Expression of the \textit{Streptomyces} Enzyme Endoglycosidase H in \textit{Escherichia coli}\textsuperscript{*}

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Endoglycosidase H is one of a large number of enzymes secreted by \textit{Streptomyces plicatus} and other \textit{Streptomyces} species. When the structural gene for this enzyme is introduced into \textit{Escherichia coli} attached to the plasmid pBR-322 or Charon 4 phage, the enzyme is synthesized and is found in the periplasmic space, culture medium, and cells. Attachment of the UV-5 \textit{loc} promoter to a site in the plasmid adjacent to the \textit{Streptomyces} insert stimulates enzyme synthesis as much as 100-fold. This result demonstrates that transcription of the \textit{Streptomyces} gene can be initiated from sequences outside of the \textit{Streptomyces} insert. Initiation of transcription on a \textit{Streptomyces} promoter is also a suggested but unproven possibility.

In contrast to the situation in \textit{Streptomyces}, where the enzyme has a molecular weight of 27,000, the enzyme made in \textit{E. coli} has a molecular weight of approximately 30,000. Possible explanations for this difference in size are lack of cleavage of the \textit{Streptomyces} secretion "signal sequence" in \textit{E. coli} or protein "processing" by enzymes secreted into the medium by \textit{Streptomyces}.

- Endoglycosidase H, an enzyme secreted by \textit{Streptomyces plicatus}, has been a major analytical tool in studies of the processing of cell surface glycoproteins. Endo H\textsuperscript{1} hydrolyzes with great efficiency the glycosidic bond between the two N-acetylglucosamine residues of "high mannose" oligosaccharides. Its interesting substrate specificity requires, in addition to the diacetyl chitobiose linkage, additional mannose residues arranged in the specific pattern found in certain eukaryotic glycopeptides (1, 2).

- Endo H was isolated from culture filtrates of \textit{S. plicatus} by Tarentino and co-workers (3, 4). They characterized it as a small stable protein (\textit{M} = 27,000). The enzyme is resistant to hydrolysis by Pronase or denaturation by SDS but is inactivated by heat above 60°C. On sizing columns the protein behaves as a globular monomeric polypeptide. Its amino acid analysis has been published and the NH\textsubscript{2}-terminal amino acid sequence has recently been determined.\textsuperscript{2}

- We became interested in endo H synthesis and secretion for several reasons. A primary aim was to begin a study of enzyme secretion by \textit{Streptomyces}. These soil organisms secrete an interesting but bewildering array of enzymes which play a major role in the breakdown and reutilization of organic compounds in soil. Very little is known concerning the factors which regulate the synthesis and secretion of these enzymes. For initial studies, it seemed preferable to choose a constitutive enzyme since a \textit{Streptomyces} enzyme with complex regulatory factors might not be expressed in \textit{Escherichia coli} as well as it seemed to be in its natural environment. It is a small, stable protein whose synthesis appears to be constitutive (3).

- A second aim in cloning endo H into \textit{E. coli} was to simplify the method for its preparation. Although the purification scheme developed by Tarentino \textit{et al.} (4) is excellent, it is time-consuming and laborious. High level expression in \textit{E. coli} and secretion into the periplasmic space should simplify preparation of the enzyme.

**EXPERIMENTAL PROCEDURES**

\textit{Growth of Streptomyces—} \textit{S. plicatus} was obtained from Dr. Frank Maley (Developmental Biochemistry Laboratories, New York State Department of Health, Albany). To maintain cultures, spore suspensions were prepared by gentle scraping in the presence of a small volume of sterile 1% Triton. Spores were sedimented for 15 min at 3000 rpm, resuspended in water, and used to inoculate liquid media or to streak on agar plates. Agar plates for routine passaging contained 0.5% soluble starch, 0.1% N-Z-amine, 0.1% nutrient broth (Difco), 0.1% yeast extract, and 1.2% agar. To encourage sporulation, plates were seeded sparsely. Since vegetative cultures do not remain viable for extended periods, it is important to induce sporulation in cultures being carried for more than a week. For growth on defined media and for \textsuperscript{35}S sulfate labeling of \textit{Streptomyces}, modifications of the medium of Jeuniaux (5) (J medium) were employed with various carbon and nitrogen sources substituted for chitin. For example, the \textsuperscript{35}S sulfate labeling medium contained (per liter): 0.5 g of NaCl, 0.5 g of NH\textsubscript{4}Cl, 0.7 g of K\textsubscript{2}HPO\textsubscript{4}, 0.3 g of KH\textsubscript{2}PO\textsubscript{4}, 0.5 g of MgCl\textsubscript{2}, 0.01 g of FeCl\textsubscript{3}, and 5 g of glucose (added after autoclaving). As noted in the text, the composition of this medium was varied and minimal \textit{E. coli} media were used from time to time for specific experiments. Minimal \textit{E. coli} media used in this study were M-9 (0.6% NaH\textsubscript{2}PO\textsubscript{4}, 0.3% K\textsubscript{2}HPO\textsubscript{4}, 0.05% NaCl, 0.1% NH\textsubscript{4}Cl) or OM (1% K\textsubscript{2}HPO\textsubscript{4}, 0.45% KH\textsubscript{2}PO\textsubscript{4}, 0.01% MgSO\textsubscript{4}, 0.1% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, and 0.05% Na\textsubscript{2}citrate). The M-9 medium was supplemented after autoclaving with 2 mM MgSO\textsubscript{4}. Both media were also usually supplemented with proline (20 μg/ml), thymine (20 μg/ml), and thiamine (0.1 μg/ml). The usual carbon source was 0.3% glucose. For \textsuperscript{35}S sulfate labeling experiments, chloride salts were used in place of the sulfates. For growth with minimal sulfate supplementation, Na\textsubscript{2}SO\textsubscript{4} (10 μg/ml) was added to either sulfate-free M-9 or OM.

\textit{Antibody Preparation—} Endoglycosidase H was assayed and prepared as described by Tarentino \textit{et al.} (4). The batch of enzyme used for antibody preparation showed no detectable impurities when analyzed by SDS-acrylamide electrophoresis. NH\textsubscript{2}-terminal amino acid analysis also indicated that the protein did not contain detectable impurities.\textsuperscript{2} Rabbit antibody to endo H was prepared by subcutaneous injection of 250 μg samples of enzyme with an interval of 3 weeks between injections. The first injection was in complete Freund’s adjuvant and the second was in incomplete Freund’s. Two weeks after the second injection, the rabbit was bled and γ-globulin was prepared as described by Tarentino \textit{et al.} (4).

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\textsuperscript{1} The abbreviations used are: endo H, endoglycosidase H; kb, kilobase pairs; SDS, sodium dodecyl sulfate.

\textsuperscript{2} F. Maley, personal communication.
preparing by the method of Livingston (6). Another rabbit was used for preparation of immune &-globulin reactive toward one of the chitinase enzymes of S. plicatus (5). The latter &-globulin was used as a control in immune precipitation studies. By titrating enzymatic activity with &-globulin in the presence of Staphylococcus A, it was found that the anti-endo H preparation had a binding capacity of 22 ng/mg secreted &-globulin and that endo H and chitinase antibodies did not cross-react.

**DNA Extraction and 20-kb Fragment Preparation**—DNA was extracted from S. plicatus by a method of the modification used by Shapiro and co-workers for DNA extraction from Caulobacterium (7). A gene library of S. plicatus DNA was constructed using the methods described by Maniatis et al. (8). All restriction enzymes were from New England Biolabs. S. plicatus DNA was digested in separate reactions with the restriction enzymes Hae III and Alu I under conditions, determined empirically for each enzyme, for partial cutting of the DNA. The optimal length of DNA fragments for the construction of a library is 20 kb. The conditions for the Hae III (Lot No. 9) were 0.125 units/10 &mgr;g of DNA digested for 1.5 h at 37 °C and those for Alu I (Lot No. 9) were 0.5 units/10 &mgr;g digested under similar conditions. The DNA was also digested with a 2-fold higher and a 2-fold lower concentration of enzyme. The digested DNA was pooled, phenol-extracted, and fractionated on a linear 15-40% sucrose gradient. Individual fractions were analyzed by agarose gel electrophoresis to determine the length of the DNA in each fraction. The fractions containing DNA of 16-22 kb in length were pooled and ethanol-precipitated. The DNA was incubated with Eco RI methylase (New England Biolabs) and Eco RI linkers to the blunt ends (8). The DNA was digested with Eco RI, and the 20-kb DNA was purified on a sucrose gradient.

**Preparation of Charon 4 Arms**—Charon 4 DNA was prepared by the method of Maniatis et al. (8). Briefly, Charon 4 DNA was digested with Eco RI, incubated at 42 °C for 2 h to allow annealing of the phage arms and fractionated on a linear sucrose gradient. Appropriate DNA fractions were pooled and concentrated by ethanol precipitation.

**Construction of Recombinant Phage—S. plicatus DNA was ligated to Charon 4 DNA at 15 °C with T4 ligase (Lot No. 16). The resulting hybrid DNA was incubated with an 8X phase packaging reaction (9). E. coli (strain DB 4548) was infected with the recombinant phage and 5 X 10^6 plaques were obtained/ &mgr;g of S. plicatus DNA. When the plaques were tested for S. plicatus DNA by the sensitive method of Woes (10) using nick-translation-labeled S. plicatus 16- to 20-kb DNA as a generic probe, approximately half of the visible plaques scored as positive. About 15% of the plaques were lac+ but this number decreased to about 1% when the library was expanded according to Maniatis et al. (8). EH-5 was picked from the expanded library.

**Screening of Phage Plaques—Phage plaques were screened by the method of Broume and Gilbert (11). Plastic discs were coated with antiserum (60 &mgr;g/ml) and incubated in contact with the plaques for 4°C for 2 h. The discs were then stained with 2% labeled anti-endo H (11) and exposed to x-ray film. Duplicate discs were examined. One plaque was picked, purified, and shown to be positive for endo H enzymatic activity by direct testing of a lysate.

Plaques or bacterial colonies were tested directly for endo H activity. Plaques were picked and extracted briefly with endo H substrate (3) in 0.15 M citrate, pH 5.0. Cells taken from colonies were extracted with 5 &mgr;l of 0.1 M Tris, 0.1 M EDTA, pH 8, before addition of substrate.

**Subcloning of EH-5 DNA—The phage has been prepared on a large scale on plates and in liquid medium. Following phage purification and ethanol precipitation (DNA) was extracted with phenol, precipitated with ethanol, used for analysis with restriction enzymes, and subcloned into pBR322. Both Bam HI and RI digests of EH-5 were cloned into pBR322 (see Figs. 1 and 2). Bam plasmids were tested for loss of resistance to tetracycline as well as endo H expression. Since all "RI" recombinant plasmid colonies were resistant to both ampicillin and tetracycline, colonies were tested directly for endo H (see above). Other plasmids were prepared from the two basic RI and Bam plasmids, pEHRI and pEHII, by restriction enzyme treatment followed by ligation. Plasmid pEHRI2 was derived by Psi I digestion of EHR followed by ligation at high dilution. The 1.6-kb fragment containing endo H was recovered from agar (12) and was subcloned into pBR322 which had been digested with Eco HI and Bam. The resulting plasmid is pEH5.6. Plasmid pEH5.6 was prepared by Ssi I digestion and relaxation of pEH5.6 at a high dilution. In each case plasmids were mapped with restriction enzymes. Endo H levels were measured quantitatively in growing HB 101 cells following transformation (see below). In all cases transformed cells were grown in the presence of ampicillin (100 &mgr;g/ml) or tetracycline (20 &mgr;g/ml). The UV-5 lac promoter was prepared from plasmid pKB258, kindly provided by Dr. M. Plaashe. The 206-base pair promoter was isolated on an acrylamide gel after Eco RI digestion of the phage and ligated to the appropriate plasmid which had been digested with Eco RI. The DNA sequence of the promoter, which reveals the presence of a Poo I restriction site at position 15 of the promoter, was determined by Dr. W. Gilbert (Biological Laboratories, Harvard University).

**Measurement of Tris EDTA-extractable Endo H Levels in Plasmid-carrying E. coli**—Overnight cultures were diluted 1:100 in Luria broth (1% tryptone 0.5% yeast extract, pH 7) containing the appropriate antibiotic; either ampicillin (100 &mgr;g/ml) or tetracycline (20 &mgr;g/ml). Cultures were shaken at 37 °C and were chilled rapidly in ice water at the appropriate time. Cells were sedimented by centrifugation at 12,000 rpm for 5 min and extracted by either a standard EDTA H2O shock or by Tris EDTA. For EDTA H2O shock, cells were washed quickly with half the original volume of 20% sucrose 0.01 M Tris-Cl, pH 7.5, and resuspended in 2% of the original volume of sucrose Tris. EDTA treatment and H2O extraction were then carried out as described by Koshland and Botstein (13). For Tris EDTA extraction, the initial cell pellet was washed quickly with 0.5 volume of PBS (0.8% NaCl, 0.02% KCl, 0.12% Na2HPO4, and 0.02% KH2PO4) and was resuspended in 1% of the original volume of 0.1 M Tris, 0.1 M EDTA, pH 8. After incubation for 10 min at 5 °C, cells were removed by centrifugation and the supernatant fluid was used for enzyme assay. The same amount of enzyme is extracted by both methods. Furthermore, SDS-acrylamide gels of the two extracts display similar protein profiles.

**RESULTS**

**Expression of Endo H in S. plicatus**—Table 1 shows the level of enzyme secreted under a number of different growth conditions. The level of enzyme is approximately the same whether the organism is growing slowly on a poor carbon source, such as glucose, or growing more rapidly in the presence of yeast extract and chitin. In contrast, the chitinase complex of enzymes (5) is synthesized only when the organism is grown on chitin as a carbon source. We have occasionally noticed a slightly higher level of endo H formation when S. plicatus is grown on N-acetylglucosamine (see Table 1). In spite of the facts that endo H hydrolyzes a diacetyl chitobiose linkage, the primary linkage in chitin, and that S. plicatus is able to elaborate high levels of chitinase activities, we have confirmed the suggestion of Tarentino and Maley (3) that endo H does not hydrolyze chitin or chitin oligosaccharides and that it is synthesized and secreted under conditions where little chitinase is synthesized.

**Table 1**

| Medium | Endo H level ng/mg secreted protein |
|--------|-----------------------------------|
| Glucose | 120 |
| Glucose, yeast extract | 90 |
| Acetyl glucosamine | 310 |
| Acetyl glucosamine, yeast extract | 90 |
| Chitin | 100 |
| Chitin, yeast extract | 160 |

P. Robbins, unpublished data.
Expression of Endo H in E. coli—Since the assay for endo H is quite sensitive and E. coli has no detectable enzymatic activity of this type itself, it was possible to measure enzyme expression in plasmid-carrying lines accurately. Approximately equal amounts of activity can be released from cells with either a standard shock treatment, as described by Koshland and Botstein (13), or by treatment of cells with 0.1 M Tris, 0.1 M EDTA, pH 8. Enzyme is also found in the growth medium and in the cells. The medium and intracellular activities are currently under investigation and will be the subject of a future publication.

Two basic plasmids were recovered by cloning the 4.3-kb RI and 4.9-kb Bam HI DNA fragments from EH-5 into pBR322 (see Fig. 1). Various derivatives of these two plasmids were prepared as described under “Experimental Procedures.” Enzyme levels were measured in growing E. coli HB-101 cells carrying all of these plasmids. Tris EDTA extract enzyme levels and restriction maps of the plasmids are presented in Fig. 2. Maps are shown opened at the Peu II site of pBR322 and an agarose gel showing plasmid DNA cut with Peu II is shown in Fig. 3. A more detailed map of the 1.6-kb RI-Bam fragment is presented in Table II. The presence of two lac promoters in pEHBl.6lac2 oriented as indicated in Fig. 2 was

Expression of Endo H During Lytic Growth of λ EH-5—EH-5, the derivative of Charon 4 with the 12-kb Streptomyces endo H insertion, grows without restriction in several strains of E. coli and expresses low levels of endo H. A partial restriction enzyme map of the Streptomyces insert in EH-5 is shown in Fig. 1, oriented left to right as usually defined for the λ genome. Given the fact that transcription for endo H formation in EH-5 is from left to right (see below) and that transcription from the major relevant promoter (Pl) is in the opposite direction, it seems possible that endo H transcription in EH-5 infected cells is being initiated from a Streptomyces promoter. Transcription from another λ promoter is also a possibility.

### Table II

Partial restriction enzyme map of Streptomyces DNA fragment EHBl.6

| Approximate map position (in thousands) from the RI origin of fragment EHBl.6 | Restriction site |
|---|---|
| 0 | Eco RI |
| 0.10 | Sma I |
| 0.29 | Sma I |
| 0.30 | Kpn I |
| 0.31 | Xho I |
| 0.36 | Smal |
| 0.56 | HinfI |
| 1.10 | Sal I |
| 1.26 | Sal I |
| 1.29 | HinfI |
| 1.33 | Peu II |
| 1.41 | Sal I |
| 1.45 | Peu I |
| 1.60 | Bam HI |

Plasmids were prepared as described in the text, digested with Peu II, separated on a 0.7% agarose gel, and stained with ethidium bromide. The order of plasmids on the gel is the same as the order of restriction maps of Fig. 2. The marker lane contains HindIII-digested λ DNA.

### FIG. 3.

Peu II restriction enzyme digests of endo H plasmids. Plasmids were prepared as described in the text, digested with Peu II, separated on a 0.7% agarose gel, and stained with ethidium bromide. The order of plasmids on the gel is the same as the order of restriction maps of Fig. 2. The marker lane contains HindIII-digested λ DNA.

### FIG. 2.

Endoglycosidase H levels in E. coli HB101 lines carrying endo H plasmids. Cells were grown to A600 of 0.5-1.0 in Luria broth and assayed for enzymatic activity as described in the text. Enzyme levels refer only to activity extractible with Tris EDTA. An activity of 0.10 corresponds to an enzyme level of 1 milliunit/ml (3) in the Tris EDTA extract. The nature and amount of enzyme in the cells and medium is currently under investigation. Restriction maps of the pBR-322 derived plasmids are linearized by opening at the Peu II site (*). Other restriction enzyme sites shown are Pst I (P), Bam HI (B), Eco RI (R), and Sal I (S). The Streptomyces DNA has a number of additional Sal I restriction sites which are not shown. Streptomyces and lac promoter DNA are shaded and the direction of transcription is indicted by arrows. The common 1.6-kb fragment which carries the endo H gene is shown by a heavy bar and deletions are indicated by dotted lines.
shown by acrylamide gel electrophoresis of Pvu II digestions of this plasmid. As expected, Pvu II cuts out the extra 205-base pair promoter (data not shown). Enzyme levels were calculated assuming equal specific activities for the $M_t = 27,000$ Streptomyces enzyme and the $M_t = 30,000$ enzyme produced in $E. coli$. We are purifying the $E. coli$ enzyme to determine both its specific activity and substrate specificity.

Transcription may be initiated on the Streptomyces insert and/or on pBR322 sequences. At least one major point is clear. Since insertion of the UV-5 lac promoter at the RI sites of either the EHB1.6 or EHR3.2 plasmids leads to an increase in enzyme synthesis to a constant very high level, it is evident that transcription occurs in the RI to Bam direction on the 1.6-kb fragment. When the UV-5 lac promoter is inserted backwards (pEHB1.6 cal and pEHR3.2 cal), promoting transcription away from the Streptomyces insert, enzyme synthesis is either left near the basal level or is depressed below the basal level. It is interesting that the same high level of enzyme is produced under the influence of the lac promoter whether transcription is taking place in a clockwise (EHB1.6) or a counter-clockwise (EHR3.2) sense on the plasmid and whether or not significant amounts of extra Streptomyces DNA are present in the plasmid. Although the plasmids with one and two lac promoters give rise to about the same level of enzyme in growing cells, significantly higher levels of endo H were observed in stationary cultures carrying the plasmid with two lac promoters (data not shown).

Since coding for the protein probably requires about 800 base pairs, the AUG for initiation of amino acid sequence coding must occur in the first 800 base pairs beyond the RI site on the EHB1.6 plasmid. Since a SalI plasmid carrying only the first 1060 base pairs, EHB1.06, does not promote the formation of complete enzyme, it seems likely that the initiating AUG lies between positions 200 and 800. Since the six NH$_2$-terminal amine acids are now known, it should be possible to locate the coding region and identify the endogenous Streptomyces promoter by direct DNA sequencing.

**Size of the Enzyme Extracted from $E. coli$—Maley and coworkers have purified $S. plicatus$ endo H to homogeneity and have shown that it behaves as a monomeric polypeptide with a molecular weight of 27,000 (3). When the enzyme is extracted from plasmid-carrying lines of $E. coli$ and sized on a Sephadex G-75 column, it behaves as a monomeric polypeptide of approximately the same size (data not shown). When the $E. coli$ enzyme is labeled with $[^35]S$SO$_4$ or $[^35]$S methionine, precipitated with anti-endo H antibody, and examined by SDS-acrylamide gel electrophoresis, however, it is clearly seen to be 2,000-3,000 daltons larger. We have labeled the protein under a variety of conditions and attempted to induce cleavage at the $M_t = 27,000$ form without success. In the experiments shown in Fig. 4, the enzyme was labeled with $[^35]$S sulfate and extracted from the periplasmic space with Triton EDTA. Aliquots were incubated alone or with $S. plicatus$ cells or culture medium for 15 h at 24°C. Half of each sample was immunoprecipitated with anti-endo H or control $\gamma$-globulin.

Although no $E. coli$ protein reactive with our anti-endo H serum smaller than 29,000-30,000 has been observed, larger polypeptides have occasionally been seen. Precipitation of labeled Streptomyces culture medium with our anti-endo H reagent also reveals larger polypeptides as well as the expected $M_t = 27,000$ enzyme. At the present time, we are attempting to determine which of these polypeptides, if any, are related to endo H.

**DISCUSSION**

The constitutive nature of endo H synthesis in Streptomyces as well as its extreme stability in the face of proteolytic enzymes and denaturing agents have probably contributed to our detection of the enzyme following cloning of Streptomyces genomic DNA in $E. coli$. The gene for endo H, after cloning in $E. coli$, may be transcribed from a promoter in the Streptomyces DNA or from promoters in the vector DNA. In the Charon 4 clone, EH-5, transcription of the endo H gene must have taken place in the direction opposite from transcription initiated at Pl, the major relevant $\lambda$ promoter. This observation suggests that transcription may occur from a Streptomyces promoter, although phage transcription obviously cannot be ruled out. The level of endo H expression, in $\lambda$ EH-5-infected cells is relatively low, in the range of 10-100 molecules/infiltrated cell (data not shown).

In the plasmid subclones, it is likely probable that pBR322 promoters play a role in endo H transcription. Of the two Streptomyces plasmids containing complete pBR322 genomes, the plasmid with the higher level of expression (EHB) has the endo H sequence placed in such a way that transcription continuing from the $\beta$-lactamase promoter at the plasmid HindIII site would immediately give a correct transcript of the enzyme. In EHB, the endo H gene is oriented in the opposite direction with respect to pBR322. In this case tran-

**FIG. 4. SDS-acrylamide gel of labeled endo H-immune precipitates from $E. coli$-carrying plasmid pEHB1.6lac2.** The plasmid-carrying cell line was grown overnight, in Luria broth (100 $\mu$g/ml of ampicillin). The culture (25 ml) was centrifuged at 10,000 rpm for 5 min, washed with sulfate-free M-9, and resuspended in 500 ml of M-9 supplemented as indicated in text. Carrier-free ($^{35}$S) sulfate (5 mCi) was added and the culture was shaken at 37°C for 7 h. Cells were recovered by centrifugation at 10,000 rpm for 5 min, washed with 100 ml of PBS (0.8% NaCl, 0.02% KCl, 0.12% Na$_2$HPO$_4$, 0.02% KH$_2$PO$_4$), and treated with 6 ml of 0.1 M Tris, 0.1 M EDTA, pH 8. After centrifugation the supernatant fluid was lyophilized, redissolved in H$_2$O to 0.5 ml, and loaded on a Sephadex G-75 column (0.7 x 48 cm). Elution at 5°C was carried out with PBS diluted with water (1:2) at a rate of about 2 ml/h. Aggregated and high molecular weight proteins, eluted between 6 and 8 ml (fraction 1). Low molecular weight proteins, including endo H (fraction 2), were eluted between 9 and 13 ml, and inorganic sulfate between 17 and 21 ml. Fraction 2 (0.2 ml) was incubated overnight at room temperature with 0.5 ml of sterile M-9 medium (b), 0.3 ml of OM medium in which $S. plicatus$ had grown (c), or 0.3 ml of suspension of mycelia (d). Lane e shows 0.2 ml of unincubated enzyme. In lanes b, c, d', and e', the control $\gamma$-globulin was used in place of anti-endo H. In all cases, volumes were adjusted to 0.5 ml, and 0.05 ml of 1.5 M NaCl, and 14 $\mu$g of immunne $\gamma$-globulin were added. After 60 min washed Staphylococcus A was added, and after washing, precipitates were boiled in SDS-mercaptoethanol prior to SDS-10% acrylamide electrophoresis. Lane a contains $^3$C marker proteins; myosin, 220,000 (200K); phosphorylase b, 100,000 (100K) and 92,500 (92.5K); bovine serum albumin, 69,000 (69K), ovalbumin, 47,000 (47K), and carbonic anhydrase, 30,000 (30K). The position of unlabeled Streptomyces endo H in this lane is indicated at 27,000 (27K).
scription of the endo H gene continuing from the plasmid promoter for the tetracycline resistance (tet) gene (located at the HindIII site) may increase gene expression as evidenced by the 3-fold lower level of expression seen when this promoter is deleted (EHB1.6). The obvious factors which could be of importance in the level of endo H expression from these plasmids (EHR, EHB, and EHB1.6) are the different levels of promotion from the β-lactamase and tet promoters and the distance between the promoter and the endo H gene. It will be interesting to determine enzyme production when EHB and EHR fragments are each reversed in the plasmid.

In pEHB1.6 the tet plasmid promoter has been deleted and the β-lactamase promoter is oriented away from the endo H gene. In spite of this, the endo H gene is expressed. This suggests promotion from a Streptomyces promoter, but, as in the phage clone, low level transcription from some yet unidentified pBR322 promoter cannot be ruled out. Another element not taken into account here is plasmid number per cell which undoubtedly varies from line to line.

The powerful promotion which results from insertion of the UV-5 lac promoter is similar to the stimulation seen when this element is inserted in front of the λ repressor gene (14). Although UV-5 contains a ribosome binding site it is clear that the ribosome binding site is probably specified by the Streptomyces genome since translation is initiated 200–800 base pairs from the point of lac insertion, and the protein made is the same size whether or not the lac promoter is present.

It is certainly not yet clear whether the larger polypeptide formed in E. coli is the result of lack of cleavage of the Streptomyces "leader" sequence (the sequence associated with secretion) or is the result of lack of cleavage by other secreted Streptomyces enzymes. To date we have not been able to bring about cleavage of the E. coli enzyme by Streptomyces cells or medium but this may simply be the result of inadequate conditions. We have recently become aware that the pattern of Streptomyces secreted proteins precipitated by our antibodies are variable and sensitive to culture conditions. We plan to examine these proteins before drawing conclusions concerning the difference between the E. coli and Streptomyces enzymes.

REFERENCES
1. Trimble, R. B., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1978) J. Biol. Chem. 253, 4508–4511
2. Tai, T., Yamashita, K., and Kobata, A. (1977) Biochim. Biophys. Res. Commun. 78, 434–441
3. Tarentino, A. L., and Maley, F. (1974) J. Biol. Chem. 249, 811–817
4. Tarentino, A. L., Trimble, R. B., and Maley, F. (1978) Methods Enzymol. 50, 574–584
5. Jeuniaux, C. (1966) Methods Enzymol. 8, 644–650
6. Livingston, D. M. (1974) Methods Enzymol. 34, 723–731
7. Nisen, P., Medford, R., Mansour, J., Parucker, M., Skalka, A., and Shapiro, L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6240–6244
8. Maniatis, T., Harϊsoo, R. C., Lacy, E., Lauer, J., O’Connell, C., Quon, D., Sim, G. K., and Efstratiadis, A. (1978) Cell 15, 687–701
9. Sternberg, N., Tiemeier, D., and Enquist, L. (1977) Gene 1, 255–262
10. Woo, S. L. C. (1979) Methods Enzymol. 88, 389–395
11. Broome, S., and Gilbert, W. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 246–2479
12. Kuhn, S., Fritz, H.-J., and Starlinger, P. (1979) Mol. Gen. Genet. 177, 235–241
13. Kosicki, D. and Botstein, D. (1980) Cell 20, 749–760
14. Backman, K., Ptashne, M., and Gilbert, W. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4174–4178
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275