TatABC Overexpression Improves Corynebacterium glutamicum Tat-Dependent Protein Secretion

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The twin-arginine translocation (Tat) pathway in Corynebacterium glutamicum has been described previously. The minimal functional Tat system in C. glutamicum required TatA and TatC but did not require TatB, although this component was required for maximal efficiency of Tat-dependent secretion. We previously demonstrated that Chryseobacterium proteolyticum pro-protein glutaminase (pro-PG) and Streptomyces mobaraensis pro-transglutaminase (pro-TG) could be secreted via the Tat pathway in C. glutamicum. Here we report that the amounts of pro-PG secreted were more than threefold larger when TatC or TatAC was overexpressed, and there was a further threefold increase when TatABC was overexpressed. These results show that the amount of TatC protein is the first bottleneck and the amount of TatB protein is the second bottleneck in Tat-dependent protein secretion in C. glutamicum. In addition, the amount of pro-TG that accumulated via the Tat pathway when TatABC was overexpressed with the TorA signal peptide in C. glutamicum was larger than the amount that accumulated via the Sec pathway. We concluded that TatABC overexpression improves Tat-dependent pro-PG and pro-TG secretion in C. glutamicum.

Protein deamidation, which hydrolyzes the amido groups on glutamine or asparagine residues in proteins, is of great interest to the food industry because it has potential applications for improving the usefulness of a variety of food proteins (11, 41). A new protein glutaminase (PG), catalyzing the deamidation of proteins, was discovered in culture supernatants of C. glutamicum (46, 47). However, the amount of PG produced by C. proteolyticum was too small for industrial applications. Streptomyces mobaraensis transglutaminase (TG) has been used in the food industry (50) to bind meat and fish and in gelled food products such as jelly, yogurt, and cheese. Moreover, this enzyme has great potential for use in the manufacture of materials found in cosmetics, thermostable microcapsules, and carriers of immobilized enzymes. Hence, there is a need to develop a more efficient system for production of TG.

Extracellular protein production has several advantages over a cytoplasmic system. In many cases, the N-terminal amino acid residue of the secreted protein is identical to that of the natural protein, because the signal peptide is completely removed by a specific signal peptidase during secretion (44). In addition, due to disulfide bond formation, the correct protein conformation is more likely to occur when the enzyme is secreted, because the extracellular environment is more oxidative than the cytoplasmic environment (26). Finally, purification of a secreted protein is likely to be easier, as there are fewer contaminating proteins in the culture medium than in the cytoplasm.

Most proteins secreted by bacteria are translocated across the cytoplasmic membrane by the Sec pathway, which acts on unfolded proteins using the energy provided by ATP hydrolysis (4, 33). Recently, the novel twin-arginine translocation (Tat) pathway was discovered (1, 3, 36); this pathway differs from the Sec pathway because it translocates folded proteins using the transmembrane proton electrochemical gradient. Various model bacterial systems have been extensively studied, including the systems of the gram-negative bacterium Escherichia coli and the gram-positive bacteria Bacillus subtilis (15, 29, 31), Streptomyces lividans (8, 39), Mycobacterium smegmatis (27, 32), and Corynebacterium glutamicum (20). The Tat pathway might have advantages over the Sec pathway for the production of heterologous proteins, because many proteins fold tightly before they reach the Sec machinery and so cannot engage with it for translocation across the cytoplasmic membrane. Although some recent reports have described use of the Tat pathway for protein production, the efficiency of this pathway seems to be too low for industrial protein production in S. lividans and B. subtilis (9, 10, 24, 40, 45).

C. glutamicum is a gram-positive, nonsporulating bacterium with a G+C content of 53.8% for the total DNA (13, 16). This bacterium has been used for several decades for industrial production of amino acids, such as glutamate and lysine for use in human and animal foods, as well as pharmaceutical products (12, 22). However, there have been few reports concerning heterologous protein secretion in C. glutamicum (2, 25, 34). Recently, we demonstrated that TG and human epidermal growth factor can be efficiently secreted in active forms by the C. glutamicum Sec pathway (5, 6, 7, 19). In addition, the Tat pathway in C. glutamicum was shown to specifically mediate the secretion of Arthrobacter globiformis isomaltodextranase (IMD) (14), as well as green fluorescent protein (GFP) (30) carrying an E. coli TorA signal peptide (20, 37). We also showed that pro-TG and pro-PG could be produced using the Tat pathway in C. glutamicum (20, 21). More recently, Tat pathway-dependent secretion of GFP has been shown to be far

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more efficient in \textit{C. glutamicum} than in two other gram-positive bacteria, \textit{B. subtilis} and \textit{Staphylococcus carnosus} (28), indicating that \textit{C. glutamicum} could be a useful host for the production of heterologous proteins.

Here we show that the amounts of pro-PG and pro-TG secreted via the Tat pathway by \textit{C. glutamicum} are dramatically increased by overexpressing \textit{tatABC}, which encode the core complex of the twin-arginine translocase. This finding demonstrates that protein production using the \textit{C. glutamicum} Tat pathway could be useful for industrial-scale protein production.

\section*{MATERIALS AND METHODS}

\subsection*{Bacterial strains, plasmids, and culture media.}

The bacterial strains and plasmids used in this study are listed in Table 1. \textit{E. coli} JM109 was grown in Luria-Bertani broth (35) and used as an intermediate host for plasmid construction. \textit{C. glutamicum} was grown at 30°C in CM2 medium (21). Protein secretion was examined using \textit{C. glutamicum} cultures grown in MMTG medium at 30°C for 72 h, as described previously (21). \textit{C. glutamicum} was transformed by electroporation using a Gene Pulser (Bio-Rad) according to the manufacturer’s protocol. Kanamycin (Km) (25 μg/ml) and chloramphenicol (Cm) (5 μg/ml) were added to the culture medium when required.

\subsection*{DNA manipulations.}

DNA manipulations were performed as described by Sambrook et al. (35). PCRs were performed with 50-μl reaction mixtures containing Pyrobest DNA polymerase (Takara Bio, Kyoto, Japan) using 5 min at 94°C, followed by 25 cycles of 10 s at 94°C, 30 s at 55°C, and 3 min at 72°C, as specified by the manufacturer. Nucleotide sequences were determined using a BigDye Terminator cycle sequencing FS Ready Reaction kit (Applied Biosystems) and a model 310 DNA sequencer (Applied Biosystems).

\subsection*{Construction of plasmids used for tatAC and tatABC overexpression.}

Plasmids used for expression of \textit{tatAC} and \textit{tatABC} were constructed by PCR. \textit{tatAC} was amplified from the chromosomal DNA of \textit{C. glutamicum} ATCC 13869 using primers TatA5 (5’-GGCGGTACCCAATGGACCTCAAAGCACGG-3’) and TatB3 (5’-GGCGTGATCATTCCTTTAAGG-3’). The amplified fragments were inserted into the SmaI site of pVC7 to obtain pVtatAC. To obtain a plasmid for \textit{tatABC} expression, we amplified a DNA fragment of the promoter and the coding regions of \textit{tatA} and \textit{tatC} by using primers primes TatBSK (5’-GGCGGTACCCAATGGACCTCAAAGCACGG-3’) and TatBSK (5’-GGCGTGATCATTCCTTTAAGG-3’). The amplified fragment was digested with KpnI, inserted into the KpnI site of pVtatAC to obtain pVtatABC (Fig. 1), and confirmed by sequencing. The nucleotide sequences were determined as described above.

\subsection*{Protein analysis.}

Proteins were separated by 4 to 20% gradient polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (25) and stained with Coomassie brilliant blue R-250. For N-terminal amino acid sequencing, proteins were transferred to polyvinylidene difluoride membranes by electroblotting after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. PG activity was assayed using Cbz-Gln-Gly (Peptide Laboratory, Osaka, Japan) as the substrate, and the enzyme units used have been described previously (46).

\begin{table}[h]
\centering
\caption{Bacterial strains and plasmids}
\begin{tabular}{|l|l|l|}
\hline
Strain or plasmid & Characteristics & Reference \\
\hline
\textit{E. coli} JM109 & recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F’ [traD36 proAB’ lacIq lacZΔM15] & 48 \\
\textit{C. glutamicum} strains & & \\
ATCC 13869 & Wild type & 19 \\
YDK010 & ATCC 13869 N-methyl-N’-nitro-N-nitrosooguanidine mutant, ΔcspB & 18 \\
YDK010B & YDK010 ΔtatB & 20 \\
YDK010C & YDK010 ΔtatC & 20 \\
YDK010E & YDK010 ΔtatE & 20 \\
YDK010AE & YDK010 ΔtatA ΔtatE & 20 \\
Plasmids & & \\
pVC7 & \textit{E. coli}-\textit{C. glutamicum} shuttle vector, Cm’ & 19 \\
pPPTGG & pPK4 carrying the pro-PG gene fused with the TorA signal peptide from \textit{E. coli} & 20 \\
pPSPTG1 & pPK4 carrying the pro-TG gene fused with the CspA signal peptide from \textit{C. ammoniagenes} & 19 \\
pPPTPTG & pPK4 carrying the pro-TG gene fused with the TorA signal peptide from \textit{E. coli} & 20 \\
pPPIPTG & pPK4 carrying the pro-TG gene fused with the IMD signal peptide from \textit{A. globiformis} & 20 \\
pVtatA & pVC7 carrying \textit{tatA} & 20 \\
pVtatB & pVC7 carrying \textit{tatB} & 20 \\
pVtatC & pVC7 carrying \textit{tatC} & 20 \\
pVtatAC & pVC7 carrying \textit{tatAC} & This study \\
pVtatABC & pVC7 carrying \textit{tatABC} & This study \\
\hline
\end{tabular}
\end{table}

\section*{RESULTS}

\subsection*{Minimum requirements for a functional Tat system for pro-PG secretion in \textit{C. glutamicum}.}

We investigated the effect of deleting individual \textit{tat} genes on the secretion of pro-PG with a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Schematic diagram of the pVtatABC construct for \textit{tat} gene expression. The coding regions of \textit{tatA} and \textit{tatC} are indicated by filled rectangles, and coding region of \textit{tatB} is indicated by an open rectangle. The filled and open triangles indicate the \textit{tatAC} promoter and the \textit{tatB} promoter, respectively. The arrows below the genes indicate the direction of transcription. Details concerning construction are described in Materials and Methods.}
\end{figure}
signal peptide in *C. glutamicum*. Plasmid pPTPPG (TorAss-pro-PG) was introduced into each *tat* deletion mutant, and supernatants of the transformants were analyzed. No pro-PG was detected in the supernatants of YDK010C (Δ*tatC*) or YDK010AE (Δ*tatAE*) mutants (Fig. 2A, lane 2; Fig. 2D, lane 2; and Fig. 2E, lane 2), a small amount (~24% of the level in wild-type strains as determined by HPLC analysis [data not shown]) was secreted by YDK010B (Δ*tatB*) (Fig. 2B, compare lanes 1 and 2), and pro-PG was secreted by YDK010E (Δ*tatE*) at levels close to the levels secreted by the wild-type strain (Fig. 2C, lane 2).

We also performed complementation experiments to examine restoration of pro-PG secretion in each *tat* mutant. The secretion of pro-PG was partially restored in the Δ*tatC* and Δ*tatAE* mutants by introducing *tatC* on pVtatC and *tatAE* on pVtatA (Fig. 2A, lane 3, and Fig. 2D, lane 3). In addition, *tatE* in the pVtatE plasmid partially complemented the defect in pro-PG secretion in the Δ*tatE* mutant (Fig. 2D, lane 4). Therefore, *TatA* and *TatE* appeared to have overlapping functions. However, introducing *tatB* on pVtatB did not restore pro-PG secretion in the Δ*tatAE* mutant (Fig. 2E, lane 3).

**Evaluation of the effect of Tat gene overexpression on Tat-dependent pro-PG secretion.** The amount of pro-PG accumulated by ATCC 13869 via the Tat pathway was approximately fourfold larger than the amount in YDK010 (21). Therefore, we used ATCC 13869 as a host for pro-PG production via the Tat pathway. ATCC 13869 carrying pPTPPG for expression of pro-PG with the *E. coli* TorA signal peptide was transformed with pVC7, pVtatA, pVtatB, pVtatC, pVtatAC, and pVtatABC. The transformants were cultured in MMTG medium (19) containing 25 mg/liter Km and 5 mg/liter Cm at 30°C for 72 h. The amounts of pro-PG secreted were assessed by analytical reverse-phase HPLC, and are shown in Fig. 3. The amount of pro-PG secreted was determined by analytical reverse-phase HPLC and were found to be 118 mg/liter for ATCC 13869/pPTPPG/pVC7 (TorAss-pro-PG), 112 mg/liter for ATCC 13869/pPTPPG/pVtatA (TorAss-pro-PG plus *tatA*), 17 mg/liter for ATCC 13869/pPTPPG/pVtatB (TorAss-pro-PG plus *tatB*), 378 mg/liter for ATCC 13869/pPTPPG/pVtatC (TorAss-pro-PG plus *tatC*), 379 mg/liter for ATCC 13869/pPTPPG/pVtatAC (TorAss-pro-PG plus *tatAC*), and 1,237 mg/liter for ATCC 13869/pPTPPG/pVtatABC (TorAss-pro-PG plus *tatABC*). The amount secreted by ATCC 13869/pPTPPG/pVtatABC (TorAss-pro-PG plus *tatABC*) was more than threefold higher than the amount secreted by any other strain (Fig. 3). The N-terminal amino acid of each secreted pro-PG was confirmed to be Asp, as expected from the native pro-PG sequence, demonstrating that the TorA signal peptide is correctly processed in *C. glutamicum*.

We have previously reported that SAM-P45 hydrolyzes the pro domain of pro-PG and converts it to an active form (21). When pro-PG secreted via the Tat pathway by ATCC 13869 carrying pPTPPG and pVtatABC was incubated with purified SAM-P45, it was cut on the C-terminal side of Thr112, leaving additional Asn and Lys residues from the pro domain in the mature protein, and the yield was ~65% (data not shown). The specific activity of the product was ~26 U/mg, which is similar to that of native PG (47). Thus, pro-PG was successfully routed to the Tat pathway in *C. glutamicum* when TatABC was overexpressed, and it was correctly folded.

**Evaluation of the effect of TatABC overexpression on Tat-dependent pro-TG secretion.** YDK010 is a better strain than ATCC 13869 for protein production via the Sec pathway, whereas ATCC 13869 is a better strain for protein production via the Tat pathway (6, 19, 21). Therefore, we transformed YDK010 carrying pPSPTG1, a Sec-dependent strain producing pro-TG with the CspA signal peptide derived from *Corynebacterium ammoniagenes* (43), with pVC7. In parallel, we transformed ATCC 13869 carrying pPTPPG or pPIPTG, each...
of which expresses pro-TG using the Tat-dependent *E. coli* TorA or *A. globiformis* IMD signal peptide in *C. glutamicum*, with pVC7 and pVtatABC. The transformants were cultured in MMTG medium (19) containing 25 mg/liter Km and 5 mg/liter Cm at 30°C for 72 h. The amounts of pro-TG that accumulated were assessed by analytical reverse-phase HPLC (Fig. 4). The amounts of pro-TG that accumulated were assessed by analytical reverse-phase HPLC for pro-TG and were found to be 427 mg/liter for YDK010/pPSPTG/pVC7 (CspAss-pro-TG), 28 mg/liter for ATCC 13869/pPTPTG/pVC7 (TorAss-pro-TG), 846 mg/liter for ATCC 13869/pPTPTG/pVtatABC (TorAss-pro-TG plus tatABC), 32 mg/liter for ATCC 13869/pPIPTG/pVtatABC, and 160 mg/liter for ATCC 13869/pPIPPG/pVtatABC (IMDss-pro-TG plus tatABC). The first N-terminal amino acid of each pro-TG secreted by the Tat pathway was Asp, as it was in native pro-TG, demonstrating that the TorA and IMD signal peptides were correctly processed in *C. glutamicum*.

We have previously reported that SAM-P45 hydrolyzes the pro domain of pro-TG and converts it to an active form (19). The pro-TG secreted via the Tat pathway by *C. glutamicum* ATCC 13869 carrying pPTPPG and pVtatABC or pPIPTG and pVtatABC was incubated with purified SAM-P45. The SAM-P45 cleaved the pro-TG on the C-terminal side of Ser41, leaving additional Phe-Arg-Ala-Pro residues from the pro domain in the mature protein, and the yield was ~100% (data not shown). The specific activity of the purified TG was ~26 U/mg, which is similar to the specific activity of native TG (19).

**DISCUSSION**

In a previous report, we showed that GFP could be secreted via the Tat pathway, but not via the Sec pathway, in *C. glutamicum*. The *tatA* and *tatC* genes are clustered, while *tatB* and *tatE* are located elsewhere on the chromosome in *C. glutamicum*. The minimal functional Tat system in *C. glutamicum* required TatA and TatC, as well as TatA and TatE, which had overlapping functions, but did not require TatB, although this protein was needed for maximal efficiency (20). In *E. coli*, *tatA*, *tatC*, *tatB*, and *tatE* are all essential for secretion (38). In *S. lividans*, only *tatC* is essential, and *tatA* and *tatB* are important but not crucial; moreover, *tatA* and *tatB* can complement each other (8). In *B. subtilis*, a functional Tat pathway requires a minimum of one TatA homolog and one TatC homolog (15). Here we showed that, like GFP secretion, pro-PG secretion via the Tat pathway in *C. glutamicum* was completely blocked in both the Δ*tatAE* and Δ*tatC* mutants and was substantially reduced in the Δ*tatB* mutant and that *tatB* could not restore pro-PG secretion to the Δ*tatAE* mutant (Fig. 2E, lane 3); as in other gram-positive bacteria, the minimal functional Tat translocase in *C. glutamicum* seems to be composed of TatA and TatC.

TatA overexpression had little effect on the amount of pro-PG secreted, while TatB overexpression reduced secretion for reasons that are not clear. Secretion increased more than threefold when TatC was overexpressed (Fig. 3). TatAC overexpression had no further effect, whereas overexpression of TatABC increased secretion an additional threefold. These results demonstrate that the amount of TatC protein is the first bottleneck in Tat-dependent protein secretion in *C. glutamicum* and the amount of TatB protein is the second bottleneck.

We reported previously that in *C. glutamicum*, pro-TG can be secreted by the Sec pathway as well as by the Tat pathway, depending only on the signal peptide used (20). The fundamental difference between the two systems is that the Sec pathway is capable of secreting only unfolded proteins, whereas the Tat machinery exports folded proteins. The amount of pro-TG secreted via the Sec pathway in YDK010 was approximately threefold larger than the amount secreted in ATCC 13869 (6, 19), whereas the amount of pro-PG accumulated by ATCC 13869 via the Tat pathway in test tube cultures was approximately fourfold larger than the amount accumulated by YDK010 (21). Therefore, we generally use YDK010 for protein secretion via the Sec pathway and ATCC 13869 for protein secretion via the Tat pathway. The quantity of pro-TG secreted via the Tat pathway (28 mg/liter for TorAss-pro-TG or 32 mg/liter for IMDSS-pro-PG) was less than the quantity secreted via the Sec pathway (427 mg/liter). However, when TatABC was overexpressed in *C. glutamicum* and pro-PG was secreted via the Tat pathway, the amounts of pro-PG accumulated using the TorA and IMD signal peptides increased by more than 30-fold (846 mg/liter) and 5-fold (160 mg/liter), respectively. As a result, the amount of pro-TG that accumulated via the Tat pathway using the TorA signal when TatABC was overexpressed was larger than the amount that accumulated via the Sec pathway.

Generally, the Tat pathway translocates folded proteins, many of which are in complexes with cofactors, and the amounts secreted are small. Some recent reports have described use of the Tat pathway for protein production in *S. lividans* and *B. subtilis*, although the efficiency seems to be too low for industrial exploitation (9, 10, 24, 40, 45). Here we assessed the effect of TatABC overexpression on secretion of pro-TG via the *C. glutamicum* Tat pathway and found that the yield could be as great as 846 mg/liter, which is greater than the yield obtained for the Sec pathway in test tube cultures. This implies that the Tat pathway has great potential for replacing the Sec pathway in industrial-scale production of proteins.

**FIG. 4.** Effect of TatABC coexpression on pro-TG secretion in *C. glutamicum*. CspAss-pro-TG, YDK010/pPSPTG/pVC7; TorAss-pro-TG, ATCC 13869/pPTPTG/pVC7; TorAss-pro-PG+tatABC, ATCC 13869/pPTPTG/pVtatABC; IMDss-pro-TG, ATCC 13869/pPIPTG; IMDss-pro-PG+ tatABC, ATCC 13869/pPIPTG/pVtatABC; IMDss-pro-PG, ATCC 13869/pPIPTG/pVtatABC; TorAss-pro-PG, ATCC 13869/pPIPTG/pVtatABC.
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