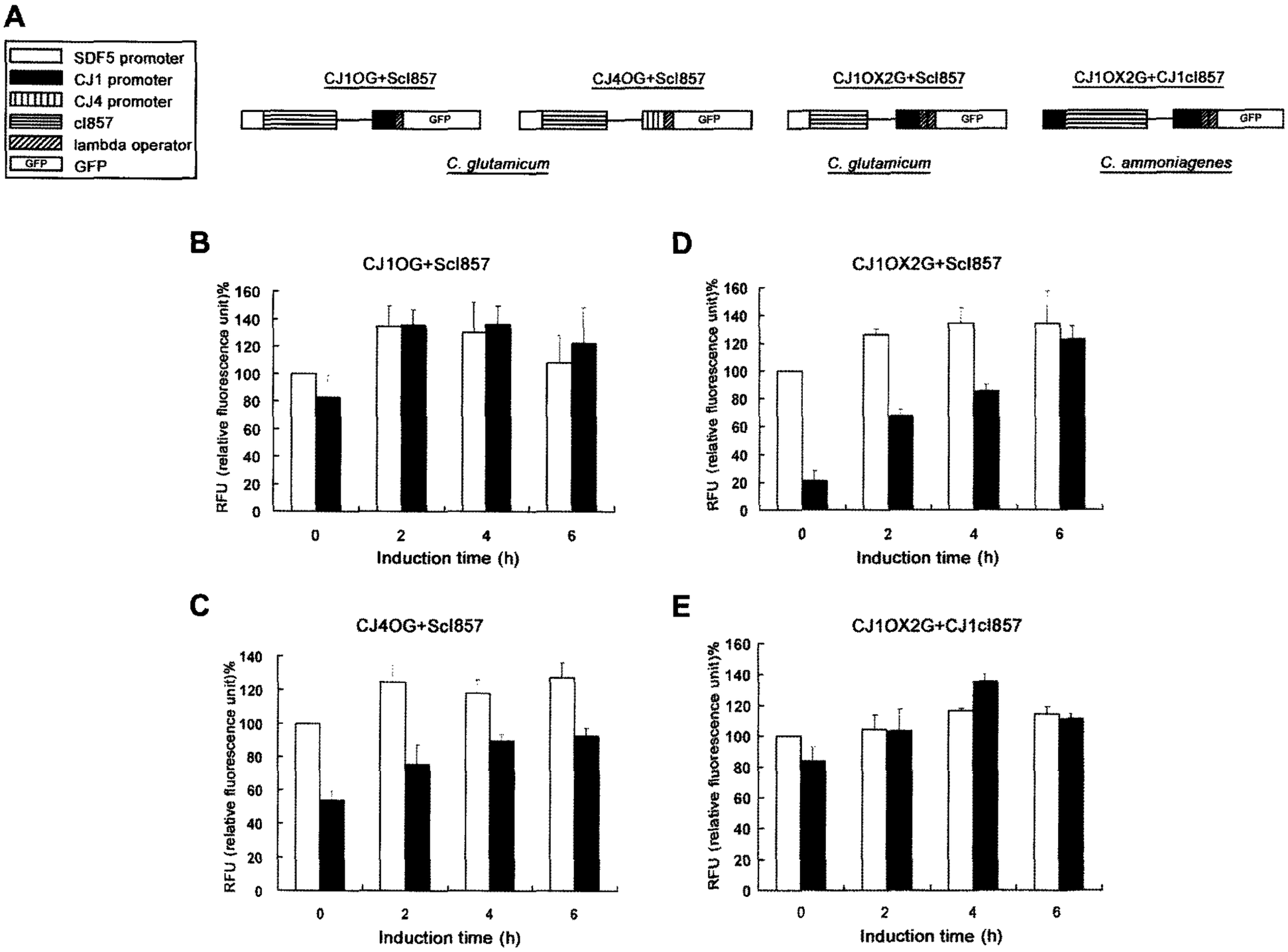

Fig. 5. Expression of GFP by temperature shift-up in *Corynebacteria* transformed by the vectors shown in (**A**). The relative fluorescences unit (RFU) from the *C. glutamicum* harboring pCJ1OG+ScI857 (**B**), pCJ4OG+ScI857 (**C**), or pCJ1OX2G+ScI857 (**D**) and from *C. ammoniagenes* harboring pCJ1OX2G+CJ1cI857 (**E**) are shown.

The fluorescence from the uninduced cells (0 h) harboring the vectors without the *cI857* gene (□) is regarded as the control (100%) for each experiment. The RFU from the cells harboring each plasmid with *cI857* (■) is shown compared with the control. All samples were adjusted to A_{600}=1.0 before disruption to measure the fluorescence as described in the Materials and Methods. Mean values±SD are plotted.

after induction by temperature shift-up. The expression and regulation of GFP by the CJ1OX2G+ScI857 and CJ1OX2G+CJ1cI857 was also investigated. The fluorescence of the *C. glutamicum* harboring pCJ1OX2G+ScI857 and that of *C. ammoniagenes* harboring pCJ1OX2G+CJ1cI857 is shown in Figs. 5D and 5E, respectively. The results showed that the cI857 repressor regulated the GFP expression by the promoters. When uninduced, the expression of GFP was repressed tightly in the case of CJ1OX2G+ScI857, but when induced, the expression was increased according to the induction time, although it was not recovered to the level of cells harboring pCJ1OX2G only. In the case of *C. ammoniagenes* harboring pCJ1OX2G+CJ1cI857, the effect of induction was not so tight by showing leaking of the GFP expression, although a high level of expression after was reached 4 h induction. These results indicated that the fusion of two successive λO_L1 to the CJ1 promoter can be generally applicable in the expression and regulation of foreign genes in *C. glutamicum* and *C. ammoniagenes* by temperature shift-up.

Construction of Generally Applicable Heat-inducible Vector and Test Expression of *pyrR* Using the Vector in Corynebacteria

The heat-inducible vector pCJ1OX2G+CJ1cI857 was added by the His-tag, enterokinase (EK) moiety, and the multiple cloning site (MCS) right after the GFP start codon for the easy purification, and detection of expressed foreign protein by Western blotting using His-tag antibody or EK antibody, elimination of fusion partner by the treatment with EK, and screening of the recombinant or nonrecombinant vector by the GFP fluorescence (pCeHEMG857, Fig. 6). Using the vector, the *pyrR* (which codes for the attenuator binding protein, PyrR) isolated from *C. glutamicum* was cloned at the XbaI and BglII sites of MCS of pCeHEMG857 (resulting in the plasmid pCeHEMGR) to test the expression, regulation, and selectivity of the recombinant vector by fluorescence. The PyrR was purified and analyzed by Western blotting before and after EK digestion. The results are shown in Fig. 7. The heat-inducible expression of *pyrR* in *C. glutamicum* is shown in Figs. 7A and 7B. After induction, the level of PyrR was increased according to the induction time, which was detected by Western blotting using the anti-His-tag antibody (Fig. 7B). The PyrR was purified by Ni^{++}-NTA chromatography followed by treating with EK to remove the fusion partner. Then, the protein was analyzed by SDS-PAGE and Western blotting using anti-EK antibody (Figs. 7C and 7D). After digestion of the PyrR fused to the His-tag and EK moiety by EK, the PyrR moved faster than the fused one (Fig. 7C, lane 3), indicating that the EK moiety was successfully removed from the fused PyrR. This was proven by the fact that, after EK treatment, the PyrR could not be detected with the anti-EK antibody by Western blotting (Fig. 7D, lane 3). These results clearly indicated that the vector pCeHEMG857, which was constructed in this work, can be successfully applied to express and regulate the foreign gene by heat induction in corynebacteria. The expressed protein can be

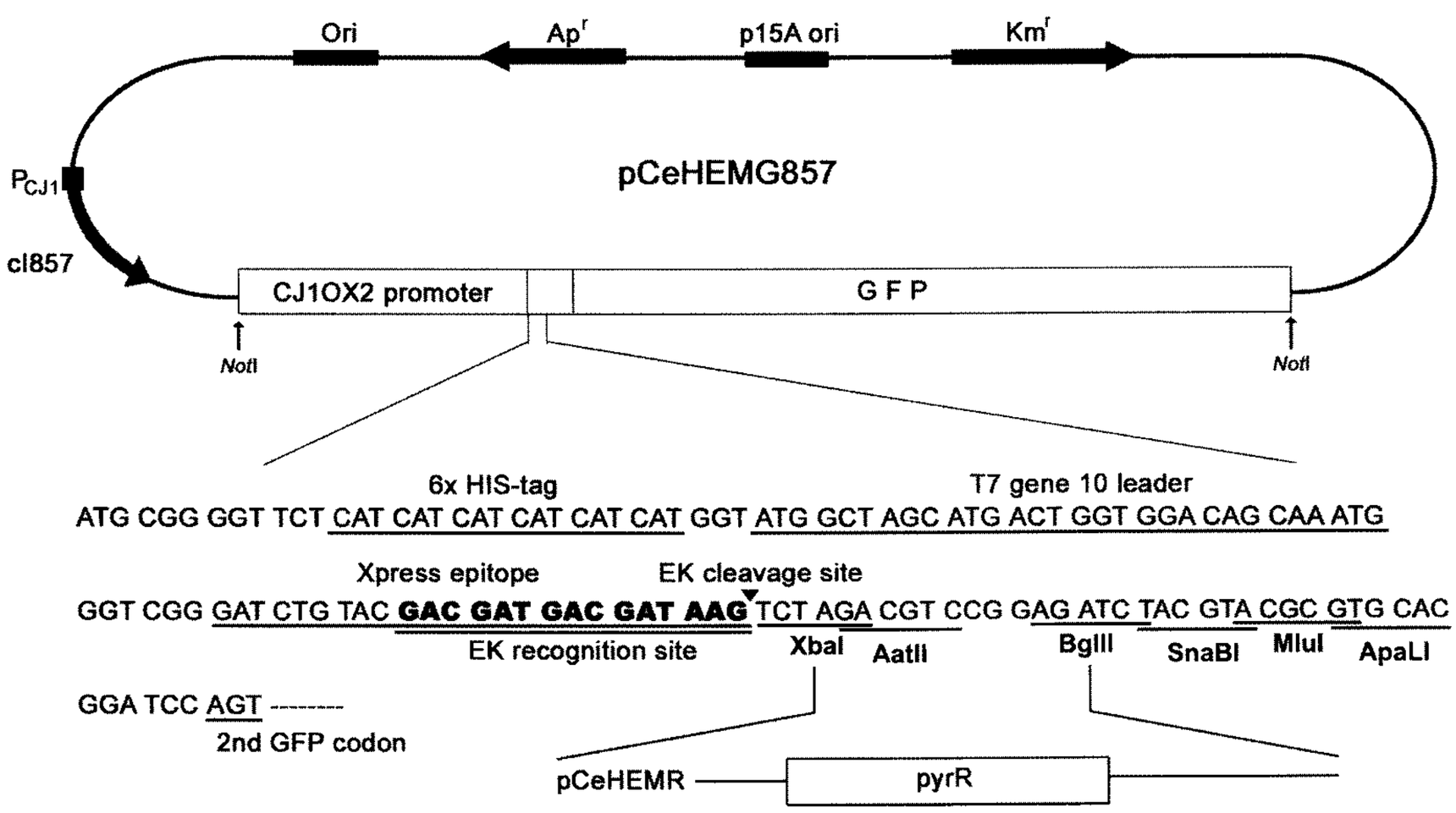

Fig. 6. Schematic diagram of recombinant vector pCeHEMG857.

The GFP is expressed by the promoter CJ1OX2, which has two successive λO_L1's, and ts *cI857* is controlled by the CJ1 promoter. Between the promoter and GFP gene, His-tag, Xpress epitope, and EK cleavage moieties are positioned. Several restriction sites (MCS) are also shown in front of the GFP gene. To construct the pCeHEMGR, *pyrR* was inserted to the XbaI and BglII sites of the vector as shown in the figure. The detailed DNA sequence is shown in the figure.

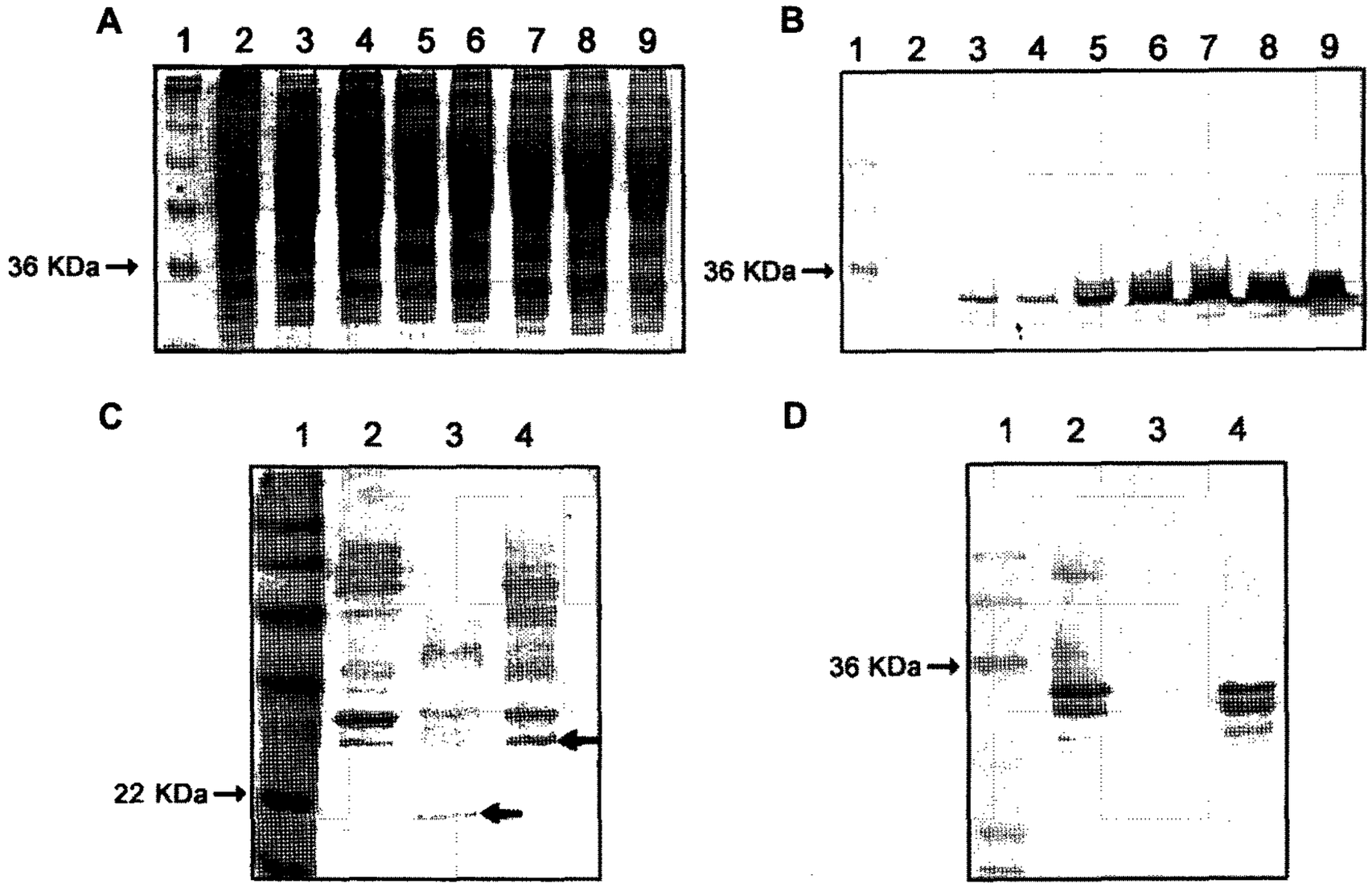

Fig. 7. Expression of *pyrR* by using the vector pCeHEMG857.
After transformation of the vector into *C. glutamicum*, the cells were grown at 30°C overnight. Then, cells were transferred to fresh WM media and expression of GFP was carried out at 42°C. The cells were harvested, disrupted, and GFP was analyzed by SDS-PAGE (**A**) and Western blotting (**B**). The samples were collected at various time intervals after induction. With increasing induction time, the amount of expressed GFP is also increasing. Lane 1, protein size marker; lane 2, *C. glutamicum* without vector as control; lane 3, *C. glutamicum* harboring pCeHEMG857 plasmid containing *pyrR* but no *cI857*; lanes 4–9, cells harboring pCeHEMGR at various time intervals after induction (lane 4, uninduction; lane 5, 30 min; lane 6, 1 h; lane 7, 2 h; lane 8, 3 h; lane 9, 4 h). After purification of the PyrR with Ni^{++}-NTA column chromatography, SDS-PAGE (**C**) and Western blotting (**D**) were performed before and after digestion of the PyrR with enterokinase. The gel was silver-stained for SDS-PAGE. Lane 1, protein size marker; lane 2, PyrR; lane 3, PyrR treated with enterokinase; lane 4, same reaction mixture as lane 3 but without addition of enterokinase.

easily purified and detected by Western blotting using anti-His-tag antibody or EK antibody. Furthermore, only the target protein can be obtained after elimination of the fusion partner by digestion with EK.

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