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High yield secretion of heterologous proteins in *Corynebacterium glutamicum* using its own Tat-type signal sequence

Hiroshi Teramoto · Keiro Watanabe · Nobuaki Suzuki · Masayuki Inui · Hideaki Yukawa

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Abstract Efficient protein secretion, the basis of large-scale production of many compounds central to the biotechnology industry, is achieved by signal peptide and propeptide optimization in addition to optimizing host factors affecting heterologous protein production. Here, we fused green fluorescent protein (GFP) to the recently identified Tat-type secretory signal peptide of CgR0949 to demonstrate a high-yield protein secretion system of *Corynebacterium glutamicum*. The resultant secretion vector facilitated effective secretion of active-form GFP (20 mg l^{-1}) into *C. glutamicum* culture medium. The expression of GFP was enhanced 2.9-fold using the Shine–Dalgarno sequence of triosephosphate isomerase in the secretion vector. Moreover, GFP drastically accumulated in the culture supernatant upon addition of calcium chloride even though Ca^{2+} addition did neither enhanced the transcription of *gfp* nor resulted in the accumulation of cytosolic GFP. Active-form GFP concentration reached 1.8 g l^{-1} after 48-h incubation in a jar fermentor. Likewise, α-amylase accumulation in *C. glutamicum* cultures was also enhanced by Ca^{2+} addition, suggesting that Ca^{2+} may affect general protein secretion in *C. glutamicum*.

Keywords Protein secretion · *Corynebacterium glutamicum* · Tat-type signal sequence · PS2 · Calcium

H. Teramoto · K. Watanabe · N. Suzuki · M. Inui · H. Yukawa (✉)
Molecular Microbiology and Biotechnology Group, Research Institute of Innovative Technology for the Earth (RITE), 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan
e-mail: mmg-lab@rite.or.jp

Introduction

Corynebacterium glutamicum is a Gram-positive, non-sporulating, and facultatively anaerobic bacterium (Kinoshita 1985; Yukawa et al. 2006; Nishimura et al. 2008) that has been used in chemical production such as lactate, glutamate, and lysine (Kinoshita 1985; Malumbers et al. 1995; Inui et al. 2004a, b; Okino et al. 2005). The fermentation conditions for mass production methods using this bacterial species are well-established (Kikuchi et al. 2008; Liebl and Sinskey 1990). However studies on protein secretions in *C. glutamicum* are only now starting to gain traction since the microorganism has long been considered able to excrete only a limited number of proteins in its culture medium. This view should change with the realization that *C. glutamicum* harbors much more protein secretion potential than previously thought (Watanabe et al. 2009). Moreover, its lack of detectable extracellular hydrolytic enzyme activity (Billman-Jacobe et al. 1995) makes *C. glutamicum* a very favorable and versatile host for heterologous protein productions, capable of much more protein secretion than the dozen or so documented to date (Billman-Jacobe et al. 1995; Date et al. 2003, 2004, 2006; Itaya and Kikuchi 2008; Kikuchi et al. 2006; Liebl et al. 1992; Meissner et al. 2007; Salim et al. 1997; Smith et al. 1986). *C. glutamicum* naturally secretes two major proteins of which PS2 is more strongly secreted than PS1 (Peyret et al. 1993). The PS2 secretion signal is therefore more popular in current extracytoplasmic production, but it has the major drawback that PS2-based protein secretion yields widely vary depending on target proteins (Kikuchi et al. 2003; Watanabe et al. 2009). This drawback has meant that overall application of *C. glutamicum* as a host in protein secretion remains comparatively limited.

Of 108 active signal sequences of *C. glutamicum*, that of CgR0949 is particularly potent, exceeding the strength of

the PS2 signal by more than two orders of magnitude (Watanabe et al. 2009). Being a Tat-type sequence, CgR0949 may transport a wide variety of pre-folded proteins, including cofactor requiring redox enzymes, multimeric proteins, and membrane proteins, across the cell membrane (Lee et al. 2006). It should therefore easily find use in protein productions in bioindustry if the current problem of significantly lower yields of Tat-exported proteins when compared to the 1 g l^{-1} yields of Sec-exported proteins (Lee et al. 2006) can be solved. Further fine tuning for the expression of Tat-type secreted protein, cell physiology, and fermentation conditions is required. In this study, an efficient Tat-type signal sequence encoded on *cgR_0949* ORF of *C. glutamicum* R in conjunction with optimized fermentation conditions and gene expression system enabled secretion of up to 1.8 g l^{-1} active green fluorescent protein (GFP) in culture medium. This is the first report showing high-level secretion of a heterologous protein in *C. glutamicum* using its own signal sequence.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are summarized in Table 1. *E. coli* was cultivated in Luria–Bartani (LB) medium at 37°C with vigorous shaking (Sambrook et al. 1989). *C. glutamicum* R was grown at 33°C in A medium [2 g yeast extract, 7 g casamino acids, 2 g urea, 7 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 6 mg $FeSO_4{\cdot}7H_2O$, 4.2 mg $MnSO_4{\cdot}H_2O$, 0.2 mg biotin, and 0.2 mg thiamine per 1 l] supplemented with 4% glucose on a rotary shaker at 200 rpm. Chloramphenicol (Wako Pure Chemical, Osaka, Japan) was used at the following concentrations: for *E. coli*, 50 μg ml^{-1} and for *C. glutamicum*, 5 μg ml^{-1}.

DNA manipulations

E. coli plasmid DNA was isolated using QIAprep spin kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *E. coli* was transformed by the $CaCl_2$ method (Sambrook et al. 1989). In the case of *C. glutamicum* plasmid extraction, cells were treated with 4 mg lysozyme/ml at 37°C for 30 min. Restriction endonucleases were purchased from TAKARA BIO Inc. (Shiga, Japan). DNA sequencing was performed on an ABI PRISM 3130xl genetic analyzer with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. DNA sequence data were analyzed using Genetyx WIN program (Genetyx, Tokyo, Japan).

Construction of plasmids for GFP production

The green fluorescence protein (GFP), AcGFP1 (27 kDa) from *Aequorea coerulescens* was used as a marker. To express and secrete GFP efficiently in *C. glutamicum*, four plasmids of which the upstream regions of the GFP gene were different from were constructed. First, a DNA fragment containing GFP gene, *gfp* was amplified from pAcGFP1 vector (Clontech Laboratories, Mountain View, CA, USA) by PCR with primers 1 and 2 (Table 2). The fragment was digested with *Xho*I and *Sph*I and ligated to the same site of pCRC900 (Table 1) to replace α-amylase gene. pCRC900 can express a gene using *tac* promoter in *C. glutamicum*. The extracted plasmid was designated pCRC901 (Table 1). DNA fragment encoding CgR0949 signal sequence was amplified by PCR with primers 3 and 4 (Table 2), purified by gel electrophoresis and extraction, digested with *Eco*RV and ligated to the same site of pCRC901 using ligation high (TOYOBO) (pCRD313).

Second, to change the Shine–Dalgarno sequence for GFP expression, the DNA fragment encoding a chimeric protein of CgR0949 and GFP was further amplified by PCR with primers 5 and 6 from pCRD313. The fragment was digested with *Mun*I and *Bam*HI and ligated to *Eco*RI and *Bam*HI site of pCRD310, which has the Shine–Dalgarno sequence of triosephosphate isomerase derived from *C. glutamicum* (pCRD314). Third and fourth, plasmids expressing GFP gene containing promoter sequences of *cspA* (pCRD315) and *cspB* (pCRD316) from *C. glutamicum* R were also constructed, respectively (Table 1). The *cspA/cspB* promoter and CgR0949 signal sequences were fused by crossover PCR. Promoter regions of *cspA* and *cspB* extracted from *C. glutamicum* R genome were 685 bp (region on genome, 3068081–3067397) and 594 bp (region on genome, 2610432–2609839), respectively. First PCR was performed to amplify each promoter or signal sequence from chromosomal DNA of *C. glutamicum* R with primers 7–12. Second PCRs were carried out to fuse the DNA fragments obtained from each corresponding first PCR. For construction of pCRD315 (316), seconnd PCR was performed with primers 8 and 9 (or 11). The resultant DNA fragments were digested with *Xho*I and *Eco*RV and ligated to the same site of pCRC901. After checking the inserted DNA sequence, the resultant plasmids were transformed into *E. coli* SCS110, and extracted plasmids were used to transform *C. glutamicum* by electroporation (Vertès et al. 1993). *C. glutamicum* cells possessing the plasmid were selected on complex solid medium plates containing 1.5% agar (Becton, Dickinson) and chloramphenicol.

Protein secretion conditions

C. glutamicum was inoculated into 40 ml of A medium in a 500-ml baffle flask and cultivated at 33°C for 16 h with

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	TAKARA
SCS110	*dam dcm endA1 supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr* Δ(*lac-proAB*)/F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	TOYOBO
C. glutamicum		
R	Wild type	Yukawa et al. (2007) FERMP-18976
R/pCRD312	Cm^r; *C. glutamicum* R bearing pCRD312	This work
R/pCRC901	Cm^r; *C. glutamicum* R bearing pCRC901	This work
R/pCRD313	Cm^r; *C. glutamicum* R bearing pCRD313	This work
R/pCRD314	Cm^r; *C. glutamicum* R bearing pCRD314	This work
Plasmids		
pAcGFP1	Amp ^{r}; pUC *ori*; 5′ MCS; AcGFP1; 3′ MCS	Clontech
pCRC900	Cm^r; MCS (with *Eco*RV); pMB1/M13 *ori*; *tac* promoter; α-amylase without secretion signal; pBL1/coryneform bacterial *ori*; shuttle vector	Watanabe et al. (2009)
pCRC901	Cm^r; MCS (with *Eco*RV); pMB1/M13 *ori*; *tac* promoter; AcGFP1 containing 3'-UTR region; pBL1/coryneform bacterial *ori*; shuttle vector	This work
pCRD310	Cm^r; MCS (with *Eco*RI and *Bam*HI); pMB1/M13 *ori*; *tac* promoter; the Shine-Dalgarno sequence of triosephosphate isomerase (*tpi*) derived from *C. glutamicum* R; pBL1/coryneform bacterial *ori*; shuttle vector	This work
pCRD311	Cm^r; pCRC900 with CgR0949 signal sequence	Watanabe et al. (2009)
pCRD312	Cm^r; pCRD310 with CgR0949 signal sequence and α-amylase	This work
pCRD313	Cm^r; pCRC901 with CgR0949 signal sequence	This work
pCRD314	Cm^r; pCRD312 with the fragment replacing α-amylase by AcGFP1 containing 3'-UTR region	This work
pCRD315	Cm^r; pCRC901 with the 685 bp fragment of *cspA* promoter region and CgR0949 signal sequence inserted into *Xho*I and *Eco*RV sites	This work
pCRD316	Cm^r; pCRC901 with the 594 bp fragment of *cspB* promoter region and CgR0949 signal sequence inserted into *Xho*I and *Eco*RV sites	This work

shaking at 200 rpm. Then, 30 ml of culture was inoculated into 300 ml of A medium (without urea) and cells incubated at 33°C with constant agitation of 1,000 rpm in a 1-l jar fermentor. To prevent glucose starvation, glucose was continuously added to the cultures at the final concentration of 6% and totally 80 g was supplemented. To monitor the glucose concentration, an aliquot culture was centrifuged, and the supernatants were analyzed by an enzyme electrode glucose sensor (BF-4, Oji Scientific instruments). Cell growth was monitored by measuring OD_{610} with a spectrophotometer (DU800, Beckman Coulter, CA, USA). Dissolved oxygen concentration was controlled not to be lower than 3.0 ppm using an oxygen gas generator. The pH was monitored using a pH controller (DT-1023, Biott Co Ltd., Tokyo, Japan) and maintained at 7.2 by supplementing with 5 N ammonia. To examine the effect of divalent cations on protein secretion, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, or $ZnCl_2$ were added to the cultures to a final concentration of 2.0 g l^{-1}. For detergent treatment, Triton X-100 and Tween 20 were used at final concentrations of 0.02% or 0.1%, respectively.

Preparation of extracellular, cell surface, and cytosolic proteins

Supernatants as extracellular fractions were prepared by centrifugation of the culture samples (10,000×*g*, 4°C; 10 min). To extract cytosolic protein, cells were harvested by centrifugation (5,000×*g*, 4°C; 10 min) and pelleted. The cells were then washed once with equal volume of extract buffer (100 mM Tris/HCl, pH 7.5, 20 mM KCl, 20 mM $MgCl_2$, 5 mM $MnSO_4$, and 0.1 mM EDTA) and centrifuged

Table 2 Oligonucleotides used in this study

Primers	
1	5′-ATGCAAATAAACCGCCGAGGCTTCTTAAAA-3′
2	5′-TGCTCCAAGGGCGTTGGCCTTTGGCATAAA-3′
3	5′-ATCCGGGTACCGGTCGCCACCATGGTGAGCAAG-3′
4	5′-ACCCAAGCTTTCACTTGTACAGCTCATCCATGCCGTGG-3′
5	5′-ATCAATTGATGCAAATAAACCGCCGAGG-3′
6	5′-ATGGATCCGGCGATTAAGTTGGGTAACG-3′
7	5′-ATGCAAATAAACCGCCGAGGC-3′
8	5′-ATGATATCTTGATTGCTCCAAGGGCGTT-3′
9	5′-ATCTCGAGTGGAGATGTCGAGAAGGTACG-3′
10	5′-GTTTATTTGCATGAAGTTTTCCTTCTCATTTTG-3′
11	5′-ATCTCGAGCTGATATTGCGCTGTGTTCC-3′
12	5′-GTTTATTTGCATAGAGGCGAGGGTCTCCTT-3′
13	5′-TCTCACGCTACCCCGATCA-3′
14	5′-AGGCATGGCGCTCTTGAA-3′
15	5′-CAGGTCTCTGGGCAGTAACTGA-3′
16	5′-CGTTTACGGCATGGACTACCA-3′

again. Since negligible quantities of proteins were obtained from cell supernatant after the second washing, only the first washing supernatant was utilized for the further experiments (as cell surface fraction). Remaining cell pellets were re-suspended with extract buffer and sonicated using an ultrasonic homogenizer (Astrason model XL2020) in an ice-water bath for three 2-min periods, interrupted by 2-min cooling intervals. Cell debris was removed by centrifugation (10,000×*g*, 4°C; 30 min). The supernatant was used as cytosolic protein samples for GFP detection. Protein concentrations were measured with a Bio-Rad protein assay kit.

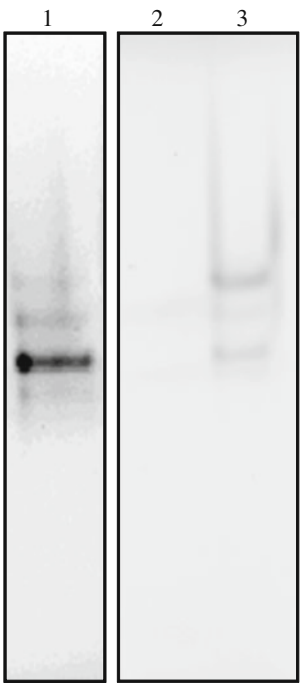

Fig. 1 GFP secretion by *C. glutamicum*. GFP secretion was detected by native PAGE analysis. Aliquots of concentrated culture supernatants were loaded on 12.5% polyacrylamide gel. *Lane 1* Purified GFP; *lane 2* the supernatant of strain R/pCRC901; *lane 3* the supernatant of strain R/pCRD313. After electrophoresis, GFP fluorescence was visualized by Typhoon TRIO image analyzer

Quantification of secreted proteins

GFP was purified from the culture supernatants by hydrophobic interaction column (HIC) chromatography method using Macro-Prep® methyl HIC support (Bio-Rad, Richmond, CA, USA) according to the manufacturer's instructions. The purity of the separated GFP was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized with CBB staining and scanned by an Image Scanner (GE Healthcare Bio-Sciences Corp.,

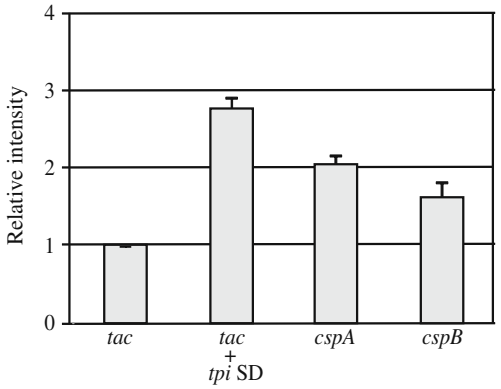

Fig. 2 GFP secretions using various expression systems via the CgR0949 signal sequence. Cells harboring the GFP expressing plasmid were cultivated for 48 h. Supernatants were subjected to SpectraMax analysis and fluorescence (excitation, 475 nm; emission, 520 nm) was measured. Data represent average values in triplicate experiments

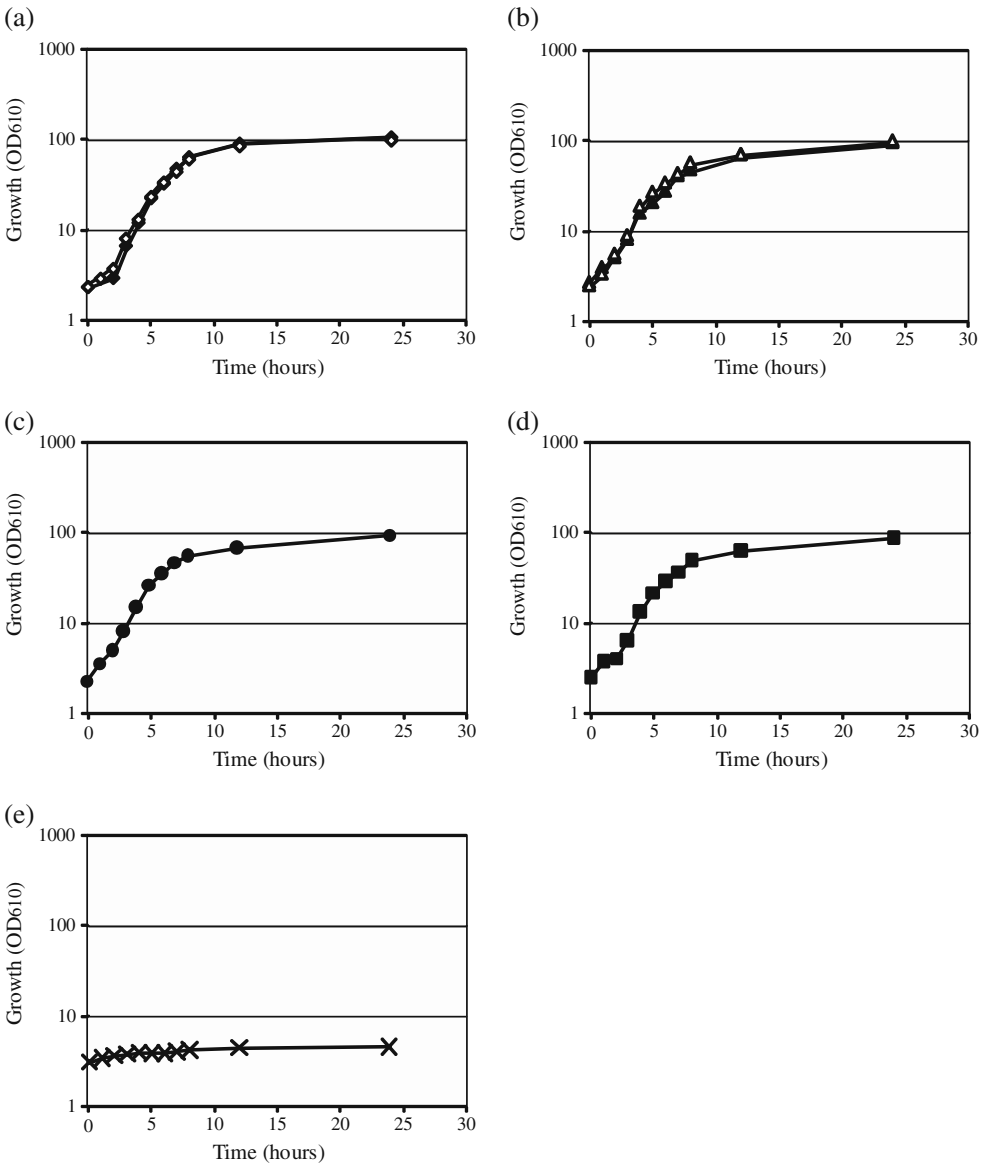

Fig. 3 Effect of several divalent cations on cell growth. Cells were incubated for 16 h at 33°C in a flask culture and aliquots were inoculated into a 1-l jar fermentor in the presence or absence of several cations. **a** R with or without Ca^{2+} (*open or closed diamond*, respectively), **b** R/pCRD314 with or without Ca^{2+} (*open or closed triangle*, respectively), **c** R/pCRD314 with Mn^{2+}, **d** R/pCRD314 with Mg^{2+}, **e** R/pCRD314 with Zn^{2+}

Piscataway, NJ, USA). The scanned images were analyzed with ImageMaster Labscan 3.0 (GE Healthcare). Active GFP was analyzed by native PAGE and visualized by the fluorescence mode of Typhoon TRIO (GE Healthcare), with a 532 nm of excitation and 526SP (short-pass) emission filter. GFP amount and its fluorescence signal in gel were linear correlation in our experimental conditions. No signal was detected from denatured (boiled) samples. The actual GFP amount of each sample was calculated using standard curve (0.2–0.8 μg purified GFP). GFP signal was also directly detected by SpectraMax® M2/M2^e Microplate Readers (Molecular Devices Corp., Sunnyvale, CA, USA). Excitation, emission, and cut-off spectra were set at 475, 520, and 515 nm respectively. Secreted α-amylase was analyzed using α-amylase assay kit according to the manufacturer's protocol (Kikkoman Corporation, Chiba, Japan).

RNA isolation

Total RNA was extracted from *C. glutamicum* R cells using Qiagen RNeasy Mini kit (Qiagen). Cultures were added to two volumes of an RNAprotect bacteria reagent (Qiagen), incubated for 10 min, centrifuged at 6,000×*g* for 10 min at room temperature. The pellet was resuspended in RLT buffer containing β-mercaptoethanol (RNeasy mini-kit; Qiagen) to a final concentration of 1 g dry cell weight per liter. A 1-ml sample of this cell suspension was subsequently mixed with 0.5 g of 0.1 mm zircon/silica beads (BioSpec Products, Bartlesville, OK, USA). Cells were mechanically disrupted for eight cycles of 45 s at a speed rating of 6.5 spaced by 5 min resting intervals in a Fastprep FP120 instrument (Qbiogene, Heidelberg, Germany). The resulting mixture was centrifuged for 10 min at 20,000×*g*. The supernatant was processed using an RNeasy system with DNase on-

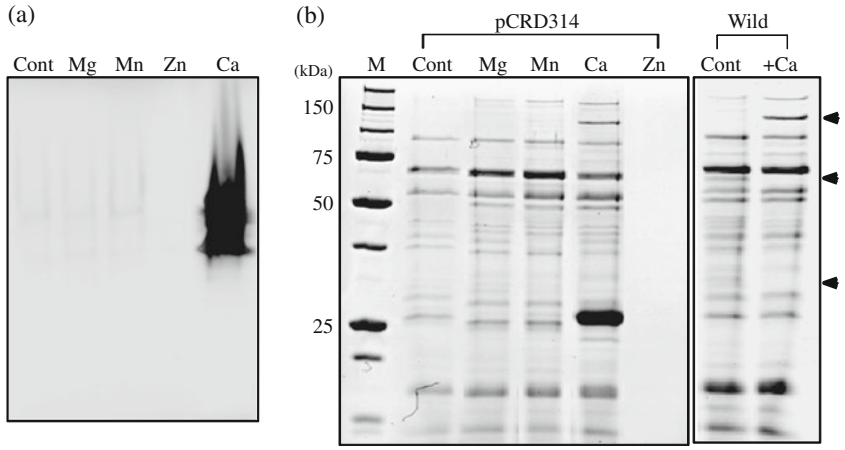

Fig. 4 Effect of divalent cations on secreted proteins. **a** Secreted GFP was detected by native PAGE. Ten-microliter aliquots of each supernatant of R/pCRD314 were loaded on 12.5% poly-acrylamide gel and visualized by Typhoon TRIO image analyzer. **b** Extracellular proteins of wild-type or R/pCRD314 were analyzed by SDS-PAGE. Five-microliter aliquots of each supernatant were loaded on 12.5% SDS poly-acrylamide gel, and proteins were stained by CBB. *Black arrows* point to the proteins whose secretion was influenced by addition of Ca^{2+}

column treatment according to manufacturer's instructions for RNA extraction. The purity of isolated RNA samples was analyzed by agarose gel electrophoresis and spectrophotometrically and stored at −80°C until ready for use.

Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (RT-PCR) was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems) and QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions. Specific primers 13–16 (Table 2) were designated by Primer Express Software v2.0 (Applied Biosystems). Each PCR reaction consisted of 10 μl 2× QuantiTect SYBR Green RT-PCR Master mix, 0.5 μM forward and reverse primers, 0.1 μl reverse transcriptase, 0.4 μl RNase inhibitor, and 20 ng total RNA in a total volume of 20 μl. PCR parameters were at 50°C for 30 min, 95°C for 15 min, and 45 cycles at 95°C for 15 s and 60°C for 1 min. The absolute quantification C_T (threshold cycle) method (Applied Biosystems) was used to quantify relative expression, with a threshold cycle being defined as the cycle at which the reporter fluorescence is distinguishable from the background in the extension phase of PCR reaction. The C_T values were computed as the average of duplicates.

Table 3 Amount of GFP detected in each fraction

Culture conditions	Calcium-deficient		Calcium-sufficient	
	24 h	48 h	24 h	48 h
Supernatant (mg l^{-1})	25±3.1 [a]	24±3.3	790±84	1,360±180
Cell surface (mg l^{-1})	7.7±2.0	N.D.[b]	64±15.0	280±13

[a] The values are mean±SD for triplicates determinations

[b] Signal was not detected by native PAGE

Results

Vector optimization enhances GFP secretion by *C. glutamicum*

In order to confirm conditions for production of GFP as secreted protein, *C. glutamicum* R was transformed with plasmid pCRD313, encoding signal sequence of *C. glutamicum* R CgR0949 and GFP on the one hand, and plasmid pCRC901 (identical to pCRD313 except for the absence of signal sequence) on the other hand. A chimeric GFP was expressed under the control of the *tac* promoter. The transformants were flask-cultured in A medium and secreted GFP was qualitatively detected by native PAGE and measurement of GFP fluorescence. As expected, only transformants bearing pCRD313 revealed the presence of GFP in their culture (Fig. 1). Furthermore, the presence of multiple bands indicated that the GFP was multimeric. Likewise, cytosolic and cell-surface fractions derived from the transformants harboring pCRD313 clearly revealed the presence of GFP, whereas only a background fluorescence was observed in pCRC901 transformants (data not shown). Attempts to enhance the GFP production by simply altering cultivation conditions of the pCRD313 transformants from

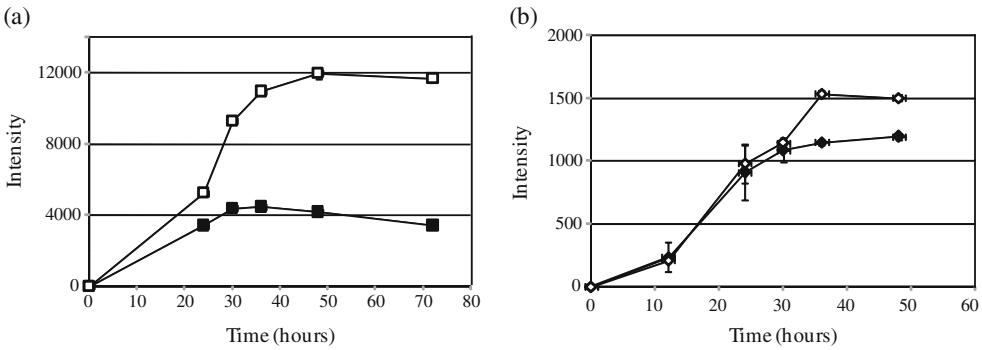

Fig. 5 Time-course analysis of extracellular and cytosolic GFP. Cells were incubated in a jar fermentor at 33°C and the GFPs (GFP signal intensities) were analyzed by SpectraMax analyzer. **a** Extracellular GFP; **b** cytosolic GFP. *Empty symbols* indicate the samples grown with Ca^{2+}, while *filled symbols* indicate samples grown without Ca^{2+}. Error bars show SD (n=3)

flask to jar fermentor were unsuccessful, as the final yield of GFP with standard A medium remained low (approximately 20 mg l^{-1}).

The other way to increase the amount of secreted GFP is by optimizing expression vectors. First, the 5′ untranslated region (UTR) region including SD sequence of *lacα* on pCRD313 was replaced with that of triosephosphate isomerase (*tpi*) gene in *C. glutamicum*. The *tac* promoter system is known to express genes effectively in *C. glutamicum* (Liebl et al. 1992), but its Shine–Dalgarno (SD) sequence is for *E. coli* and therefore not optimized for *C. glutamicum*. We previously found that the SD sequence of *tpi* showed high translation efficiency in *C. glutamicum*. Therefore, vector pCRD314 containing *tac* promoter, SD sequence of *tpi*, and *gfp* gene was constructed. Second, *tac* promoter was replaced with promoters of PS1-encoding *cspA* or PS2-encoding *cspB* of *C. glutamicum*, as enhanced heterologous protein secretion was previously achieved using *cspB* promoter and *cspA* signal sequences (Date et al. 2004). Therefore, the vectors, pCRD315 and pCRD316, containing *cspA* promoter and *cspB* promoter, respectively, were also constructed.

C. glutamicum R was separately transformed with each of these three vectors and the transformants were cultured in A medium at 33°C for 48 h. The fluorescence of the supernatants was measured by SpectraMax. All transformants showed increased fluorescence intensities compared to that of pCRD313 (Fig. 2). However, since the pCRD314 transformants showed highest fluorescence intensity (2.9 times higher), they were used for further analysis.

Calcium ions induce secretion of GFP

Changes to cultivation conditions, including treatment with detergents and addition of metal ions to the growth medium, are known to increase protein secretion in bacteria (Boekema et al. 2007; Kikuchi et al. 2002; Yang et al. 1998). Their effects on protein secretion by pCRD314 *C. glutamicum* transformants were hence investigated. First, Triton X-100 and Tween 20 at various concentrations were added to flask cultures of the transformants, but no increase

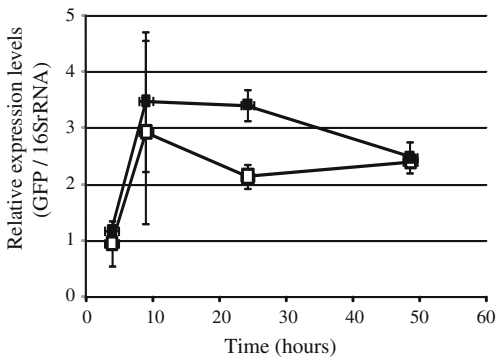

Fig. 6 GFP mRNA accumulation. Cells were incubated at 33°C in a jar fermentor, and mRNAs were isolated and analyzed by qRT-PCR. *Filled and empty circles* show GFP/16S rRNA ratio obtained from cells in calcium-sufficient or in calcium-deficient cultures, respectively

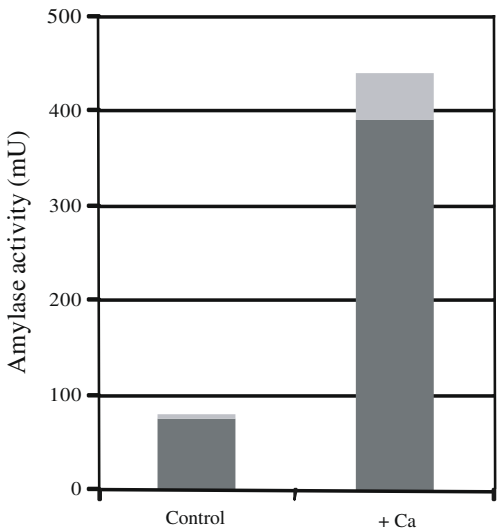

Fig. 7 Secretion of α-amylase by Ca^{2+} addition. Strain R/pCRD312 was cultivated in a jar fermentor at 33°C with or without Ca^{2+}. After 48 h, secreted α-amylase activity in culture medium and cell surface was analyzed. *Gray and black boxes* indicate amylase activity in cell surface and supernatant fraction, respectively

in GFP secretions was observed (data not shown). On the contrary, Triton X-100 strongly inhibited cell growth at 0.02% concentration. Second, the effect of the divalent cations Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+} were investigated by monitoring cell growth and protein secretion of appropriately supplemented cultures (Figs. 3 and 4). Growth was not inhibited by the addition of Mg^{2+}, Mn^{2+}, or Ca^{2+}, but was strongly inhibited (95% inhibition) by Zn^{2+} (Fig. 3). GFP secretion in media was very slightly increased by Mg^{2+} (1.1-fold) and Mn^{2+} (1.2-fold); however, the drastic induction of GFP secretion was detected by Ca^{2+} (over 30-fold; Fig. 4a, Table 3). SDS-PAGE analysis showed that the addition of Ca^{2+} increased the secretion of several proteins besides GFP, even though the predominant protein induced by Ca^{2+} addition was GFP (Fig. 4b).

Using A medium supplemented Ca^{2+}, concentration of secreted GFP reached 790±84 mg l^{-1} after 24-h incubation in a jar fermentor (Table 3). A longer period of cultivation further enhanced GFP accumulation (Fig. 5a, Table 3). The maximum amount of secreted GFP (sum of GFP amount in supernatant and cell surface fractions) reached 1640± 193 mg l^{-1} after 48-h incubation in the presence of Ca^{2+} (Table 3). In contract, the amount was only 24±3.3 mg l^{-1} at the same time in the absence of Ca^{2+} (Table 3). Interestingly, the secretion of entire spectrum of secreted proteins of wild type was not enhanced by Ca^{2+} addition (total extracellular protein after 24-h incubation in calcium-minus culture; 400 mg l^{-1}, calcium-containing culture; 360 mg l^{-1}). The GFP in cytosolic fraction was slightly increased by Ca^{2+} addition (Fig. 5b). Total cellular proteins amounts were same with or without Ca^{2+} (11.5 mg protein/g cell weight after 24 h of incubation).

Ca^{2+} addition has no effect on *gfp* gene expression

To investigate the effect of Ca^{2+} on the GFP secretion, the transcription levels of *gfp* were analyzed by quantitative RT-PCR. Total RNAs were extracted from cells harvested at mid-, late log (after 4 h and 9 h of incubation), and stationary phases (after 24 and 48 h of incubation), and the accumulation of messenger RNA (mRNA) was compared. The amount of 16S ribosomal RNA was used as internal standard. The results indicated that *gfp* transcription levels of the cells supplemented Ca^{2+} were only 1.6 times higher than those of control during the cultivation period (Fig. 6), despite a tremendous induction of GFP secretion that occurred upon the addition of Ca^{2+}.

The effect of Ca^{2+} addition on α-amylase secretion

In order to determine the effect of Ca^{2+} addition on different secretory proteins, α-amylase-producing *C. glutamicum* strain was constructed. Using pCRD312 vector, α-amylase was expressed under the control of *tac* promoter, followed by SD sequence of *tpi* in *C. glutamicum*. The transformant was cultivated in a jar fermentor for 48 h, and the α-amylase in the supernatant and cell surface fractions were determined by amylase assay kit. The enzyme activity was found in both supernatant and cell surface fractions, and consequently, the activity of α-amylase in calcium-sufficient culture increased five times over that of calcium-deficient culture (Fig. 7).

Discussion

Because *C. glutamicum* does not to have broad-spectrum proteolytic activity, it has been widely considered to secrete only a limited number of proteins, e.g., PS1 and PS2 (Billman-Jacobe et al. 1995). Recent studies have, however, indicated that *C. glutamicum* indeed possesses a variety of secretory proteins (Watanabe et al. 2009; Hermann et al. 2001), meriting investigation into whether the bacterium might make be good host for protein production. In this report, enhanced secretion of heterologous proteins was studied using GFP and α-amylase as model secreted proteins. Using the signal sequence of the to-date-uncharacterized CgR0949 and optimizing gene expression and fermentation conditions, the concentration of secreted GFP in the medium reached 1.8 g l^{-1}. This GFP yield is double the 0.9 g l^{-1} of *Streptomyces transglutaminase* secreted by *C. glutamicum* using the signal sequence of PS1 and promoter of PS2 (Date et al. 2004); PS1 and PS2 constitute the two major secreted *C. glutamicum* proteins (Jollif et al. 1992). It also exceeds the 1 g l^{-1} yields achieved using Sec-type signal sequences (Lee et al. 2006), demonstrating that enhanced *C. glutamicum* secretion using Tat-type signals like CgR0949 is feasible. In essence, our secretion system married the signal sequence of CgR0949 to an expression system, which fused *E. coli tac* promoter to the SD sequence of *C. glutamicum* triose phosphate isomerase-encoding *tpi*. Replacement of the 5′ UTR sequence of *lac* gene with that of *tpi* contributed significantly to the improved translation efficiency. This makes sense considering that efficient expression of *tpi* is important for cell viability (Solem et al. 2008).

In bacteria, Sec and Tat pathways constitute the two major systems for protein secretion. By virtue of being the pathway through which most bacterial secretions occur, the Sec pathway is the more-studied and consequently more often employed pathway for heterologous protein secretion. Proteins are secreted through the Sec pathway in an unfolded form (Muller and Klosgen 2005; Tjalsma et al. 2000) and may sometimes fail to fold properly out of plasma membrane. Moreover, some proteins can be

secreted through both systems. For example, in *E. coli*, GFP proteins can heterologously excrete through both Sec and Tat pathways, but the GFP secreted through the Sec pathway is inactive (Feilmeier et al. 2000). In contrast, GFP is always secreted in an active form across the plasma membrane by the Tat system (Thomas et al. 2001). The always active Tat-secreted protein has helped heighten interest in the pathway. In one evaluation of Tat-dependent secretion of GFP in the three Gram-positive bacteria *Staphylococcus carnosus*, *B. subtilis*, and *C. glutamicum*, only *C. glutamicum* was able to excrete active form GFP into its medium (Meissner et al. 2007), suggesting that not all Tat pathways are the same. Other proteins secreted via the *C. glutamicum* Tat pathway include isomaltodextranase and protein glutaminase (Kikuchi et al. 2006, 2008). This study demonstrated optimizations in Tat-dependent secretion that improve the attractiveness of *C. glutamicum* as a host for protein production.

In optimizing fermentation conditions, calcium ions, but not magnesium, manganese, or zinc ions, enhanced GFP secretion. The profile of secreted proteins in the presence of calcium differed from that in its absence, with several extracellular proteins up-regulated while others were down-regulated by Ca^{2+} addition (Fig. 4b). The overall effect of calcium addition was that it led to a 10% decline in the total extracellular protein pool. Irrespective of this, the amount of active form GFP secreted upon Ca^{2+} addition was over 50 times greater than that in the absence of calcium (Table 3). In contrast, cytosolic GFP increased only 1.3 times in the presence of calcium (Fig. 5), matching the corresponding differences in mRNA levels (1.6 times higher) upon calcium addition (Fig. 6). This may be explained as most of synthesized GFP being immediately released into the culture medium across the plasma membrane due to increased secretion efficiency upon Ca^{2+} addition. The increased efficiency may be accompanied by a cellular protection mechanism that degrades any excess GFP. GFP mRNA levels were higher in stationary-phase cultures than log-phase cultures, and their variation in log-phase cultures was very low (Fig. 6). Since *tac* promoter is constitutively expressed in *C. glutamicum* (Liebl et al. 1992), the expression of GFP by *tac* promoter may have little effect during jar fermentation with or without Ca^{2+}.

Secretion of α-amylase in our system led to activities that were over five times higher upon Ca^{2+} addition (Fig. 7). Kikuchi et al. previously demonstrated that calcium-independent pro-transglutaminase was secreted in medium using PS2 signal sequence, which utilizes Sec pathway, and its activity was up-regulated by the addition of 2.0 g l^{-1} Ca^{2+} (Kikuchi et al. 2002). In multicellular organisms, Ca^{2+} is a well-known factor facilitating exocytotic secretion through recruitment of components involved in vesicular and membrane fusion. In eukaryotic cells, SNARE proteins, the cytoplasmic protein cargo for secretion, also drive Ca^{2+}-triggered membrane fusion in exocytosis (Kesavan et al. 2007). In bacteria, however, this type of Ca^{2+}-mediated secretion has not been reported, yet. It is still difficult to explain the drastic increase of GFP accumulation in calcium-sufficient cultures (Table 3), but at least Ca^{2+} can induce protein secretions involving in PS2 and CgR0949 signal sequences.

Obviously, we cannot eliminate the possibility of calcium influence on transcriptome of *C. glutamicum* because it is a well-known signal molecule that triggers a wide variety of cellular events in bacteria (Dominguez 2004). Calcium might affect the expression of genes involved in secretion pathway. Further investigation, such as transcriptomic and proteomic analyses, should further clarify the effects of Ca^{2+} on protein secretion.

C. glutamicum possesses a variety of cell surface proteins by proteomic analysis (Hansmeier et al. 2006). In our secretory system, GFP was additionally detected in the cell surface fraction, suggesting that a part of secreted GFP was trapped on the cell wall of *C. glutamicum* (Table 3). The *C. glutamicum* cell envelope containing the mycolic acid layer is known to act as a permeability barrier (Daffé 2005). Of the total GFP, about 20% was located in the cell surface fractions (Table 3), indicating that improving the permeability of cell envelope is needed to increase protein secretion when using Gram-positive bacteria as a host.

In summary, calcium was an essential component for the mass production of heterologous protein using secretion system in this study. To date, the amount of secreted GFP by signal sequences ranges from 0.1 to 12 mg l^{-1} (Eiden-Plach et al. 2004; Kjaerulff and Jensen 2005; Su et al. 2004). By optimizing culture conditions as well as plasmids, high level secretion of GFP in *C. glutamicum* was achieved. To improve the secretion efficiency, further tuning is under investigation.

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References

Billman-Jacobe H, Wang L, Kortt A, Stewart D, Radford A (1995) Expression and secretion of heterologous proteases by *Corynebacterium glutamicum*. Appl Environ Microbiol 61:1610–1613

Boekema BK, Beselin A, Breuer M, Hauer B, Koster M, Rosenau F, Jaeger KE, Tommassen J (2007) Hexadecane and Tween 80 stimulate lipase production in *Burkholderia glumae* by different mechanisms. Appl Environ Microbiol 73:3838–3844

Daffé M (2005) The cell envelope of corynebacteria. In: Eggeling L, Bott M (eds) Handbook of *Corynebacterium glutamicum*. CRC Press, USA, pp 121–148

Date M, Yokoyama K, Umezawa Y, Matsui H, Kikuchi Y (2003) Production of native-type *Streptoverticillium mobaraense* transglutaminase in *Corynebacterium glutamicum*. Appl Environ Microbiol 69:3011–3014

Date M, Yokoyama K, Umezawa Y, Matsui H, Kikuchi Y (2004) High level expression of *Streptomyces mobaraensis* transglutaminase in *Corynebacterium glutamicum* using a chimeric pro-region from *Streptomyces cinnamoneus* transglutaminase. J Biotechnol 110:219–226

Date M, Itaya H, Matsui H, Kikuchi Y (2006) Secretion of human epidermal growth factor by *Corynebacterium glutamicum*. Lett Appl Microbiol 42:66–70

Dominguez DC (2004) Calcium signalling in bacteria. Mol Microbiol 54:291–297

Eiden-Plach A, Zagorc T, Heintel T, Carius Y, Breinig F, Schmitt MJ (2004) Viral preprotoxin signal sequence allows efficient secretion of green fluorescent protein by *Candida glabrata*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Appl Environ Microbiol 70:961–966

Feilmeier BJ, Iseminger G, Schroeder D, Webber H, Phillips GJ (2000) Green fluorescent protein functions as a reporter for protein localization in *Escherichia coli*. J Bacteriol 182:4068–4076

Hansmeier N, Chao TC, Pühler A, Tauch A, Kalinowski J (2006) The cytosolic, cell surface and extracellular proteomes of the biotechnologically important soil bacterium *Corynebacterium efficiens* YS-314 in comparison to those of *Corynebacterium glutamicum* ATCC 13032. Proteomics 6:233–250

Hermann T, Pfefferle W, Baumann C, Busker E, Schaffer S, Bott M, Sahm H, Dusch N, Kalinowski J, Puhler A, Bendt AK, Kramer R, Burkovski A (2001) Proteome analysis of *Corynebacterium glutamicum*. Electrophoresis 22:1712–1723

Inui M, Kawaguchi H, Murakami S, Vertès AA, Yukawa H (2004a) Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. J Mol Microbiol Biotechnol 8:243–254

Inui M, Murakami S, Okino S, Kawaguchi H, Vertès AA, Yukawa H (2004b) Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J Mol Microbiol Biotechnol 7:182–196

Itaya H, Kikuchi Y (2008) Secretion of *Streptomyces mobaraensis* pro-transglutaminase by coryneform bacteria. Appl Microbiol Biotechnol 78:621–625

Jollif G, Mathieu L, Hahn V, Bayan N, Duchiron F, Renaud M, Shechter E, Leblon G (1992) Cloning and nucleotide sequence of the *csp1* gene encoding PS1, one of the two major secreted proteins of *Corynebacterium glutamicum*: the deduced NH_2-terminal region of PS1 is similar to the *Mycobacterium* antigen 85 complex. Mol Microbiol 6:2349–2362

Kesavan J, Borisovska M, Bruns D (2007) v-SNARE actions during Ca(2+)-triggered exocytosis. Cell 131:351–363

Kikuchi Y, Date M, Umezawa Y, Yokoyama K, Heima H, Matsui H (2002) Method for the secretion and production of protein. International Patent Cooperation Treaty patent WO02/081694

Kikuchi Y, Date M, Yokoyama K, Umezawa Y, Matsui H (2003) Secretion of active-form *Streptoverticillium mobaraense* transglutaminase by *Corynebacterium glutamicum*: processing of the pro-transglutaminase by a cosecreted subtilisin-Like protease from *Streptomyces albogriseolus*. Appl Environ Microbiol 69:358–366

Kikuchi Y, Date M, Itaya H, Matsui K, Wu LF (2006) Functional analysis of the twin-arginine translocation pathway in *Corynebacterium glutamicum* ATCC 13869. Appl Environ Microbiol 72:7183–7192

Kikuchi Y, Itaya H, Date M, Matsui K, Wu LF (2008) Production of *Chryseobacterium proteolyticum* protein-glutaminase using the twin-arginine translocation pathway in *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 78:67–74

Kinoshita S (1985) Glutamic acid bacteria. In: Demain AL, Solomon NA (eds) Biology of industrial microorganisms. Cummings, London, pp 115–146

Kjaerulff S, Jensen MR (2005) Comparison of different signal peptides for secretion of heterologous proteins in fission yeast. Biochem Biophys Res Commun 336:974–982

Lee PA, Tullman-Ercek D, Georgiou G (2006) The bacterial twin-arginine translocation pathway. Annu Rev Microbiol 60:373–395

Liebl W, Sinskey AJ (1990) Coryneform expression and secretion system. US patent 4,965,197

Liebl W, Sinskey AJ, Schleifer KH (1992) Expression, secretion, and processing of staphylococcal nuclease by *Corynebacterium glutamicum*. J Bacteriol 174:1854–1861

Malumbers M, Mateos ML, Martin FJ (1995) Microorganisms for amino acid production: *Escherichia coli* and Corynebacteria. In: Hui YH, Khachatorians GG (eds) Food biotechnology microorganisms, vol. 2. V.C.H Publishers, New York, pp 423–469

Meissner D, Vollstedt A, van Dijl JM, Freudl R (2007) Comparative analysis of twin-arginine (Tat)-dependent protein secretion of a heterologous model protein (GFP) in three different Gram-positive bacteria. Appl Microbiol Biotechnol 76:633–642

Muller M, Klosgen RB (2005) The Tat pathway in bacteria and chloroplasts (review). Mol Membr Biol 22:113–121

Nishimura T, Teramoto H, Vertès AA, Inui M, Yukawa H (2008) ArnR, a novel transcriptional regulator, represses expression of the *narKGHJI* operon in *Corynebacterium glutamicum*. J Bacteriol 190:3264–3273

Okino S, Inui M, Yukawa H (2005) Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation. Appl Microbiol Biotechnol 68:475–480

Peyret JL, Bayan N, Joliff G, Gulik-Krzywicki T, Mathieu L, Schechter E, Leblon G (1993) Characterization of the *cspB* gene encoding PS2, an ordered surface-layer protein in *Corynebacterium glutamicum*. Mol Microbiol 9:97–109

Salim K, Haedens V, Content J, Leblon G, Huygen K (1997) Heterologous expression of the *Mycobacterium tuberculosis* gene encoding antigen 85A in *Corynebacterium glutamicum*. Appl Environ Microbiol 63:4392–4400

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Smith MD, Flickinger JL, Lineberger DW, Schmidt B (1986) Protoplast transformation in coryneform bacteria and introduction of an alpha-amylase gene from *Bacillus amyloliquefaciens* into *Brevibacterium lactofermentum*. Appl Environ Microbiol 51:634–639

Solem C, Koebmann B, Jensen PR (2008) Control analysis of the role of triosephosphate isomerase in glucose metabolism in *Lactococcus lactis*. IET Syst Boil 2:64–72

Su WW, Guan P, Bugos RC (2004) High-level secretion of functional green fluorescent protein from transgenic tobacco cell cultures: characterization and sensing. Biotechnol Bioeng 85:610–619

Thomas JD, Daniel RA, Errington J, Robinson C (2001) Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli*. Mol Microbiol 39:47–53

Tjalsma H, Bolhuis A, Jongbloed JD, Bron S, van Dijl JM (2000) Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol Mol Biol Rev 64:515–547

Vertès AA, Inui M, Kobayashi M, Kurusu Y, Yukawa H (1993) Presence of *mrr*- and *mcr*-like restriction systems in coryneform bacteria. Res Microbiol 144:181–185

Watanabe K, Tsuchida Y, Okibe N, Teramoto H, Suzuki N, Inui M, Yukawa H (2009) Scanning the *Corynebacterium glutamicum*

R genome for high efficiency secretion signal sequences. Microbiology 155:741–750

Yang J, Moyana T, MacKenzie S, Xia Q, Xiang J (1998) One hundred seventy-fold increase in excretion of an FV fragment-tumor necrosis factor alpha fusion protein (sFV/TNF-alpha) from *Escherichia coli* caused by the synergistic effects of glycine and triton X-100. Appl Environ Microbiol 64:2869–2874

Yukawa H, Inui M, Vertès AA (2006) Genomes and genome-level engineering of amino acid-producing bacteria. In: Wendisch VF (ed) Amino acid biosynthesis, vol. 5. Springer, Berlin, pp 349–401

Yukawa H, Omumasaba CA, Nonaka H, Kós P, Okai N, Suzuki N, Suda M, Tsuge Y, Watanabe J, Ikeda Y, Vertès AA, Inui M (2007) Comparative analysis of the *Corynebacterium glutamicum* group and complete genome sequence of strain R. Microbiology 153:1042–1058