JOURNAL OF BACTERIOLOGY, Sept. 1988, p. 3924–3929
0021-9193/88/093924-06$02.00/0

Vol. 170, No. 9

Secretion of Interleukin-1β and *Escherichia coli* Galactokinase by *Streptomyces lividans*

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Received 28 March 1988/Accepted 22 June 1988

The functionality of the *Streptomyces lividans* β-galactosidase signal peptide to direct heterologous protein export was examined. The signal peptide plus eight amino acids of mature protein were sufficient to export not only a naturally exported protein, interleukin-1β, but also a naturally occurring cytoplasmic protein, *Escherichia coli* galactokinase. Interestingly, cells which expressed yet exported galactokinase were phenotypically Gal⁻. The potential use of the exported galactokinase system for the isolation and characterization of mutations within signal peptides and the export machinery of the host is discussed.

Streptomyces species naturally produce a variety of extracellular proteases (17, 28), amylases (14), and glycosidases (31) which are used to modify complex organic compounds into readily utilizable forms (40). Since these gram-positive bacteria are surrounded by only a plasma membrane and a cell wall (18), exported proteins are most commonly localized into the culture supernatant. This situation is in contrast to the situation with many proteins exported by *Escherichia coli*, which are typically localized into either the periplasmic space or the outer membrane. These features have prompted considerable interest in the use of *Streptomyces* species as hosts for the expression and secretion of heterologous gene products. At present, however, our ability to fully exploit the streptomycetes as expression and secretion hosts is limited since very little is known about the export process in these bacteria.

Our initial studies into *Streptomyces* protein export involved the isolation and partial characterization of the gene which encodes an extracellular β-galactosidase activity (6, 9). Similar to other secreted proteins, the *Streptomyces* β-galactosidase protein is produced as a precursor whose signal peptide is proteolytically removed during export. Although the β-galactosidase signal peptide is unusually long (56 amino acids), it does have similarities to other signal peptides (9) since it contains a number of positively charged amino acids near its amino terminus followed by a region of hydrophobic amino acids.

To assess the functional role of the β-galactosidase signal peptide, it was fused to the coding sequence of interleukin-1β and *E. coli* galactokinase. These proteins are useful indicator tags of export activity since they naturally have different cellular locations and are easily assayed. The ability of the β-galactosidase signal peptide to direct protein export was initially examined by fusing it to interleukin-1β, which is naturally secreted by monocytes and macrophages (8). In contrast, the *E. coli galK* gene product, both in *E. coli* and *Streptomyces lividans*, is localized within the cytoplasm. Therefore, this protein allowed us to examine directly whether the β-galactosidase signal peptide is sufficient for localization of a naturally occurring cytoplasmic protein into the culture supernatant.

In this study we showed that the β-galactosidase signal peptide plus eight amino acids from the mature protein are necessary and sufficient for secretion of both interleukin-1β and *E. coli* galactokinase. Both of these proteins retained their biological activity on export into the culture supernatant. Interestingly, extracellular localization of galactokinase resulted in cells that were unable to utilize galactose as the sole carbon source. The exported galactokinase, therefore, represents an indicator system which can be used to identify the sequences and factors which mediate protein export.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study were JM101 (21) and TB1 (Focus **6**:7, 1984). The *S. lividans* strains used were wild-type *S. lividans* 1326 (19) and a galactokinase-deficient mutant, *S. lividans* 12K (5).

Plasmids and phages. The *E. coli* plasmids used in this study included an interleukin-1β expression plasmid pMGil1-β (22); an *E. coli-Saccharomyces* shuttle vector pYSK9-12, which contains the *E. coli galK* gene (32); placN5, which contains the *E. coli* LacZ gene (T. Eckhardt, unpublished data); pbgal4, which contains the *Streptomyces* β-galactosidase gene (9); pSK03-φCD (32); and pUC19 (42). Phage DNA from M13mp10 (38) was used to create a signal sequence deletion derivative (43). The *Streptomyces* plasmid used in this study was pIJ350 (15), which provided the replication functions for the *Streptomyces* expression vectors described below. Previously published procedures were used for plasmid isolation from and transformation of *E. coli* (7, 37) and *S. lividans* (4, 36).

Media. *E. coli* TB1 was grown in LB (23) liquid medium or LB agar containing ampicillin at 50 μg/ml, when needed for plasmid selection. YT liquid medium or agar (23) was used for M13 phage propagation. *Streptomyces* cultures were grown in YEME (4) plus 34% sucrose for plasmid isolation, protoplast preparation, and expression studies. MBSM agar containing 1% galactose or 1% glucose was used to test for carbon source utilization (5).

Plasmid constructions. p3SSX8, p3SSX10, and p3SSX12 are *E. coli* plasmids that contain the β-galactosidase promoter and the coding sequence which encodes for the signal peptide plus eight amino acids from the amino terminus of

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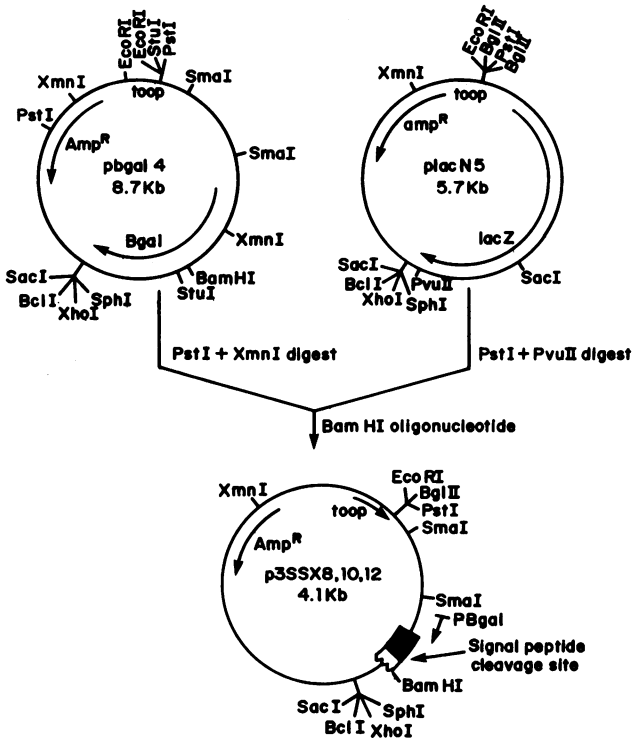

FIG. 1. Schematic drawing of p3SSX8, p3SSX10, and p3SSX12 vector constructions. These plasmids were constructed such that translational fusions could be constructed between the β-galactosidase signal peptide and the gene of interest. The β-galactosidase signal sequence is represented by the filled-in box. The direction of β-galactosidase transcription and the position of the β-galactosidase (βgal) signal peptide cleavage site are shown by arrows.

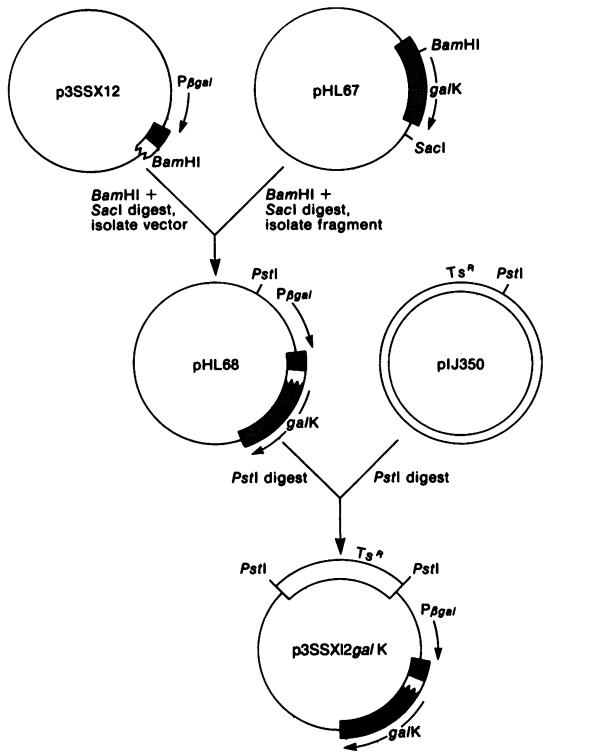

FIG. 2. Schematic drawing of p3SSX12-*galK* construction. The *E. coli* galactokinase gene was fused to the β-galactosidase (βgal) gene as described in the text. The *Streptomyces* replication functions were provided by pIJ350, which is flanked by the *Pst*I sites within p3SSX12-*galK*.

the mature protein. The 1.35-kilobase-pair (kb) *Pst*I-*Xmn*I fragment from pbgal4 and an oligonucleotide containing a *Bam*HI recognition site were ligated to the 3.3-kb *Pst*I-*Pvu*II fragment from placN5, which contains toop and the β-lactamase gene (Fig. 1). Three different oligonucleotides (adaptors 1003, 1015, and 1017; New England BioLabs, Inc., Beverly, Mass.) were used so that translational fusions could be made to the β-galactosidase signal sequence in all three reading frames.

p3SSX12-*galK* is an *E. coli-S. lividans* shuttle vector which contains a β-galactosidase–galactokinase gene fusion (Fig. 2). The galactokinase gene was isolated from plasmid pYSK9-12 as a 1.56-kb *Bam*HI-*Xmn*I fragment. This fragment was inserted into pUC19 via the *Bam*HI and *Sma*I sites to create pHL67. The *galK* gene was then isolated on a *Bam*HI-*Sac*I fragment which was inserted into p3SSX12 by using the *Bam*HI and *Sac*I sites. This plasmid, pHL68, was converted into p3SSX12 *galK* by inserting pIJ350 digested with *Pst*I into the *Pst*I site of pHL68.

The β-galactosidase–interleukin-1β gene fusion shuttle vector p3SSX2il1-β was constructed (Fig. 3) as described above for p3SSX12-*galK*. The coding sequence for interleukin-1β was isolated as a 0.7-kb *Ban*I fragment from pMGil1-β. The staggered ends of this fragment and those of *Bam*HI-digested p3SSX12 were repaired with the Klenow fragment of DNA polymerase I and ligated to create pHL25. p3SSX12il1-β was obtained by inserting *Pst*I-digested pIJ350 into the *Pst*I site of pHL25.

A pIJ350 derivative containing the β-galactosidase gene was also constructed to serve as a control plasmid to monitor any cross-reactivity between *S. lividans*-produced proteins and the interleukin-1β antiserum. The β-galactosidase gene was removed from pbgal4 on a *Pst*I-*Sac*I fragment (Fig. 1). This fragment was inserted into p3SSX12, which was digested with *Pst*I-*Sac*I. pIJ350 was then cloned into p3SSXβgal by using the unique *Pst*I sites on both plasmids to create pIJ350βgal.

Enzyme assays and immunoblots. *S. lividans* was grown in YEME containing 34% sucrose, 1% galactose, 50 mM 3(*N*-morpholino)propanesulfonic acid (MOPS; pH 7), and 2 μg of thiostrepton (E. R. Squibb & Sons, Princeton, N.J.) per ml. The cultures were grown at 28°C for 48 to 72 h. Ten milliliters of culture was concentrated by centrifugation. The cells were lysed by sonication in GalK sonication buffer (5), and cell debris was removed by centrifugation in a microfuge (Beckman Instruments, Inc., Fullerton, Calif.). Intracellular protein extract (10 μg) and culture supernatant (15 μl) were analyzed for galactokinase and interleukin-1β production. These amounts represented equal proportions of cells and culture supernatant; that is, 10 μg of protein would be obtained from cells in 15 μl of culture. Galactokinase activity was assayed as described by Wilson and Hogness (41) and as modified by McKenney et al. (20). Interleukin-1β activity was detected as described by Simon et al. (33). These samples were also analyzed by immunoblotting as described previously (5). The antisera were prepared against *E. coli* galactokinase or interleukin-1β that was expressed in *E. coli* and purified to homogeneity.

Protein sequencing. The *S. lividans* cultures were grown as described above for the enzyme assays, except that 50 μg of (2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanolyl)-L-leucine

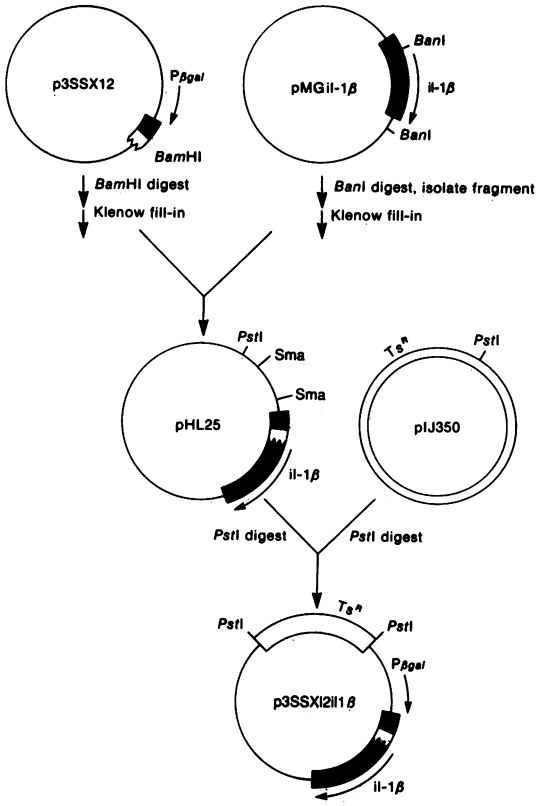

FIG. 3. Schematic drawing of p3SSX12il1-β construction. The coding sequence for mature interleukin-1β(il-1β) was fused to the β-galactosidase (βgal) signal sequence as described in the text.

(Bestatin; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml was included as an amino peptidase inhibitor. A high-pressure liquid chromotography system (model 344; Beckman) equipped with a variable wavelength detector (165; Beckman) was used for all chromotographic runs. One milliliter of the culture supernatant was applied directly to a C4 column (4.5 by 250 mm; 300 A; Vydak) equilibrated with 0.05% trifluoroacetic acid containing 15% CH_3CN (buffer A). After a 10- to 15-min wash in buffer A (flow rate, 1 ml/min), a linear gradient was developed by the addition of 0.05% trifluoroacetic acid containing 60% CH_3CN (buffer B) over 45 min. Peaks were monitored at 214 nm.

The interleukin-1β collected from the C4 column (570 pmol) was applied directly to the spinning cup of a protein

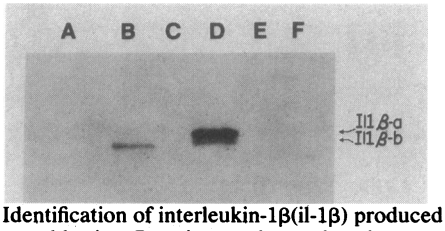

FIG. 5. Identification of interleukin-1β(il-1β) produced in *S. lividans* by immunoblotting. Protein samples analyzed were prestained, low-range molecular weight standards (molecular weights, 3,000 to 43,000; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (lane A), 50 mg of purified recombinant interleukin-1β produced in *E. coli* (lane B), pIJ350βgal culture supernatant (lane C), p3SSX12il1-β culture supernatant (lane D), pIJ350βgal cell extract (lane E), and p3SSX12il1-β cell extract (lane F).

sequenator (model 890M; Beckman) that was used for automated Edman degradation. PTH amino acids were identified and quantitated by reverse-phase liquid chromatography by using an ultrasphere octadecylsilane column (2.0 by 250 mm; Beckman) and a computing integrator (model 7000S; SICA).

RESULTS

Export of interleukin-1β by the β-galactosidase signal peptide. The ability of the β-galactosidase signal peptide to direct protein export was examined initially by fusing the β-galactosidase promoter and the signal peptide-coding sequence to the coding sequence for mature interleukin-1β. The interleukin-1β-coding sequence was fused to a site within the β-galactosidase gene which was eight amino acids downstream of the natural signal peptide cleavage site (Fig. 4). This site was chosen to conserve the signal peptide cleavage site and, therefore, to ensure accurate processing of the β-galactosidase–interleukin-1β fusion protein.

The presence of the β-galactosidase–interleukin-1β fusion protein within either culture supernatants or soluble cell extracts was initially detected by immunoblotting with antiserum prepared against interleukin-1β. The β-galactosidase–interleukin-1β fusion protein was detected only within the culture supernatant (Fig. 5), which suggests that it was completely localized outside of the cell. However, once the culture supernatant and cell extracts were assayed by using the more sensitive activity measurements, it was apparent that small amounts of interleukin-1β were also present internally (Table 1). Since the intracellular interleukin-1β fusion protein was not detected by the immunoblot, it was not possible to determine whether the intracellular material could have represented the precursor β-galactosidase–inter-

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p3SSX12111-β

--GCG  GCC  GAC  GAG  CCG  CCG  GAG  TGG  AAC  GCG  CGG  ATC  GCA  CCT  GTA
  Ala  Ala  Asp  Glu  Pro  Pro  Glu  Trp  Asn  ALA  ARG  ILE  ALA  PRO  VAL
  -1   +1                                                  +2il1-β


p3SSX12galK

--GCG  GCC  GAC  GAG  CCG  CCG  GAG  TGG  AAC  GCG  CGG  ATC  CGG  GAA  TTC  CAA  GAA
  Ala  Ala  Asp  Glu  Pro  Pro  Glu  Trp  Asn  ALA  ARG  ILE  ARG  GLU  PHE  GLN  GLU
  -1   +1                                                                 +5 GalK
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FIG. 4. Nucleotide sequence and corresponding amino acid sequence at gene fusion junctions. The amino terminus for each fusion protein, indicated as +1, was predicted from the known β-galactosidase signal peptide cleavage site (9). Amino acids designated in lowercase letters were derived from the *S. lividans* β-galactosidase gene. The nucleotides within the *Bam*HI fusion site are underlined. Also identified are the +2 amino acid of interleukin-1β(il-1β) and the +5 amino acid of galactokinase.

TABLE 1. Interleukin-1β activity

Plasmid	Interleukin-1β units in[a]:	
	Intracellular protein	Culture supernatants
p3SSX12il1-β	6.3×10^4	3.8×10^6
p3ΔSSil1-β	6.0×10^4	9.0×10^2
p3βgal	3.0×10^2	2.0×10^2

[a] Interleukin-1β units were obtained from a previously published report (33).

leukin-1β fusion protein, which would have retained the β-galactosidase signal peptide.

Within the culture supernatant there were two distinct species of the β-galactosidase–interleukin-1β fusion protein, il1-βa and il1-βb, both of which appeared to be larger than that purified from *E. coli*. We reasoned that il1-βa was larger than the *E. coli*-produced protein, because of the eight additional amino acids between the signal peptide cleavage site and the amino terminus of interleukin-1β. In fact, this conclusion was substantiated by amino-terminal sequence analysis of il1-βa (see below). Finally, the il1-βb species appeared to be a degradation product of the il1-βa species since il1-βa was converted to il1-βb on prolonged incubation in *S. lividans* culture supernatants (data not shown). It is unclear, however, where the proteolytic cleavage occurred to yield il1-βa.

The β-galactosidase–interleukin-1β gene fusion was prepared such that the β-galactosidase signal peptide cleavage site was conserved. We reasoned, therefore, that if interleukin-1β transport into the culture supernatant is dependent on the signal sequence, accurate processing of the signal peptide would occur. Reverse-phase chromatography was used to purify the il1-βa species from the culture supernatant. Within this species there were three forms of the β-galactosidase–interleukin-1β fusion protein. The amino terminus for approximately 63% of the il1-βa species corresponded to the amino terminus of mature β-galactosidase. The other forms had an amino terminus which corresponded to the +2 or the +3 amino acid of mature β-galactosidase. The presence of these minor forms was not surprising, since preparations of mature β-galactosidase also contained species with an amino terminus corresponding to the +2 or the +3 position (9). It is unclear whether these minor forms arose during signal peptide cleavage or from an amino peptidase.

As was noted above, interleukin-1β is naturally secreted by monocytes and macrophages. Unlike most secreted proteins, however, the precursor form of interleukin-1β does not have an amino terminus which resembles a prototypical signal peptide (39). Consequently, it is unclear whether interleukin-1β secretion occurs via a transport mechanism which is dependent on a signal peptide. To ensure that extracellular localization of interleukin-1β in *S. lividans* was indeed dependent on the β-galactosidase signal peptide, oligonucleotide-directed mutagenesis was used to delete the DNA region coding for amino acids 2 to 56 in the β-galactosidase signal sequence to create p3ΔSSil1β. Assays of cell extracts and culture supernatants showed that interleukin-1β is present only in the cell extracts (Table 1). This result, in combination with the accurate processing of the β-galactosidase signal peptide from the β-galactosidase–interleukin-1β fusion protein, suggests that the extracellular localization of interleukin-1β is indeed dependent on the β-galactosidase signal peptide.

Export of *E. coli* galactokinase by the β-galactosidase signal peptide. We next sought to determine whether the β-galactosidase signal peptide was also sufficient for the secretion of a naturally occurring cytoplasmic protein, *E. coli* galactokinase. As with interleukin-1β, the galactokinase-coding sequence was inserted within the β-galactosidase gene at a site that was located eight amino acids downstream of the signal peptide cleavage site (Fig. 4).

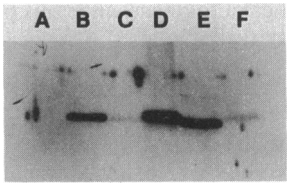

FIG. 6. Identification of *E. coli* galactokinase produced in *S. lividans* by immunoblotting. Protein samples analyzed were prestained, high-range molecular weight standards (molecular weights, 14,300 to 200,000; Bethesda Research Laboratories) (lane A), 20 ng of purified *E. coli* galactokinase (lane B), pSK03-φCD culture supernatant (lane C), p3SSX12l-*galK* culture supernatant (lane D), pSK03-φCD cell extract (lane E), and p3SSX12-*galK* cell extract (lane F).

Cell extracts and culture supernatants were examined for the presence of galactokinase by immunoblotting with antiserum prepared against *E. coli* galactokinase. When authentic *E. coli* galactokinase was expressed in *S. lividans* from pSK03-φCD, it was detected only in the cell extracts (Fig. 6). Attachment of the β-galactosidase signal sequence to the amino terminus of galactokinase resulted in a fusion protein which was present primarily within the culture supernatant. The β-galactosidase–galactokinase fusion protein was not totally localized to the culture supernatant, since galactokinase was also detected within the soluble cell extracts by the immunoblot (Fig. 6, lane F) and enzyme assays. Using the more sensitive galactokinase assay, we found that for p3SSX12-*galK* the galactokinase activity was present in culture supernatants at 345 U (5), whereas only 120 U was detected in the soluble intracellular protein. Although pSK03-φCD produced 975 U of intracellular galactokinase, there was no detectable galactokinase in the culture supernatant.

The β-galactosidase–*E. coli* galactokinase fusion protein appeared to be larger than the galactokinase purified from *E. coli* since the fusion protein migrated with a slower mobility on a sodium dodecyl sulfate-polyacrylamide gels. It seems likely that the fusion protein is larger, since eight additional amino acids were fused to the amino acid of galactokinase when the signal peptide sequence–*E. coli galK* gene fusion was constructed.

The *S. lividans* strain used in this experiment is a *galK* mutant which is unable to grow on minimal galactose medium. When galactokinase expression is restored in this mutant by supplying the *E. coli galK* gene on a plasmid expression vector, the transformed host in then able to grow on minimal galactose medium (5). Although the β-galactosidase–*E. coli* galactokinase fusion restored galactokinase expression, it seemed unlikely that cells which expressed this fusion protein would be able to utilize galactose, since the fusion protein was localized into the culture supernatant. In fact, while the β-galactosidase–*E. coli galK* (*S. lividans* 12K) transformants were able to grow on minimal glucose medium, these cells did not grow on minimal galactose medium. Furthermore, the Gal^- phenotype of these transformants did indeed appear to be due to the extracellu-

lar localization of galactokinase since this protein retained its enzymatic activity (see above).

DISCUSSION

We examined the functionality of the *S. lividans* β-galactosidase signal peptide by fusing the signal sequence plus eight amino acids from the mature β-galactosidase protein to the coding sequences of *E. coli* galactokinase and mature interleukin-1β. Results of these studies showed that this region is sufficient for the localization of interleukin-1β and *E. coli* galactokinase from the cytoplasm into the culture medium. Both proteins retained their biological activity on export. In addition, the β-galactosidase–interleukin-1β fusion protein was processed correctly at the signal peptide cleavage site.

Although the signal peptide is required for protein export in *E. coli* (10), frequently information within the mature protein is also essential for export and correct localization (2, 10, 13, 24). For example, the LamB protein, which acts as the receptor for λ phage, requires both its signal peptide and the first 39 amino acids of the mature protein for export (3). LamB localization into the outer membrane also requires a region between residues 39 and 49 of the mature protein. One consequence of this complexity is that attachment of the LamB and other signal peptides to a nonexported protein is not sufficient for protein export.

Since interleukin-1β is naturally secreted by monocytes and macrophages, we were not totally surprised that it was exported in *S. lividans*. In fact, this observation is reminiscent of chicken ovalbumin (11) and rat preproinsulin (34, 35) export in *E. coli* and interferon export in *Bacillus subtilis* (27). It was surprising, however, to find that the β-galactosidase signal peptide was sufficient to direct the export of *E. coli* galactokinase. Since galactokinase is naturally found within the cytoplasm, we did not expect it to contain export information or to have a structure which was competent for passage through the cytoplasmic membrane (30). We cannot eliminate the possibility that galactokinase is a unique example. It is entirely possible that the β-galactosidase signal peptide or other *Streptomyces* signal peptides are unable to direct the export of other cytoplasmic proteins. This situation would occur if their composition was incompatible with transfer across the membrane or if regions within the mature protein were essential for localization into the culture supernatant.

In *E. coli*, gene fusions have proven invaluable in the study of protein export (1, 2). Most fusions contain amino termini of various lengths from exported proteins plus the enzymatically active carboxy terminus of *E. coli* β-galactosidae. Analysis of these hybrid proteins has localized regions within the exported proteins which are required for their export. In addition, the fusion proteins have been used to isolate a variety of export-deficient mutations within the exported proteins and the host secretory machinery (16, 25, 26). Our ability to export *E. coli* galactokinase suggests that this gene would be useful for studying protein export in *S. lividans*. For example, export of the functional β-galactosidase–galactokinase fusion protein resulted in a galactose-negative phenotype. Mutations which block protein export would internalize galactokinase and could confer a galactose-positive phenotype, provided that the intercellular galactokinase retained its enzymatic activity. Such mutations should include alterations within the signal peptide and the protein export machinery of the host. Mutations within the secretory apparatus of the cell should allow for a functional analysis of the export mechanism. This analysis would be invaluable, since one could then compare this process with the export mechanism in *E. coli* and eucaryotic cells.

Finally, members of the genus *Streptomyces* have recently grown in popularity as an alternative host system for the expression and secretion of heterologous gene products (12, 29). Our results have shown that *S. lividans* can indeed export two heterologous proteins. However, the levels of *E. coli* galactokinase and interleukin-1β secreted by *S. lividans* were approximately 100-fold lower than secreted levels of the cloned *S. lividans* β-galactosidase. More experiments are required to elucidate the factors which contribute to reducing the efficiency of secreting heterologous proteins in *S. lividans*.

Protein secretion in *Streptomyces* species offers the advantage that secreted proteins, like β-galactosidase, are localized almost exclusively to the culture supernatant rather than the periplasmic space or the outer membrane, as is usually the case in *E. coli*. This feature may simplify purification procedures. Although the gene fusions described here included a small region from the mature β-galactosidase protein, results of additional studies have shown that a *Streptomyces* signal peptide alone is sufficient for the export of the heterologous gene product (J. Fornwald, R. Keys, D. Taylor, and M. Brawner, unpublished data). Therefore, by using only the signal peptide, it should be possible to produce authentic gene products. Although our experience in heterologous gene expression in *Streptomyces* species is still rather limited, it seems likely that we can improve its utility as an expression and secretion host by better understanding the export process in *S. lividans*.

ACKNOWLEDGMENTS

We thank A. Shatzman and D. Taylor for comments on the manuscript, Deborah Murdoch for secretarial assistance, and E. R. Squibb & Sons for the thiostrepton.

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