JOURNAL OF BACTERIOLOGY, Mar. 1992, p. 1854–1861
0021-9193/92/061854-08$02.00/0

Vol. 174, No. 6

Expression, Secretion, and Processing of Staphylococcal Nuclease by *Corynebacterium glutamicum*

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Received 13 September 1991/Accepted 13 January 1992

The gene for staphylococcal nuclease (SNase), an extracellular enzyme of *Staphylococcus aureus*, was introduced into *Corynebacterium glutamicum*. The heterologous gene was expressed in this host organism, and SNase was efficiently exported to the culture medium. Amino-terminal sequencing of SNase secreted by *C. glutamicum* revealed that the signal peptide was apparently cleaved off at precisely the same position as in the original host, *S. aureus*. As with *S. aureus*, a second smaller form of SNase (A form), whose appearance is presumably the result of a secondary processing step, was found in the culture medium of the recombinant *C. glutamicum* strain. The A form was one residue shorter than the mature nuclease A produced by *S. aureus*. Variation of the sodium chloride concentration in the growth medium had a marked influence on the location and the processing of SNase by *C. glutamicum*. In a complex growth medium containing 4% sodium chloride, SNase was exclusively located in the supernatant, but a significant amount of the enzyme remained cell associated if the strain was grown in a low-salt medium. Also, high salt concentrations seemed to inhibit processing of the high-molecular-weight form of SNase (B form) to the smaller A form. Similarities and differences in the export and modes of processing of SNase by three different, nonrelated gram-positive host organisms are discussed. Finally, a versatile *Escherichia coli-C. glutamicum tac-lacI*^{q} expression shuttle vector was constructed. With this vector, it was possible to achieve isopropyl-β-D-galactopyranoside (IPTG)-inducible overexpression and secretion of SNase in *C. glutamicum*, whereby the expression level was dependent on the concentration of the inducer.

Corynebacterium glutamicum is a gram-positive bacterium with a high G+C content of DNA and is closely related to other so-called "amino acid-producing corynebacteria" (12). These organisms are used for the industrial production of certain amino acids. Recent progress in the development of more sophisticated genetic tools for *C. glutamicum* makes this species an interesting alternative gram-positive cloning host. Compared with other established high-G+C gram-positive hosts such as certain *Streptomyces* species, *C. glutamicum* has some advantages; e.g., there are no complex cellular differentiation steps such as mycelium formation or sporulation during growth. Also, *C. glutamicum* does not have the disadvantage of severe genetic instabilities, as are known to occur in *Streptomyces* species. One major advantage over low-G+C gram-positive hosts such as *Bacillus* species or *Staphylococcus* species is the rather broad acceptance of heterologous expression signals, including gram-negative promoters (23), observed for *C. glutamicum* ("*Brevibacterium lactofermentum*").

It is generally accepted that gram-positive bacteria are superior to other bacteria as protein secreters, the most prominent examples being certain *Bacillus* species. Nevertheless, knowledge about protein export by gram-positive bacteria is still very poor. This is especially true for the branch of gram-positive bacteria with a high moles percent G+C. Therefore, we have initiated studies on protein export by a member of this group of bacteria, *C. glutamicum* (15, 16). Practically nothing is known about this organism's capability for and mechanism of protein export. In one case, however, weak expression of a *Bacillus amyloliquefaciens* α-amylase and its appearance in the culture medium of "*B. lactofermentum*" has been reported (26). Also, we were able to isolate a gene encoding an extracellular nuclease from *C. glutamicum* AS019 (16). These studies have made clear that *C. glutamicum* is capable of directing the secretion of proteins synthesized within the cell.

In order to gather more detailed information about protein secretion by *C. glutamicum*, we have now developed a model system in which we use staphylococcal nuclease (also called heat-stable nuclease, thermonuclease, and micrococcal nuclease and hereafter referred to as SNase) as a secreted enzyme. SNase is a small, biochemically well-characterized enzyme. From culture supernatants of *Staphylococcus aureus*, the natural producer of this nuclease, two different forms of nuclease have been recovered: nuclease A (149 amino acids), the mature form, and nuclease B, which differs from nuclease A by an amino-terminal extension of 19 amino acids. Nuclease A is believed to be a processed form of the initially exported nuclease B (5). The gene encoding SNase has been isolated from *S. aureus* strain Foggie (25). This paper deals with the heterologous expression of *S. aureus* SNase in *C. glutamicum* and the use of this system for investigating protein export and processing in this host organism.

MATERIALS AND METHODS

Strains and plasmids. The *C. glutamicum* strains used throughout this study were AS019 (30) and R163, a restriction-deficient mutant of AS019 (14). The *Escherichia coli* strain used for plasmid constructions was JM83 (29). *S. aureus* Foggie was an American Type Culture Collection strain (ATCC 27735). pFOG301 (25), a kind gift from D. Shortle (Baltimore, Md.), was the source of the gene encoding SNase. The *tac* expression vector pJF118ut (2), a deriv-

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TABLE 1. Bacterial plasmids

Plasmid	Relevant characteristics[a]	Propagation in:	Reference
pCA43	Cm^r(S)	*S. carnosus*	9
pNuc1	Cm^r(S) *nuc*	*S. carnosus*	This study
pFOG301	Ap^r(E) *nuc*	*E. coli*	25
pJF118EH	Ap^r(E) *lacI*^{q} P_{tac}	*E. coli*	6
pJF118ut	Ap^r(E) *lacI*^{q} p_{tac}	*E. coli*	2
pWST1	Ap^r(E) Km^r(C)	*E. coli, C. glutamicum*	15
pWLT1	Ap^r(E) Km^r(E,C)	*E. coli, C. glutamicum*	This study
pWLT1d	Ap^r(E) Km^r(E,C)	*E. coli, C. glutamicum*	This study
pWLQ1	Ap^r(E) Km^r(E,C) *lacI*^{q} P_{tac}	*E. coli, C. glutamicum*	This study
pWLQ2	Ap^r(E) Km^r(E,C) *lacI*^{q} P_{tac}	*E. coli, C. glutamicum*	This study
pWNuc1	Ap^r(E) Km^r(C) *nuc*	*E. coli, C. glutamicum*	This study
pWNuc5	Ap^r(E) Km^r(C) *nuc*	*E. coli, C. glutamicum*	This study
pWLQN2	Ap^r(E) Km^r(E,C) *lacI*^{q} P_{tac} *nuc*	*E. coli, C. glutamicum*	This study
pWLQN4	Ap^r(E) Km^r(E,C) *lacI*^{q} P_{tac} *nuc*	*E. coli, C. glutamicum*	This study
pWLQN10	Ap^r(E) Km^r(E,C) *lacI*^{q} P_{tac} *nuc*	*E. coli, C. glutamicum*	This study
pWLQN12	Ap^r(E) Km^r(E,C) *lacI*^{q} P_{tac} *nuc*	*E. coli, C. glutamicum*	This study
pWLQN14	Ap^r(E) Km^r(E,C) *lacI*^{q} P_{tac} *nuc*	*E. coli, C. glutamicum*	This study

[a] Abbreviations: Ap^r, ampicillin resistance marker; Km^r, kanamycin resistance marker; Cm^r, chloramphenicol resistance marker; letters in parentheses indicate if the markers function in *E. coli* (E), *S. aureus* (S), or *C. glutamicum* (C); *lacI*^{q}, *lac* repressor gene; P_{tac}, *tac* promoter; *nuc*, SNase gene.

ative of pJF118EH (6), was kindly supplied by A. Böck (München, Germany). The features and, where appropriate, the construction of all plasmids used can be found in Table 1 and in Results, respectively.

Strains were normally propagated in LB medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride, pH 7.2 to 7.3) supplemented with selective antibiotics when appropriate (20 μg of kanamycin per ml for *C. glutamicum* and 50 μg of kanamycin per ml or 100 μg of ampicillin per ml for *E. coli*). The medium used for most SNase expression studies and for the purification of nuclease produced by *C. glutamicum* was LB2, which is LB medium containing 2% tryptone instead of 1%.

Recombinant DNA methods. Restriction endonucleases and DNA modification enzymes were purchased from Boehringer (Mannheim, Germany) and used according to the supplier's instructions. Standard recombinant DNA techniques and the isolation of plasmid DNA from *E. coli* were done as described by Ausubel et al. (1). Plasmid DNA from *C. glutamicum* was extracted according to the method of Yoshihama et al. (30). The introduction of plasmid DNA into *C. glutamicum* was performed with protoplast transformation or electroporation as described previously (11, 30). For transformation of *E. coli*, either a calcium chloride procedure described by Maniatis et al. (18) or the electroporation protocol described by Ausubel et al. (1) was followed.

DNase assays. Production of DNase by recombinant clones was checked on DNase agar (Oxoid) plates as described previously (15). DNase activity in the supernatant of cultures was determined spectrophotometrically by monitoring DNA hydrolysis, which is accompanied by an increase in absorbance at 260 nm, by the method described by Cuatrecasas et al. (4). Chromosomally encoded extracellular DNase activity of *C. glutamicum* AS019 (16) was not detectable under these assay conditions.

Purification of SNase from the supernatant of *C. glutamicum* AS019/pWNuc5. A logarithmic culture of *C. glutamicum* AS019/pWNuc5 was inoculated (0.5%) into LB2 broth supplemented with 1 mM calcium chloride and 20 μg of kanamycin per ml and was grown aerobically at 30°C. The culture (10 liters total) was harvested after 18 h at an optical density (600 nm) of 3.7, and the cells were discarded. The supernatant was mixed with 0.1 volume of 0.2 M Tris-HCl buffer (pH 7) and then passed through a ZetaPrep 250 (SB cartridge; LKB) mass ion exchange unit preequilibrated with 20 mM Tris-HCl (pH 7) at a flow rate of 40 ml/min. Proteins bound to the ion exchange matrix were eluted with 500 ml of 0.1 M Tris-HCl (pH 9)–0.5 M NaCl. The eluate fractions with DNase activity (160 ml) were pooled and concentrated by ultrafiltration, first with an Amicon model 52 unit using a PM10 filter and subsequently with a Centricon 10 spin concentrator (Amicon, Beverly, Mass.).

Protein analysis and immunological detection of SNase. Active nuclease proteins could be visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and removal of the SDS from the gel by an activity-staining procedure. Protein samples were separated on an SDS–12.5% polyacrylamide gel, and half of the gel was cut off and stained with Coomassie brilliant blue. The SDS was removed from the rest of the gel by washing it for 60 min in 40 mM Tris-HCl (pH 7)–25% isopropanol and for another 60 min in 40 mM Tris-HCl, pH 7 (buffer changed every 15 min). The gel was then laid on the agar surface of a petri dish containing 10 ml of toluidine blue agar (10) (50 mM Tris-HCl [pH 9.6], 0.01 mM calcium chloride, 1% agar, 1% sodium chloride, 0.03% boiled salmon testes DNA [Sigma], 0.3 mM toluidine blue O [Sigma]) and incubated at 37°C for 2 to 3 h. Protein species with DNase activity become visible as pink bands against a dark blue background.

For immunoblot experiments, proteins separated by SDS-PAGE were blotted electrophoretically onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) as described previously (13). The specific anti-SNase antibodies used were a polyclonal preparation from a rabbit, kindly supplied by D. Shortle. The second antiserum used for detection was alkaline phosphatase-conjugated anti-rabbit immunoglobulin G [F(ab')$_2$ fragment; Sigma]. The commercial SNase preparation ("micrococcal nuclease"; Sigma) used as a standard in SDS-PAGE and Western blot (immunoblot) experiments represents mature nuclease A from the supernatant of *S. aureus* Foggie.

Amino-terminal protein sequencing. Sequencing of amino-terminal peptide residues was done with a microsequencing method. For this purpose, protein samples (approximately 100 pmol of each protein of interest) were electrophoresed on a 12.5% denaturing polyacrylamide gel and then electro-

blotted onto an Immobilon membrane (Millipore, Bedford, Mass.), as described by Matsudaira (20), and sequenced with an Applied Biosystems 470A gas-phase sequencer. These analyses were carried out by W. Lane of the protein microsequencing facility at Harvard University, Cambridge, Mass.

RESULTS

Introduction of the SNase gene into *C. glutamicum*. The source of the nuclease gene for the construction of nuclease shuttle plasmids pWNuc1 and pWNuc5 was plasmid pNuc1. This plasmid is a derivative of staphylococcal vector pCA43 (9) carrying the *S. aureus* nuclease gene. It was constructed by ligating the nuclease gene as a 1.8-kb *Eco*RI-*Sal*I fragment of pFOG301 with *Xba*I-cut pCA43 (both DNA fragments blunt ended with Klenow fragment of DNA polymerase I) and directly transforming the ligation products into *Staphylococcus carnosus* protoplasts. A spontaneous deletion of approximately 1 kb downstream of the SNase gene yielded plasmid pNuc1 (Fig. 1). The SNase gene was excised from pNuc1 with *Eco*RV and ligated into the *E. coli-C. glutamicum* shuttle vector pWST1 (15) linearized with *Sma*I (Fig. 1). The resulting plasmids carrying the nuclease insert in both possible orientations, designated pWNuc1 and pWNuc5, were transformed into *C. glutamicum* AS019.

Expression and secretion of SNase by *C. glutamicum*. *C. glutamicum* AS019 or R163 transformants bearing the plasmids pWNuc1 or pWNuc5 displayed a DNase-positive phenotype in the plate assay described in Materials and Methods (see Fig. 6) (15), indicating that staphylococcal DNA sequences upstream of the SNase-coding region had promoter activity in *C. glutamicum*. Extracellular SNase production during growth of *C. glutamicum* AS019/pWNuc5 in LB2 liquid medium containing 20 μg of kanamycin per ml was monitored by measuring the DNase activity present in the supernatant at various stages of growth (Fig. 2A). The growth curve and the DNase activity profile had practically identical shapes. The extracellular activity reached a plateau when the bacteria entered the stationary phase. An increase in nuclease activity occurred during growth only. The concentration of SNase in the supernatant of a stationary culture of this strain was measured (not shown) by a semiquantitative agar diffusion assay (10) and was estimated to be roughly 1 to 2 mg/liter.

Localization of SNase produced by *C. glutamicum*. The intracellular precursor form of SNase, which should be 25.1 kDa in size (21), was never detected in crude extracts of either exponential- or stationary-phase *C. glutamicum* AS019/pWNuc5 cells by Western blot experiments (not shown), indicating efficient export across the cellular membrane. Active SNase enzyme was found in the supernatant of growing *C. glutamicum* AS019/pWNuc5 cultures (see above). The presence of SNase in the culture medium was also confirmed by immunoblot experiments. The anti-SNase serum reacted specifically with two bands of approximately 18 and 20.5 kDa. *S. aureus*, the original producer of SNase, has also been observed to produce two different forms of nuclease, termed nuclease A and nuclease B, with molecular masses of about 16.8 and 18.8 kDa, respectively (5). By analogy with the SNase species produced by *S. aureus*, the two forms of SNase secreted by *C. glutamicum* AS019/pWNuc5 are hereafter referred to as the A form and the B form, respectively. In SDS-PAGE, the A and B forms migrated similarly to nuclease A and nuclease B, respectively, of *S. aureus* Foggie (Fig. 3 and 4; data for the B form not shown).

When the supernatant of a stationary-phase culture grown in low-salt (0 or 1% sodium chloride) medium and the crude extract from washed cells corresponding to the same volume of culture were analyzed by SDS-PAGE and immunoblotting, it was found that a substantial amount (up to an estimated 40%, as judged from various Western blots) of the SNase produced was cell associated (Fig. 5). Interestingly, the cell-bound SNase was predominantly the lower-molecular-weight A form. In this and several other Western blots (not shown), it was repeatedly found that during growth in low-salt medium the ratio of B form to A form was significantly smaller for the cell-bound than for the extracellular SNase species. It was not possible to significantly decrease the amount of cell-associated SNase by simply washing the cells with dilute (10 mM) phosphate buffer (pH 7) with or without 4% sodium chloride (not shown). Similarly, sonication or treatment of the cells with lysozyme did not effect release of the cell-associated SNase (not shown). However, when *C. glutamicum* AS019/pWNuc5 was grown to stationary phase in LB2 broth containing 4% sodium chloride, the location of SNase was exclusively extracellular, and cell-associated enzyme could no longer be detected in Western blot experiments (Fig. 5).

Processing of SNase by *C. glutamicum*. SNase secreted to the culture medium by *C. glutamicum* AS019/pWNuc5 was purified from the culture supernatant as described in Materials and Methods. The final concentrate was analyzed by SDS-PAGE. Two major bands at about 18 and 20.5 kDa and a weak band at 38 kDa were visible after protein staining with Coomassie brilliant blue (Fig. 3). Staining of DNase activity after removal of the SDS from the gel revealed that both the 18- and 20.5-kDa bands had DNase activity and thus represent the A and B forms, respectively.

The amino-terminal amino acid sequences of both active peptides were determined. For this purpose, a sample containing approximately 80 pmol of the A form and 40 pmol of the B form was separated by SDS-PAGE and electrophoretically blotted onto a polyvinylidene fluoride membrane (20). Amino-terminal sequence analysis was performed with an Applied Biosystems 470A gas-phase sequencer. The sequence obtained for the B form was H_2N-Ser-Gln-Thr-Asp-Asn-Gly, which corresponds to amino acids −19 to −14 in the sequence published by Shortle (25). This sequence is the same as the N terminus of nuclease B secreted by *S. aureus* (5). Since serine −19 is the first amino acid after the putative SNase signal peptide (25), *C. glutamicum* clearly uses the same signal peptide cleavage site as *S. aureus*. The A form of SNase produced by pWNuc5-bearing *C. glutamicum* had the sequence H_2N-Thr-Ser-Thr-Lys-Lys-Leu, which corresponds to positions 2 through 7 in the SNase sequence of Shortle (25). Thus, the A form found in the culture supernatant of the recombinant *C. glutamicum* strain is one residue shorter than the mature nuclease A of the natural producer *S. aureus* Foggie (3). During sequence determination of the A form, a small degree of "premove" was found, meaning that a small Ser peak was found in the first cycle, a small Thr peak was found in the second cycle, and so on. This indicates that processing of the A form is not strictly specific, thus leading to slight heterogeneity of the amino terminus of the polypeptide.

Overexpression of SNase in *C. glutamicum*. After it was determined that *C. glutamicum* is capable of secreting and processing SNase, it was of interest to try to assess the potential of this organism for high-level overexpression of an

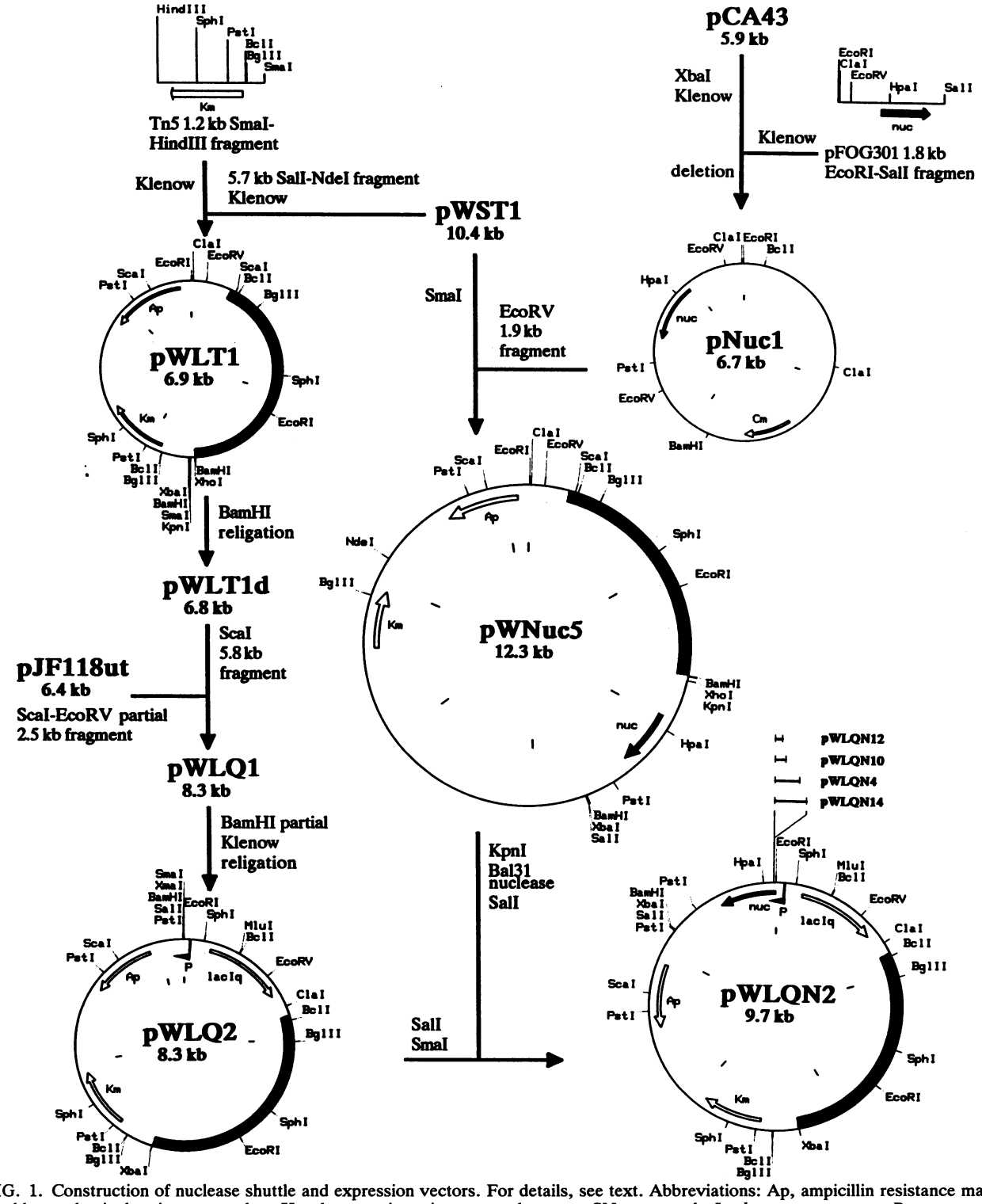

FIG. 1. Construction of nuclease shuttle and expression vectors. For details, see text. Abbreviations: Ap, ampicillin resistance marker; Cm, chloramphenicol resistance marker; Km, kanamycin resistance marker; nuc, SNase gene; lacIq, *lac* repressor gene; P, *tac* promoter. Black boxes represent the corynebacterial replicon pSR1 (Yoshihama et al. [30]). Restriction maps and construction details for the plasmids pCA43, pWST1, pJF118EH, and pJF118ut have been published elsewhere (2, 6, 9, 15).

exported protein. Thus, we sought to place the SNase gene under the transcriptional control of a strong promoter in *C. glutamicum*. Since the *E. coli tac* promoter-*lac* operator-*lacI*^{q} repressor system has been shown to function in *C. glutamicum* (28), we decided to use this system as the basis for the construction of a regulable expression vector.

In order to keep the size of the envisaged expression plasmid small, we first constructed a new, handy *E. coli-C.*

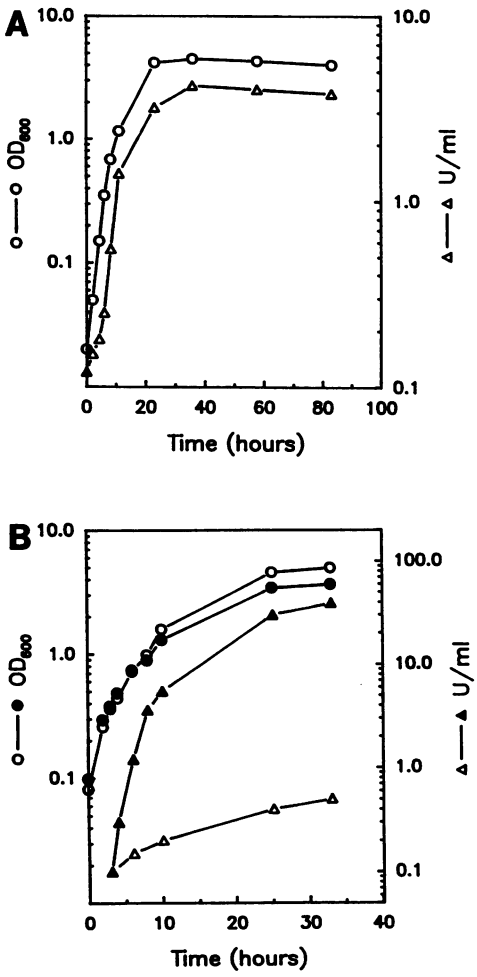

FIG. 2. Production of SNase during growth of *C. glutamicum* AS019/pWNuc5 (A) and *C. glutamicum* R163/pWLQN10 (B) in LB2 broth at 30°C. Note the different scales of the *x* and *y* axes of the two graphs. Circles represent the optical densities of the cultures, and triangles represent SNase activity in the culture supernatants. In the experiment represented in panel B, 1 mM IPTG was added to one of the cultures after 3 h (filled symbols), while the other culture was left without IPTG (open symbols). OD_{600}, optical density at 600 nm.

glutamicum shuttle vector, designated pWLT1 (Fig. 1). For this purpose, the kanamycin resistance gene of transposon Tn*5* was isolated as a 1.2-kb *Sma*I-*Hin*dIII fragment, treated with Klenow fragment, and ligated with the 5.7-kb *Sal*I-*Nde*I fragment (also blunt ended) of pWST1. The kanamycin resistance gene of this new *E. coli-C. glutamicum* shuttle plasmid functions in both host organisms, while the ampicillin resistance marker is useful only for *E. coli*. The *tac* expression shuttle vector pWLQ1 was then derived from pWLT1 by first deleting most of the multiple cloning site (pWLT1d; Fig. 1) and subsequently inserting the *tac* promoter and *lacI*^{q} gene of pJF118ut (2), as outlined in Fig. 1. Plasmid pWLQ2 is an improved expression vector, with the *Bam*HI site in its polylinker the single *Bam*HI site in the plasmid, but otherwise it is identical to pWLQ1.

The SNase gene was inserted downstream of the *tac* promoter-*lac* operator region of pWLQ2 in the following way. pWNuc5 was linearized with *Kpn*I, which is located approximately 600 bp upstream of the SNase-coding region.

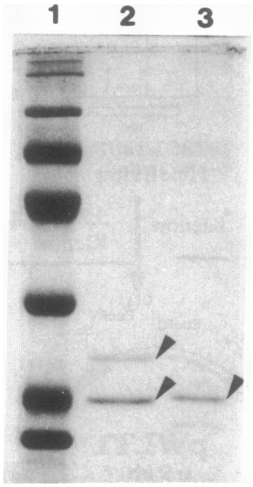

FIG. 3. SDS-PAGE of SNase produced by *C. glutamicum* AS019/pWNuc5. SNase was isolated from the culture supernatant as described in Materials and Methods. Lane 1, molecular mass markers: lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), α-chymotrypsinogen (25.7 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase *b* (97.4 kDa), and myosin (200 kDa); lane 2, SNase isolated from *C. glutamicum* AS019/pWNuc5 supernatant; lane 3, commercial SNase preparation ("micrococcal nuclease" [Sigma] representing mature *S. aureus* nuclease A). After electrophoresis, the gel was stained with Coomassie brilliant blue. The bands with DNase activity (determined with the activity-staining procedure described in Materials and Methods) are marked with arrowheads. Commercial nuclease A (16.8 kDa) displayed unusual mobility in relation to the 18.4-kDa marker protein.

In order to place the SNase open reading frame in close proximity to the *tac* promoter and thus eliminate any possible interference of intermitting sequences, the DNA ends were shortened by nuclease *Bal* 31 treatment. Upon restriction with *Sal*I, the SNase-encoding fragments were ligated

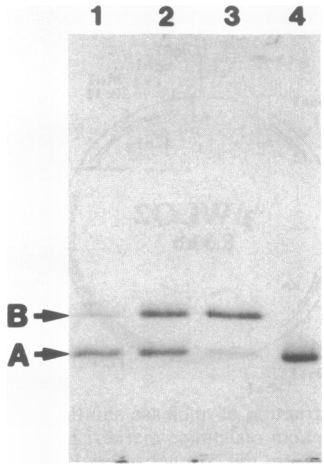

FIG. 4. Effect of different sodium chloride concentrations in the growth medium on the ratio of A to B forms of SNase produced by *C. glutamicum* AS019/pWNuc5 (lanes 1 to 3). The strain was grown for 18 h in LB2 broth containing 0% (lane 1), 1% (lane 2), or 4% (lane 3) sodium chloride, and 5-μl samples of supernatant were applied per slot. Lane 4, 0.01 μg of commercial SNase preparation (Sigma). Arrows on the left-hand margin indicate the positions of the A and B forms of SNase.

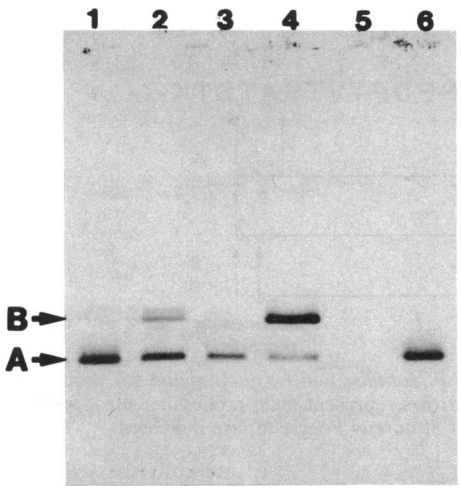

FIG. 5. Location and processing of SNase by *C. glutamicum* AS019/pWNuc5. Supernatants (lanes 2 and 4) and crude extract samples (lanes 3 and 5) were obtained from stationary cultures of the strain grown either in LB2 without sodium chloride (lanes 2 and 3) or in LB2 with 4% sodium chloride (lanes 4 and 5). The different salt concentrations in the culture broth had no significant effect on the final optical density of the cultures (not shown). Crude extracts were prepared from phosphate-buffered saline (10 mM phosphate buffer [pH 7.6]–0.85% sodium chloride)-washed cells by passage through a French pressure cell at 6.9 MPa. For each experiment, the supernatant (12.5 μl) and crude extract (about 9 μg of protein) samples loaded correspond to the same culture volume. Lanes 1 and 6, 0.01 μg of commercial SNase preparation (Sigma). Arrows on the left-hand margin indicate the positions of the A and B forms of SNase.

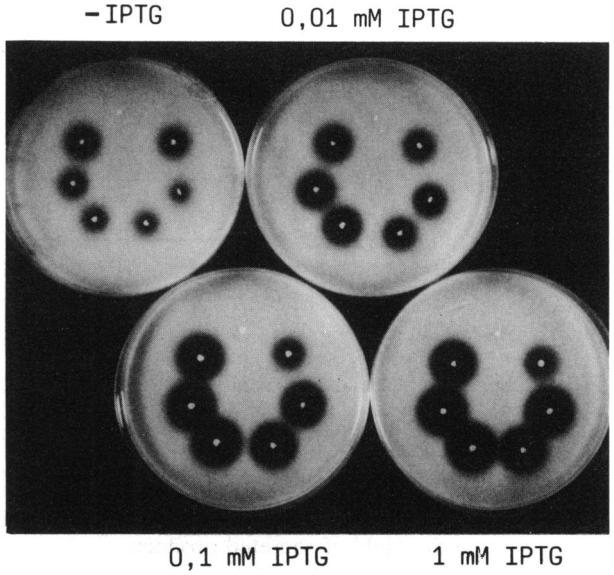

FIG. 6. SNase expression by various *C. glutamicum* strains. The strains were inoculated onto DNase test agar (Oxoid) plates containing 20 μg of kanamycin per ml. Some of the plates contained IPTG at the concentrations indicated. After incubation at 30°C for 18 h, the plates were flooded with 1 N hydrochloric acid. Zones of clearing against a white background of precipitated DNA are indicative of DNase activity. The recombinant *C. glutamicum* strains, starting from the top center of each plate and going in clockwise order, are R163/pWST1, R163/pWNuc5, R163/pWLQN2, R163/pWLQN4, R163/pWLQN10, R163/pWLQN12, and R163/pWLQN14.

into *Sma*I-*Sal*I-digested pWLQ2 (Fig. 1). In the resulting SNase expression plasmids pWLQN2, pWLQN12, pWLQN10, pWLQN4, and pWLQN14, the distances between the vector-borne *Eco*RI site 41 bp downstream of the *tac* promoter −10 region and the putative SNase start codon (amino acid position −79 in the sequence of Shortle [25]) are 20, 50, 150, 200, and 230 bp, respectively (approximate values [±20 bp]; determined by fine restriction mapping).

SNase expression in *C. glutamicum* R163 transformed with the various pWLQN plasmids was examined. Since the *lacI*^{q} repressor gene is present in *cis* on the vector constructs, these plasmids directed isopropyl-β-D-galactopyranoside (IPTG)-inducible SNase production by the *C. glutamicum* transformants (Fig. 6). The amount of SNase secreted during growth increased with rising IPTG concentrations, reaching a plateau of maximum expression at about 0.5 to 1 mM IPTG. Thus, with this system it is possible to adjust the expression level in *C. glutamicum* at will simply by changing the IPTG concentration in the culture medium.

The fact that strains R163/pWLQN12 and R163/pWLQN14 displayed substantial DNase activity even in the absence of inducer (Fig. 6) indicated that the stretch of staphylococcal DNA upstream of about 50 bp of the SNase-coding region contains sequences with promoter activity in *C. glutamicum*. In these cases, induction by IPTG was not as pronounced as with R163/pWLQN2 and R163/pWLQN4, where repression in the absence of inducer appears to be relatively, albeit not entirely, complete. It is not clear why repression in *C. glutamicum* AS019/pWLQN4 seems to be fairly complete whereas strain R163/pWLQN14 produces a relatively large halo on DNase test plates without IPTG (Fig. 6) even though the DNA sequences upstream of the SNase genes of pWLQN4 and pWLQN14 differ only slightly from one another (about a 30-bp difference). The situation observed with strains R163/pWLQN2 and R163/pWLQN12 was similar (Fig. 6). Perhaps the proximity to the *lac* operator of certain sequences in the SNase inserts of the constructs pWLQN12 and pWLQN14 interferes with the effective binding of the *lac* repressor to its site of action.

For one of the SNase expression strains, *C. glutamicum* R163/pWLQN10, nuclease production was monitored during growth in the presence or absence of 1 mM IPTG. The addition of IPTG to the growing culture 3 h after inoculation led to a massive induction of SNase expression and secretion (Fig. 2B). After 33 h of growth, the supernatant of the culture supplemented with IPTG displayed about 80-fold higher SNase activity than the culture lacking inducer.

DISCUSSION

A heterologous gene coding for a secreted enzyme, SNase from the low-G+C gram-positive bacterium *S. aureus*, has been introduced into the high-G+C gram-positive host *C. glutamicum*. The level of SNase expression in *C. glutamicum* AS019/pWNuc5 was not very high (an estimated 1 to 2 mg/liter), but it could be increased about 10-fold by placing the SNase gene under the transcriptional control of the *tac* promoter, as in the expression plasmid pWLQN10 (Fig. 2B and 6). This probably is not yet the upper limit for overexpression of a secreted protein in this host organism, since the G+C content of the SNase open reading frame (34.5 mol%) and thus the codon usage is not optimal for *C. glutamicum*. Additionally, maximum-strength promoters for *C. glutamicum* have yet to be found. Such promoters, in

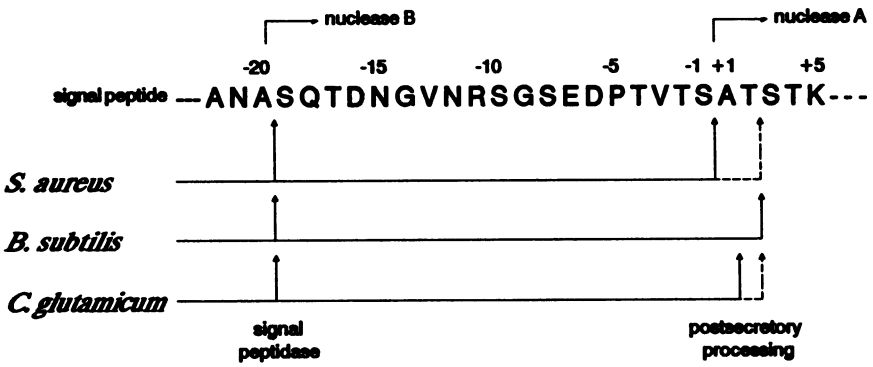

FIG. 7. Signal peptidase and secondary cleavage sites utilized by *S. aureus*, *B. subtilis*, and *C. glutamicum* for processing of SNase. Numbering of the amino acid residues was done as described in reference 25. Arrows represent main processing sites, and broken arrows represent minor processing sites. The N termini of nuclease A and nuclease B of *S. aureus* Foggie (5) are indicated.

conjunction with an optimized translation initiation site, may further improve the yield of secreted product.

The export and processing of SNase have now been studied in three phylogenetically extremely different bacteria: *S. aureus*, *Bacillus subtilis*, and *C. glutamicum* (references 5, 8, and 21 and this report). All of these bacteria translocate SNase across their cytoplasmic membrane, even though the precursor's signal peptide is much longer (60 amino acids) than average (15 to 30 amino acids [7]) and exhibits an unusual structure (8). The data known about the processing of SNase upon export by *S. aureus*, *B. subtilis*, and *C. glutamicum* are summarized in Fig. 7. For *C. glutamicum* and *S. aureus*, the amino termini of the B forms of nuclease have been determined and were found to be identical (reference 5 and this report). For *B. subtilis*, the B form was not present in steady-state cultures, but an approximately 20-kDa protein comigrating with SNase B was found to be the first nuclease form released to the medium by this host organism too (21). Thus, primary processing of the SNase precursor seems to occur at the same site, which is a typical signal peptidase cleavage site (Ala-Asn-Ala ↓), in all these organisms (Fig. 7). The findings mentioned above are in accordance with the hypothesis concerning the conserved nature among all organisms of protein transport in general, and they indicate that the signal peptidases of the three gram-positive host organisms have similar specificities.

Curiously, at least all three gram-positive bacteria studied to date (no data are available for *E. coli* in which SNase has also been expressed [17, 27]) carry out a second processing step, presumably subsequent to signal peptide cleavage. In *S. aureus* (5) and *C. glutamicum* (this work), the degree of secondary processing varies with the culture conditions. In both organisms, the proportion of A form to B form can be shifted by changing the concentration of sodium chloride in the culture medium (not shown for *S. aureus*; see Fig. 4 for *C. glutamicum*). It seems that variations in the growth conditions leading to the observed differences in the proportions of the A forms to B forms recovered from the medium cause this effect, mainly via a change of reaction conditions which either enhance or inhibit the activity of the protease(s) participating in the secondary processing event(s). This observation may be of importance for future expression of other heterologous extracellular enzymes in *C. glutamicum*, because it shows that the proteolytic activity responsible for secondary processing can be suppressed simply by increasing the salt concentration in the culture broth.

The amino terminus of most of the A form secreted by *C. glutamicum* is one residue shorter than nuclease A of *S. aureus* Foggie (3), and the A form produced by *B. subtilis* is even an amino acid shorter (21). Heterogeneity of the amino terminus of *S. aureus* nuclease A has also been observed (19). The most likely explanation for the mechanism of secondary processing carried out by the three different host organisms seems to be that more or less nonspecific proteases are involved which cause the trimming of the perhaps particularly exposed amino termini of the common B forms to the respective A forms. Supportive of this notion are suggestions that the 19 extra amino acids of nuclease B as well as residues 1 to 5 of nuclease A are flexible (5).

Under certain growth conditions, a significant proportion of SNase produced by *C. glutamicum* AS019/pWNuc5 was cell associated (Fig. 5). Similar observations have been made about the expression of SNase and a SNase hybrid protein in *S. aureus* and *E. coli*, respectively (24, 27). Repeated washing of the recombinant *C. glutamicum* cells with dilute phosphate buffer, sonication, or protoplasting by treatment with lysozyme did not effect release of cell-bound SNase (not shown). Possibly, binding of SNase to *C. glutamicum* cells is due to electrostatic effects, since upon growth in LB2 with 4% sodium chloride no SNase protein remained cell bound (Fig. 5). On the other hand, simple salt washes with a 4% sodium chloride solution were not effective in removing cell-associated SNase from normal LB2-grown cells (not shown), but the hydrophobic mycolic acid layer typical of *Corynebacterium* cell walls (22) may have prevented effective elution under the conditions used.

The SNase expression system used in this paper provides a useful tool for more detailed experimental studies on protein export by *C. glutamicum*. Inducible overexpression of SNase in *C. glutamicum* has been achieved, but production of foreign extracellular proteins by this host may be further improved by the optimization of expression signal structures as well as the conditions of induction and growth.

ACKNOWLEDGMENTS

This work was supported in part by the Deutsche Forschungsgemeinschaft (grant B9 of SFB145).

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