BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Secretion of *Streptomyces mobaraensis* pro-transglutaminase by coryneform bacteria

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Abstract We previously reported on the secretion of *Streptomyces mobaraensis* transglutaminase by *Corynebacterium glutamicum* ATCC13869 (formerly classified as *Brevibacterium lactofermentum*). In the present work, we investigated whether any other coryneform bacteria showed higher productivity than *C. glutamicum* ATCC13869. We found that most coryneform species secreted pro-transglutaminase efficiently. Moreover, we confirmed that *Corynebacterium ammoniagenes* ATCC6872 produced about 2.5 g/l pro-transglutaminase over a 71-h period in a jar fermentor. Our findings suggest that some other coryneform bacteria, especially *C. ammoniagenes* ATCC6872, are potential hosts for industrial scale protein production.

Keywords *Corynebacterium glutamicum* ·
Corynebacterium ammoniagenes · Transglutaminase ·
Protein secretion

Introduction

The secretion of heterologous proteins into culture medium is a potentially useful method of production (Choi and Lee 2004), although the amounts obtained through this approach are generally small. Thus, there remains a need to develop an efficient secretion system for industrial proteins. *Streptomyces mobaraensis* transglutaminase (TG) has been used in the food industry to modify proteins (Yokoyama

et al. 2004). It is utilized in binding meat and fish and in gelled food products such as jelly, yogurt, and cheese. Moreover, it has great potential for use in manufacturing the materials found in cosmetics, thermostable microcapsules, and carriers for immobilized enzymes (Yokoyama et al. 2004). However, so far, an efficient production system for TG has been lacking.

A large number of different coryneform bacteria have been isolated from soil, animals, and plants (Liebl 2005). Some are used in biotechnological production processes; for example, *Corynebacterium glutamicum* and *Corynebacterium ammoniagenes* are well-known industrial producers of amino acids and nucleotides, respectively (Liebl 2005). *C. glutamicum* is a Gram-positive, non-sporulating bacterium with about 53.8% guanine–cytosine (GC) DNA content and its genome has been sequenced (Ikeda and Nakagawa 2003; Kalinowski et al. 2003). This species is extensively used in the industrial production of amino acids, such as glutamate and lysine, which have been applied in human food, animal feed, and pharmaceutical products for several decades (Hermann 2003; Krämer 1994; Liebl 2005). However, there had been only few reports concerning heterologous protein secretion in *C. glutamicum* (Billman-Jacobe et al. 1995; Liebl et al. 1992; Salim et al. 1997). Recently, we demonstrated that TG could be efficiently secreted in an active form using a signal peptide derived from a cell-surface protein of coryneform bacteria in *C. glutamicum* ATCC13869 (Date et al. 2003, 2004; Kikuchi et al. 2003). We showed that *C. glutamicum* ATCC13869 could also secrete an active form of human epidermal growth factor, which consists of 53 amino acid residues, including six cysteine residues that form three disulfide bonds (Date et al. 2006). More recently, it has been shown that the twin-arginine translocation (Tat) pathway, which is a recently detected protein secretion

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pathway, is active in *C. glutamicum* ATCC13869 and that the *Chryseobacterium proteolyticum* pro-protein glutaminase, which is not secreted via the Sec pathway in *C. glutamicum* ATCC13869, is efficiently secreted by the Tat pathway (Kikuchi et al. 2006, 2007). These results demonstrate that protein production using *C. glutamicum* might be useful on an industrial scale. However, little is known about heterologous protein production by other species of coryneform bacteria.

In this report, we show that most other coryneform bacteria also secrete pro-TG efficiently and that *C. ammoniagenes* ATCC6872 is the best producer species, accumulating about 2.5 g/l pro-TG over a period of 71 h in a jar fermentor.

Materials and methods

Bacterial strains, plasmids, and culture medium

The bacterial strains used are shown in Table 1. The plasmids were as follows: pPSPTG1 (Kikuchi et al. 2003), which contains the *cspB* promoter derived from *C. glutamicum* ATCC13869; the CspA signal peptide sequence derived from a cell-surface protein of *C. ammoniagenes* ATCC6872; and the coding region of pro-TG, derived from *S. mobaraensis*, and pPKPTG1 (Kikuchi et al. 2003), in which the signal peptide for pro-TG secretion

Table 1 Pro-TG accumulation by coryneform bacteria carrying pPSPTG1 in MMTG medium at 30°C for 72 h

Strain	Pro-TG (mg/l)
Corynebacterium ammoniagenes ATCC6872	454
Corynebacterium glutamicum ATCC13032	147
Brevibacterium taipei ATCC13744	201
Corynebacterium glutamicum (*Micrococcus glutamicus*) ATCC13761	66
Brevibacterium roseum ATCC13825	269
Corynebacterium glutamicum (*Brevibacterium flavum*) ATCC13826	314
Corynebacterium herculis ATCC13868	54
Corynebacterium glutamicum (*Brevibacterium lactofermentum*) ATCC13869	118
Corynebacterium acetoacidophilum ATCC13870	369
Corynebacterium glutamicum (*Brevibacterium divaricatum*) ATCC14020	391
Brevibacterium saccharolyticum ATCC14066	238
Brevibacterium immariophilium ATCC14068	257
Microbacterium ammoniaphilum ATCC15354	423
Corynebacterium glutamicum (*Corynebacterium lilium*) ATCC15990	246
Corynebacterium callunae ATCC15991	759
Brevibacterium thiogenitalis ATCC19240	156

is replaced by the CspB signal peptide sequence from *C. glutamicum* ATCC13869. The coryneform bacteria were aerobically grown in CM2G medium (Kikuchi et al. 2003) at 30°C in a shaking test tube. Plasmids were introduced by electroporation with a Gene Pulser (Bio-Rad) using the standard protocol for *Corynebacterium* species. For pro-TG production in a test tube culture, we used MMTG medium containing 60 g glucose, 1 g $MgSO_4 \cdot 7H_2O$, 30 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4H_2O$, 450 μg thiamine hydrochloride, 450 μg biotin, 0.15 g dl-methionine, and 50 g $CaCO_3$ per l distilled water, adjusted to pH 7.5 (Kikuchi et al. 2003). Kanamycin (25 mg/l) was added to the culture medium when required.

Jar fermentation

Fermentor growth was carried out in a 1-l jar fermentor (300 ml working capacity). Glycerol stock suspensions of bacteria were stored at −80°C in 20 *v*/*v* glycerol. Glycerol stock suspension (50 μl) was inoculated into 50 ml CM2G medium in a 500-ml Sakaguchi flask, and cultured at 27°C for 24 h. Then, 15 ml of the culture was inoculated into 300 ml MMTG-J medium containing 120 g glucose, 1 g $MgSO_4 \cdot 7H_2O$, 30 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4H_2O$, 450 μg thiamine hydrochloride, 450 μg biotin, 0.15 g dl-methionine, and 0.2 g total nitrogen of soybean hydrolyzate per l distilled water, adjusted to pH 7.2. The jar fermentor operating conditions were as follows: agitation rate=600 rpm, airflow rate=1/2 vvm; temperature=27°C. The culture was automatically maintained at pH 7.2 by the controlled addition of ammonia gas with airflow. The glucose concentrations were analyzed using an AS-210 glucose analyzer (Sakura Seiki, Tokyo, Japan), and the optical densities were measured at 625 nm with a DU640 photometer (Beckman Coulter).

Protein analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 4–20% gradient polyacrylamide gel was carried out as described by Laemmli (1970), with Coomassie brilliant blue R-250 staining. The secreted pro-TG was measured by reverse-phase high-performance liquid chromatography, using purified native TG as a standard (Yokoyama et al. 2002). For processing the pro-TG to convert to an active form, the secreted pro-TG in a culture supernatant was incubated with purified SAM-P45 (Suzuki et al. 1997) at 30°C for 2 h, at a 100:1 ratio of pro-PG to SAM-P45. The purified SAM-P45 was a gift from Dr. S. Taguchi. The active-form TG was purified according to the method described previously (Yokoyama et al. 2002). TG activity was measured by the calorimetric hydroxamate procedure using *N*-carbobenzoxy-L-glutaminylglycine as

described previously (Yokoyama et al. 2002). For determining the *N*-terminal amino acid sequence of the pro-TG, after separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane by electroblotting. The membrane carrying the pro-TG was directly subjected to a gas phase protein sequencer (model PSQ; Shimadzu, Kyoto, Japan) equipped with an in-line amino acid analyzer (model RF-550; Shimadzu) as described previously (Kikuchi et al. 1997).

Results and discussion

Secretion of pro-TG by coryneform bacteria using *C. ammoniagenes* ATCC6872 CspA signal peptide

We transformed the bacteria listed in Table 1 with pPSPTG1, cultured each transformant in MMTG medium at 30°C for 72 h, and then subjected the supernatants to SDS-PAGE. As shown in Fig. 1, each transformant could stably secrete the pro-TG, and high levels of pro-TG with the anticipated molecular weight (42 kDa) were detected in the supernatants for all of the bacteria tested (Table 1). The pro-TG was most efficiently secreted by *C. ammoniagenes* ATCC6872, *Corynebacterium acetoacidophilum* ATCC13870, *C. glutamicum* ATCC14020 (formerly classified as *Brevibacterium divaricatum*), *Microbacterium ammoniaphilum* ATCC15354, and *Corynebacterium callunae* ATCC15991.

We tested 16 strains of coryneform bacteria for the secretion of *S. mobaraensis* pro-TG. As shown in Fig. 1 and Table 1, all of the bacteria secreted pro-TG. We have previously shown that the *C. glutamicum* system has two advantages for heterologous protein secretion: a lack of proteolytic activity in *C. glutamicum* culture supernatants and a small amount of protein secretion by the bacteria themselves (Date et al. 2006; Kikuchi et al. 2003). As a result, the purification of secreted heterologous proteins is relatively easy. From the results shown in Fig. 1, it is clear that the other coryneform bacteria share these advantages for heterologous protein secretion: their culture supernatants contain little endogenous protein and no degradation of pro-TG is detected in the supernatants.

Effect of signal peptide sequence on pro-TG secretion

Next, to investigate whether the secretion efficiency depended on the signal peptide sequence, we examined the effect of using the CspA signal peptide from *C. ammoniagenes* ATCC6872 (Fig. 2a) or the CspB signal peptide from *C. glutamicum* ATCC13869 (Fig. 2a) for the pro-TG secretion. *C. ammoniagenes* ATCC6872, *C. acetoacidophilum* ATCC13870, *C. glutamicum* ATCC14020, *M. ammoniaphilum* ATCC15354, and *C. callunae*
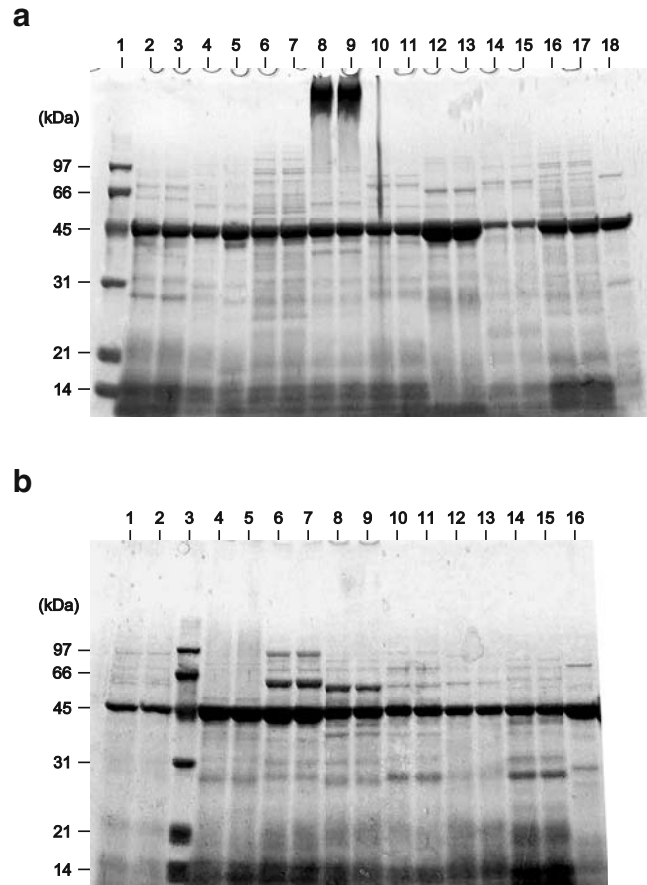

Fig. 1 SDS-PAGE analysis of pro-TG secretion by coryneform bacteria carrying pPSPTG1. Each track contained a 10-μl aliquot of supernatant mixed with an equal volume of sample buffer. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. **a** *Lane 1*, molecular weight markers; *lanes 2* and *3*, *B. saccharolyticum* ATCC14066; *lanes 4* and *5*, *B. roseum* ATCC13825; *lanes 6* and *7*, *B. immariophilium* ATCC14068; *lanes 8* and *9*, *C. glutamicum* ATCC13826; *lanes 10* and *11*, *C. glutamicum* ATCC15990; *lanes 12* and *13*, *C. callunae* ATCC15991; *lanes 14* and *15*, *C. herculis* ATCC13868; *lanes 16* and *17*, *C. acetoacidophilum* ATCC13870; *lanes 18*, *C. ammoniagenes* ATCC6872. **b** *Lanes 1* and *2*, *C. glutamicum* ATCC13761; *lane 3*, molecular weight markers; *lanes 4* and *5*, *C. glutamicum* ATCC14020; *lanes 6* and *7*, *M. ammoniaphilum* ATCC15354; *lanes 8* and *9*, *B. taipei* ATCC13744; *lanes 10* and *11*, *B. thiogenitalis* ATCC19240; *lanes 12* and *13*, *C. glutamicum* ATCC13869; *lanes 14* and *15*, *C. glutamicum* ATCC13032; *lane 16*, *C. ammoniagenes* ATCC6872

ATCC15991 could efficiently secrete the pro-TG using the CspA signal peptide from *C. ammoniagenes* ATCC6872 in this study. These strains and *C. glutamicum* ATCC13869 were transformed with pPKPTG1 harboring the CspB signal peptide from *C. glutamicum* ATCC13869. As shown in Fig. 2b, the substitution of the CspB signal peptide had little effect on pro-TG accumulation in *C. acetoacidophilum* ATCC13870, *C. glutamicum* ATCC14020, and *M. ammoniaphilum* ATCC15354. However, in *C. callunae* ATCC15991, the pro-TG accumulation (403 mg/l) was approximately halved (746 mg/l) while in

Fig. 2 Effect of signal peptide sequence on pro-TG secretion in coryneform bacteria. **a** Amino acid sequence alignment of *C. ammoniagenes* CspA signal peptide and *C. glutamicum* CspB signal peptide using Vector NTI software. Identical and similar residues are shown on *black* and *gray backgrounds*, respectively. **b** Accumulation of secreted pro-TG by coryneform bacteria carrying pPSPTG1 or pPKPTG1. The *solid bars* indicate pro-TG accumulation using the CspA signal peptide from *C. ammoniagenes* ATCC6872, and the *open bars* indicate pro-TG accumulation using the CspB signal peptide from *C. glutamicum* ATCC13869
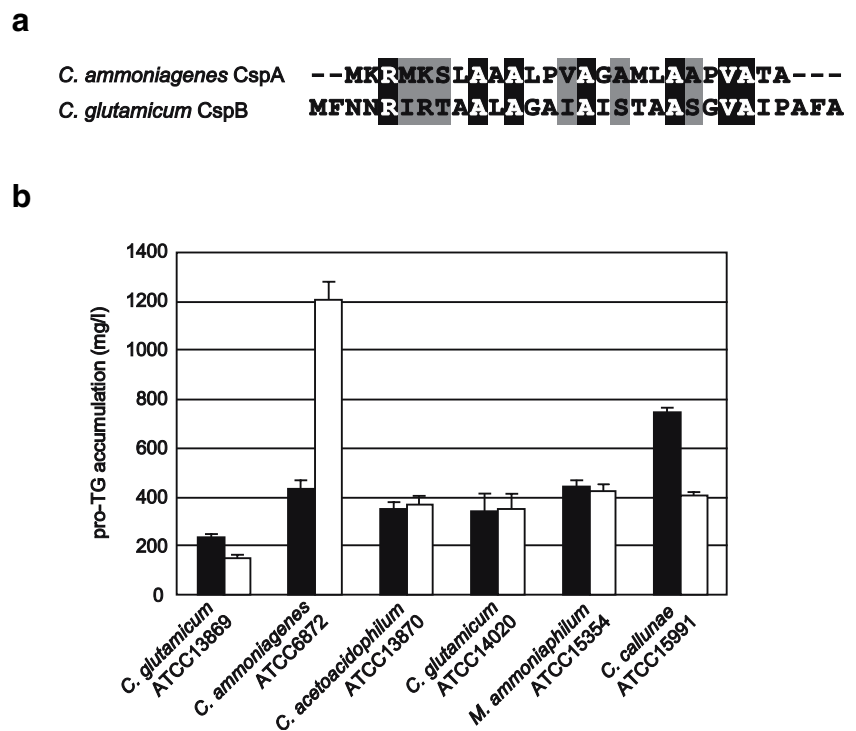
C. ammoniagenes ATCC6872, it was about 2.8-fold higher.

The amount of pro-TG accumulation by *C. ammoniagenes* ATCC6872 using the CspB signal peptide from *C. glutamicum* ATCC13869 was about 2.8-fold higher than with its own CspA signal peptide (Fig. 2b). Interestingly, the difference between the effects of the two signal peptides was the opposite in *C. glutamicum* ATCC13869: the amount of pro-TG accumulation using the CspA signal peptide from *C. ammoniagenes* ATCC6872 was about 1.5-fold higher than with the CspB signal peptide from *C. glutamicum* ATCC13869 (Kikuchi et al. 2003). We found the same to be true for all of the heterologous proteins examined so far (data not shown). The reason for the differing effects of these signal peptides is unclear.

Cultivation of *C. ammoniagenes* ATCC6872 carrying pPKPTG1 in a jar fermentor

As *C. ammoniagenes* ATCC6872 carrying pPKPTG1 was the best producer strain in our study, we subjected it to a fermentation test in a jar fermentor under the conditions described in the "Materials and methods" section. The MMTG-J medium contained 120 g/l glucose; after the consumption of glucose for 71 h under these conditions, the optical density at 625 nm reached approximately 140, and the dry cell concentration was approximately 50 g/l. During the exponential growth phase, little pro-TG accumulated in the culture supernatant; however, the rate of

accumulation increased linearly after entry into the stationary phase, and approximately 2.5 g/l pro-TG was produced (Fig. 3). The *N*-terminal amino acid of the secreted pro-TG was Asp, as in native pro-TG (Kikuchi et al. 2003), demonstrating that the CspB signal peptide had been correctly processed in *C. ammoniagenes* ATCC6872. We then incubated the culture supernatant with purified SAM-P45, which is a subtilisin-like protease from *Streptomyces albogriseolus*, for 2 h at a 100:1 ratio of pro-TG to SAM-P45 in order to process the pro-domain (Kikuchi et al. 2003). The pro-TG secreted by *C. ammoniagenes*
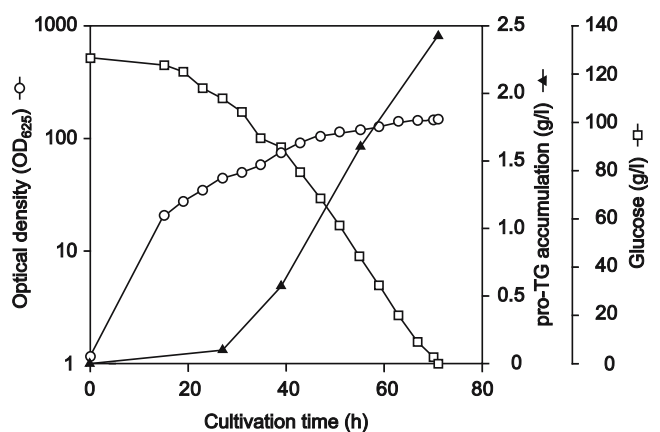

Fig. 3 Jar fermentation experiment employing *C. ammoniagenes* ATCC6872 carrying pPKPTG1. Growth (optical density at 625 nm), glucose concentration, and pro-TG accumulation are indicated by *open circles*, *open squares*, and *closed triangles*, respectively

ATCC6872 carrying pPKPTG1 was cleaved by purified SAM-P45 to the C-side of 41-Ser of the pro-domain and converted to an active form and the conversion yield of this enzymatic process was approximately 100%. The specific activity of the purified active-form TG, with additional Phe-Arg-Ala-Pro residues, was similar to that of the native TG (about 23 U/mg; Kikuchi et al. 2003).

The results of the fermentation experiments in the 1-l jar fermentors showed that the pro-TG secreted by *C. ammoniagenes* ATCC6872 was correctly folded. Thus, protein production using *C. ammoniagenes* ATCC6872 could be useful for industrial scale protein production.

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