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Cloning, Sequencing, and Regulation of Expression of an Extracellular Esterase Gene from the Plant Pathogen *Streptomyces scabies*

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The gene that encodes the extracellular esterase produced by *Streptomyces scabies* has been cloned and sequenced. The gene was identified by hybridization to a synthetic oligonucleotide that corresponds to the amino-terminal amino acid sequence determined for the secreted form of the esterase. Nucleotide sequence analysis predicted a 345-amino-acid open reading frame, a putative ribosome-binding site, and 39 amino acids at the amino terminus of the sequence that is not found in the secreted protein. This 39-amino-acid sequence has many of the characteristics common to known signal peptides. End mapping the esterase transcript revealed a single 5′ end of the mRNA located 51 nucleotides upstream from the start point for translation. Northern (RNA) hybridization analysis of the esterase message by using the cloned esterase gene as a probe indicated that the esterase mRNA is about 1,440 nucleotides in length and was detected only when the cells were grown in the presence of zinc. These results suggest that the level of esterase mRNA detected in the cells is regulated by zinc.

Streptomyces scabies is an aerobic gram-positive soil bacterium that is the causative organism of scab disease on potato, radish, and other underground vegetables (7). For a plant pathogen to infect its host cell, it must first penetrate the plant's outer protective layers (28). The pathogen can do this by entering through a natural opening such as the lenticel, by entering through an existing wound, or by creating an entrance through the protective layer. One possible method of creating an entrance is for the pathogen to secrete one or more enzymes that could degrade the protective barrier. Cutin and suberin are the waxy polyesters that cover the external portions of plants and form the protective barrier against moisture loss and pathogen invasion (26, 27). Both compounds contain an assortment of long-chain fatty acids, fatty alcohols, and dicarboxylic acids. In addition, a major component of suberin is phenolic compounds polymerized via ester and ether linkages (26, 27).

Previous workers have isolated extracellular esterases from many organisms with the ability to depolymerize cutin (9, 28, 32, 46, 50, 53) and have shown that for some, the cutin esterase is necessary for pathogen penetration of the intact cuticle (30, 31, 35). Penetration of the protective suberin layer covering underground tubers by *S. scabies* may also involve the production and secretion of suberin-degrading enzymes. A suberin-specific esterase could be used by *S. scabies* to breach the host's surface integrity and allow penetration.

In earlier studies, we observed production and secretion of an esterase activity in cultures of pathogenic strains of *S. scabies* grown with a suberin-enriched fraction of potato peel as the sole carbon source. Zinc was determined to be the critical component of the suberin-enriched fraction that was necessary for induction of this esterase (38). Zinc is not required by the esterase for enzymatic activity (38), suggesting that it may instead be necessary for production of the enzyme during transcriptional or posttranscriptional events. To investigate the molecular mechanism of zinc induction of esterase production, the esterase gene from *S. scabies* was cloned and sequenced. The length of the esterase mRNA and the location of its 5′ end were determined by Northern (RNA) hybridization and nuclease S1 mapping, using DNA probes made from restriction fragments of the cloned gene. Hybridization studies have indicated that zinc affects the level of esterase mRNA produced by the pathogen and may be a mechanism for modulating transcription of the esterase gene.

(A preliminary account of some of this work has been reported previously [49].)

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. scabies* FLI (37) was maintained as a frozen spore suspension in 20% glycerol. pUC118 and pUC119 were used as cloning vehicles and for the generation of single-stranded DNA for sequencing (54). Plasmids were maintained in *Escherichia coli* DH5αF′ (33). M13K07 was used as helper phage for the production of single-stranded DNA for sequencing reactions (54).

Media and growth conditions. *S. scabies* was grown on oatmeal agar (52) at 30°C for spore preparation. Seed cultures were grown at 30°C in tryptic soy broth (Difco) and used to inoculate a modified minimal medium containing 1% glucose, 4 mM asparagine, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 2 μM $FeSO_4$, and 2 μM $ZnSO_4$ when needed (18). *E. coli* strains were grown on antibiotic medium 2 agar plates (Difco) and in LB (40) liquid cultures at 37°C.

DNA preparations. *S. scabies* genomic DNA was isolated by the lysozyme-sodium dodecyl sulfate (SDS) method of Hopwood et al. (20), with the following changes. The cell pellet was resuspended in 40 ml of 10% glycerol and centrifuged for 30 min at 12,000 × *g*. This pellet was resuspended in 50 mM Tris (pH 8.0)–25% sucrose to a final volume of 6

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ml. Lysozyme was added to a final concentration of 1 mg/ml, and the mixture was incubated at 30°C. Then 1.34 ml of a freshly prepared STEP solution (0.4 M EDTA, 50 mM Tris [pH 7.5], 0.5% SDS, 1 mg of proteinase K per ml) was added, and the mixture was incubated at 50°C for 60 min with occasional gentle mixing. Phenol (6 ml) was added and mixed by inversion for 5 to 10 min. Chloroform (6 ml) was then added and also mixed by inversion for 5 min. This mixture was centrifuged at 11,000 × *g* for 2 min, and the aqueous layer was transferred to a new tube. The aqueous phase was extracted with a 1:1 phenol-chloroform mixture, followed by a final extraction with chloroform. Sodium acetate (pH 7) was added to a final concentration of 0.3 M, and the DNA was precipitated in 2 volumes of ethanol. The precipitated nucleic acid was spooled out of suspension on a glass rod, transferred to a 5-ml solution of 50 mM Tris (pH 7.5)–1 mM EDTA–200 μg of boiled RNase A per ml, and gently mixed at 4°C. The DNA was extracted with an equal volume of phenol-chloroform (1:1), extracted with chloroform, and ethanol precipitated. The precipitated DNA was spooled out of suspension on a glass rod and gently dissolved in 50 mM Tris (pH 7.5)–1 mM EDTA.

E. coli plasmid DNA and lambda DNA were purified essentially as described previously (36), and single-stranded DNA was purified as reported elsewhere (54). Individual DNA restriction fragments were purified by separation on agarose gels, followed by electroelution of the desired band in an IBI model UEA electroelution apparatus according to the manufacturer's directions.

Preparation and screening of the genomic DNA library. *S. scabies* genomic DNA was partially digested with *Sau*3A1 such that the average fragment length was about 10 kb, as estimated by electrophoresis on a 0.7% agarose gel. This partially digested DNA was ligated to λEMBL 3 arms and packaged by using Packagene extract (Promega). The number of PFU per milliliter was determined by infection of *E. coli* LE392 (42) and plating as described previously (36).

Duplicate screenings of each plate (2,500 plaques per plate; total of 10^4 plaques) were prepared by transfer to nitrocellulose paper as described previously (36). Hybridization with a labeled 33-mer oligonucleotide was in 6× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2× Denhardt solution (8)–25 mM sodium dihydrogen phosphate–0.1% sodium pyrophosphate–30% formamide–0.1% SDS–100 μg of boiled calf thymus DNA per ml for 15 h at 30°C. The filters were washed in 2× SSC–0.1% SDS once for 5 min at room temperature and then twice for 30 min at 50°C. The final wash was in 0.2× SSC–0.1% SDS for 30 min at room temperature.

Transformations. Competent cells of *E. coli* DH5αF′ (33) were prepared as previously reported (17; protocol 8) and used for all transformation procedures.

DNA sequencing. Both strands of the DNA were sequenced by the dideoxy-chain termination method (48), using a Sequenase kit purchased from United States Biochemical. In all cases, the primer used was the 17-mer (no. 1211; purchased from New England BioLabs). The only exception was for one portion of the sequence in which a synthetic oligonucleotide (33-mer) was used as a primer for a double-stranded sequencing reaction (instructions supplied by United States Biochemical). For all templates, both dG and dI reactions were used.

RNA isolation. RNA was isolated by the hot phenol method, using cells that had been filtered over ice and broken by vortexing with 4-mm glass beads (20). The isolated nucleic acid was incubated with 2.3 U of DNase per ml in 50 mM Tris (pH 7.8)–50 mM $MgCl_2$ at room temperature for 30 min. RNA concentration was determined by measuring A_{260}.

Northern analysis. The RNA samples were denatured in 50% formamide–6% formaldehyde–10 mM morpholinepropanesulfonic acid (MOPS; pH 7)–2.5 mM sodium acetate–0.25 mM EDTA at 65°C for 15 min and electrophoresed in 1% agarose gels containing 6% formaldehyde (36). RNA was capillary blotted to a Zeta Probe nylon membrane (Bio-Rad) in 10 mM NaOH and baked at 80°C for 1 h. Prehybridization and hybridization were carried out in 1% SDS–1 M NaCl–10% dextran sulfate at 50°C. The probe used for hybridization was a 330-bp *Bgl*I fragment that is completely contained within the open reading frame of the esterase gene. This fragment was labeled by the random-priming method (13). The filter was washed in 2× SSC–1% SDS at 55°C and in 0.1× SSC–1% SDS at 40°C.

Nuclease S1 5′-end mapping. The 5′ end of the esterase mRNA was mapped by nuclease S1 protection. The DNA fragment used as a probe was an 1,100-bp *Sma*I-*Eco*RI fragment corresponding to the 5′ end of the esterase gene (see Fig. 2). The fragment was 5′ end labeled at the *Eco*RI site with polynucleotide kinase and [γ-^{32}P]ATP (36) after treatment with calf intestine alkaline phosphatase, according to the manufacturers' recommendations. The hybridization mixture containing probe and 50 μg of total *S. scabies* RNA was heated to 85°C and cooled slowly over 1 h. The hybrids were treated with 150 U of nuclease S1 for 30 min at 25°C. The reaction products were analyzed on a 6% polyacrylamide–8 M urea gel next to a dideoxy DNA sequencing ladder of M13mp18 (57).

DNA and protein sequence analysis. All sequence analysis was carried out through the Molecular Biology Computing Center (College of Biological Sciences, University of Minnesota, St. Paul), using the IntelliGenetics suite of programs (IntelliGenetics, Inc., Mountain View, Calif.).

Chemicals and enzymes. [γ-^{32}P]ATP (5,000 mCi/mmol), [α-^{32}P]dCTP (300 mCi/mmol), and [α-^{35}S]dATP (650 mCi/mmol) were obtained from Amersham. DNA polymerase I (Klenow fragment) was obtained from United States Biochemical. RNasin was obtained from Promega, RNase and DNase were purchased from Worthington, nitrocellulose filters were obtained from Schleicher & Schuell, and T4 polynucleotide kinase and mung bean nuclease were purchased from New England BioLabs. RNA size markers were synthesized in vitro from the Riboprobe Gemini Positive Control Template (Promega). Random primers and all other enzymes were obtained from Boehringer Mannheim Biochemicals and used as specified by the manufacturer. Other chemicals were obtained from Sigma Chemical Co.

Nucleotide sequence accession number. The DNA sequence reported here has been submitted to GenBank and assigned accession number M57297.

RESULTS

Cloning of the *S. scabies* esterase gene. The genomic library of *S. scabies* DNA produced in phage lambda was probed with a 33-mer mixed synthetic oligonucleotide. The choice of this particular oligonucleotide was based on the DNA sequence predicted from the amino-terminal amino acid sequence of the secreted form of the esterase, as determined by sequential Edman degradation (56). The first 30 amino acids were determined to be:

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1        10        20        30
APADPVPTVFFGDSYTANFGIAPVTNQDSE
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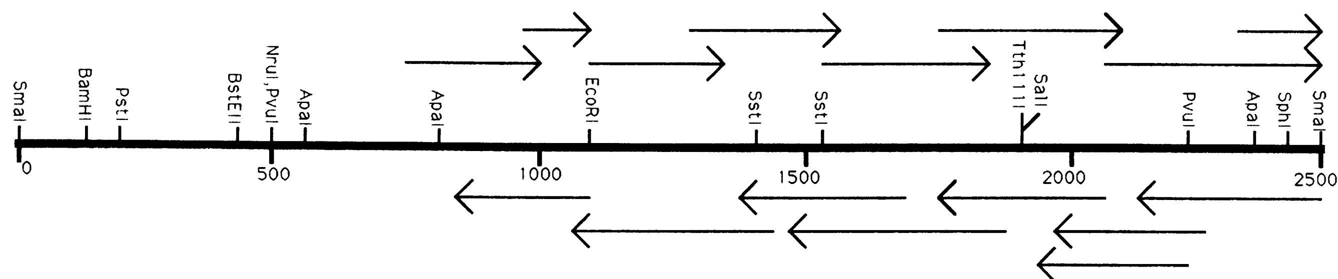

FIG. 1. Restriction endonuclease map of the esterase gene and sequencing strategy. The positions and lengths of all nucleotide sequences determined for the 2.5-kb *Sma*I esterase gene fragment are indicated by the arrows and represent overlapping segments on both DNA strands. The locations of certain endonuclease cleavage sites within this fragment are shown.

The DNA sequence selected for the mixed synthetic oligonucleotide corresponds to amino acid residues 18 through 28. The sequence of the 33-mer mixed oligonucleotide used is 5'-AACTTCGG(C+G)ATCGC(C+G)CC(C+G)GT(C+G)AC(C+G)AACCAGGAC-3'. The rationale for the selection of the mixed synthetic oligonucleotide synthesized was based on the high G+C content of streptomycete DNA. In every position of degeneracy where there was a choice between a T or C residue, the C was chosen. At every position where there was a choice among all four residues, a mixture of G and C was chosen. The 33-nucleotide mixed probe was radioactively labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (36).

The DNA from a positively hybridizing plaque was purified and cleaved with the restriction enzyme *Sal*I. The length of the *S. scabies* DNA insert was approximately 10 kb. To analyze the insert further, the lambda genomic clone was cleaved with several different restriction enzymes and probed with the 33-mer mixed synthetic oligonucleotide. The *Sma*I digest revealed a 2.5-kb fragment that hybridized to the probe (data not shown). This *Sma*I fragment was purified and subcloned in both orientations into the *Sma*I site of pUC119 (54). Single and double digestion of these plasmids with various restriction enzymes resulted in the construction of a restriction map of the 2.5-kb fragment (Fig. 1).

Sequence analysis of the esterase gene. Using the restriction map developed for the 2.5-kb cloned fragment, it was possible to construct a series of plasmids for which various regions had been deleted. Deletants were made by removal of specific restriction fragments or by the action of exonuclease III or *Bal* 31 nuclease. The overlapping sequences on both strands that were chosen for DNA sequencing are illustrated in Fig. 1. The DNA and predicted amino acid sequences of the open reading frame encoding the esterase are shown in Fig. 2. This open reading frame encodes 345 amino acids. Amino acids 40 to 69 correspond exactly with the amino-terminal amino acid sequence of the purified extracellular esterase as determined by Edman degradation. Also, the DNA sequence of nucleotides 483 to 515 corresponds to the sequence chosen for the mixed synthetic oligonucleotide that was used as the probe for cloning the esterase gene. These sequence matches indicate that the gene which was cloned actually encodes the *S. scabies* extracellular esterase. The coding sequence of the esterase gene is 69% G+C in the first position of the codons, only 46% in the second, and 92% in the third. The entire coding sequence has an overall G+C content of 69%, compared with about 73% G+C for the entire genome of most *Streptomyces* species (3).

The DNA sequence predicts the presence of two in-frame AUG codons, either of which could be recognized for initiation of translation. An indication that one or both of these codons is the actual start codon is the presence of a sequence, centered about 11 nucleotides 5' to the first methionine codon, that is complementary to the 3' end of 16S rRNA of *Streptomyces lividans* (2). This sequence (5'-GAAAGG-3') could serve as the ribosome-binding site. Extending from the first AUG start codon to the N-terminal alanine of the extracellular form of the esterase, the DNA sequence predicts the synthesis of 39 additional amino acids. This region of the amino acid sequence may comprise a signal sequence that is removed from the esterase during the process of secretion.

The predicted amino acid composition of the esterase open reading frame from amino acids 40 to 345 (Fig. 2) was compared with the amino acid composition experimentally determined for the purified extracellular form of the esterase. The representations of the amino acids were similar when we compared the predicted composition with the determined composition (data not shown), once again indicating that the cloned gene encodes the extracellular esterase that was purified from *S. scabies* FL1 (38).

Identification of the 5' terminus of the esterase message. End mapping experiments were carried out with nuclease S1 to determine the location of the transcriptional start site of the esterase mRNA. An 1,100-bp *Sma*I-*Eco*RI fragment from the cloned esterase gene was 5' end labeled at the *Eco*RI site and used as the probe. This 1,100-bp fragment extends from the *Sma*I site at one end of the subcloned esterase gene fragment to the *Eco*RI site within the coding sequence of the esterase gene (Fig. 1). One major fragment was protected from S1 digestion by hybridization with the probe (Fig. 3). This fragment was estimated to be 82 nucleotides in length by comparison with an M13mp18 sequencing ladder. This corresponds to a transcriptional start site at the G residue at position 264 of the esterase sequence, which is 51 nucleotides upstream from the first possible translational start site at position 315. This proposed transcriptional start site is indicated in Fig. 2.

Zinc regulation. Production of the extracellular esterase by *S. scabies* is dependent on the presence of zinc in the growth medium (38). To determine whether zinc regulates enzyme production by affecting the level of esterase mRNA, total RNA was extracted from cells grown in the presence and absence of 2 μM zinc. Esterase-specific mRNA was detected by Northern hybridization with a 330-bp *Bgl*I fragment that is contained completely within the open reading frame of the esterase gene. There was very little esterase mRNA in the cells grown without zinc compared with the RNA extracted from the cells grown in the presence of zinc (Fig. 4). The

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        10         20         30         40         50         60         70         80         90        100
GACGGCCGCT GCTCTGTACG GCAGGCCGGC CGCATTCCGG ACGCCTCCGC CCCGTCGGCA CATGCCCACG CCGCCGGGCC CGCGTGGCGG TCGGGCAGGC
       110        120        130        140        150        160        170        180        190        200
TCCTCCCCTT TCGGGTCGTG ATCACATCGG TACTCATCGG CCACACCTCC CCAGCACCCC TGCTGAACAG GTGAGATGGA CACACGTGAG GGGTTGGCCA
       210        220        230        240        250        260      * 270        280        290        300
GGAACACCCG GTTTGATCAC CTCCATTGGA TTATCACGCT CCGTATGTGA CTGAATGCAG AGCGTTCCAT CTTGCTCGGC TCCTCCTCGT GAGCCTCGGT

       310          320             329             338             347             356             365             374             383
GAAAGGGCAC  AGCC ATG TCT TCG GCC ATG CGT AAG ACG ACG AAT TCT CCG GTG GTA CGG CGG CTG ACC GCC GCG GCC GTC GCA
                 MET Ser Ser Ala MET Arg Lys Thr Thr Asn Ser Pro Val Val Arg Arg Leu Thr Ala Ala Ala Val Ala

        392         401         410         419         428    ↓    437         446         455         464
CTG GGC AGC TGC CTG GCG CTG GCC GGT CCG GCG GGA AGC GCG GGC GCG GCG CCC GCC GAC CCC GTA CCG ACC GTC TTC TTC
Leu Gly Ser Cys Leu Ala Leu Ala Gly Pro Ala Gly Ser Ala Gly Ala Ala Pro Ala Asp Pro Val Pro Thr Val Phe Phe

        473         482         491         500         509         518         527         536         545
GGC GAC TCC TAC ACC GCC AAC TTC GGC ATC GCT CCC GTG ACC AAC CAG GAC AGC GAG AGG GGC TGG TGT TTC CAG GCG AAG
Gly Asp Ser Tyr Thr Ala Asn Phe Gly Ile Ala Pro Val Thr Asn Gln Asp Ser Glu Arg Gly Trp Cys Phe Gln Ala Lys

        554         563         572         581         590         599         608         617         626
GAG AAC TAT CCC GCC GTC GCC ACC CGG AGC CTG GCC GAC AAG GGC ATC ACG CTC GAC GTC CAG GCG GAC GTC TCC TGC GGA
Glu Asn Tyr Pro Ala Val Ala Thr Arg Ser Leu Ala Asp Lys Gly Ile Thr Leu Asp Val Gln Ala Asp Val Ser Cys Gly

        635         644         653         662         671         680         689         698         707
GGC GCG CTC ATC CAC CAC TTC TGG GAG AAG CAG GAG CTG CCC TTC GGC GCC GGC GAG CTC CCG CCG CAG CAG GAC GCG CTC
Gly Ala Leu Ile His His Phe Trp Glu Lys Gln Glu Leu Pro Phe Gly Ala Gly Glu Leu Pro Pro Gln Gln Asp Ala Leu

        716         725         734         743         752         761         770         779         788
AAG CAG GAC ACC CAG CTG ACC GTG GGA AGC CTG GGC GGC AAC ACA CTG GGT TTC AAC CGC ATC CTG AAG CAG TGT TCC GAC
Lys Gln Asp Thr Gln Leu Thr Val Gly Ser Leu Gly Gly Asn Thr Leu Gly Phe Asn Arg Ile Leu Lys Gln Cys Ser Asp

        797         806         815         824         833         842         851         860         869
GAG CTC CGG AAG CCC TCT CTG CTG CCG GGA GAC CCG GTG GAC GGG GAC GAG CCG GCC GCC AAG TGC GGT GAG TTC TTC GGG
Glu Leu Arg Lys Pro Ser Leu Leu Pro Gly Asp Pro Val Asp Gly Asp Glu Pro Ala Ala Lys Cys Gly Glu Phe Phe Gly

        878         887         896         905         914         923         932         941         950
ACC GGG GAC GGA AAG CAG TGG CTG GAC GAC CAG TTC GAG CGG GTC GGT GCG GAG CTG GAG GAG CTG CTC GAC CGC ATC GGC
Thr Gly Asp Gly Lys Gln Trp Leu Asp Asp Gln Phe Glu Arg Val Gly Ala Glu Leu Glu Glu Leu Leu Asp Arg Ile Gly

        959         968         977         986         995        1004        1013        1022        1031
TAC TTC GCC CCC GAC GCC AAG CGC GTC CTG GTC GGC TAC CCC CGG CTC GTG CCC GAG GAC ACC ACC AAG TGC CTG ACC GCG
Tyr Phe Ala Pro Asp Ala Lys Arg Val Leu Val Gly Tyr Pro Arg Leu Val Pro Glu Asp Thr Thr Lys Cys Leu Thr Ala

       1040        1049        1058        1067        1076        1085        1094        1103        1112
GCG CCC GGT CAG ACG CAA CTG CCG TTC GCC GAC ATC CCC CAG GAC GCC CTG CCG GTC CTG GAC CAG ATC CAG AAG CGG CTG
Ala Pro Gly Gln Thr Gln Leu Pro Phe Ala Asp Ile Pro Gln Asp Ala Leu Pro Val Leu Asp Gln Ile Gln Lys Arg Leu

       1121        1130        1139        1148        1157        1166        1175        1184        1193
AAC GAT GCC ATG AAG AAG GCC GCC GCC GAC GGC GGC GCC GAC TTC GTC GAC CTC TAC GCC GGC ACC GGC GCC AAC ACG GCG
Asn Asp Ala Met Lys Lys Ala Ala Ala Asp Gly Gly Ala Asp Phe Val Asp Leu Tyr Ala Gly Thr Gly Ala Asn Thr Ala

       1202        1211        1220        1229        1238        1247        1256        1265        1274
TGC GAC GGC GCC GAC CGG GGC ATC GGC GGC CTG CTG GAG GAC TCC CAG CTC GAA CTC CTC GGC ACC AAG ATC CCC TGG TAC
Cys Asp Gly Ala Asp Arg Gly Ile Gly Gly Leu Leu Glu Asp Ser Gln Leu Glu Leu Leu Gly Thr Lys Ile Pro Trp Tyr

       1283        1292        1301        1310        1319        1328        1337        1346        1355
GCG CAC CCC AAT GAC AAG GGC CGC GAC ATC CAG GCC AAG CAG GTG GCC GAC AAG ATC GAG GAG ATC CTC AAC CGG TAA GAG
Ala His Pro Asn Asp Lys Gly Arg Asp Ile Gln Ala Lys Gln Val Ala Asp Lys Ile Glu Glu Ile Leu Asn Arg  ●

      1365       1375       1385       1395       1405       1415       1425       1435       1445       1455
GCACAGTCTC CCCGCTGGTC GTCCAAGGGC AGAAGGGTCC GTAGGCGGAC TAGGGTCCGT CTTCAAAGAT CATCGGGTGG TGGATCATGG TGGGGTGATA
      1465       1475       1485       1495       1505       1515       1525       1535       1545       1555
CGCCGGCCATG AACTCACCGA TCGGGATTGG GAGTTGCTCG CTCCACTGAT ACCGCGGGCC GCGACGGGCC GGCCGCGAGT GTCGGACCCG CAGGTCATCA
      1565       1575       1585       1595       1605       1615       1625       1635       1645       1655
ACGGGATGGT CTACAAGATC TTCGAGGCCC CGCTCGCTGG CGAGGGGGCC CACGTCGGTG TCCTCGTCCA GCGGGTTCCC GACCTTCAGC GCCCGCATGC
      1665       1675       1685       1695       1705       1715       1725       1735       1745
CCTCGACGAA CCGCTCGGCG AACGCCTCGT ACACGTCGGC GTGCACGATG AACCGCTTGG CGGCGATGCA CGACTGACCG GTGTTCTGCA CCC
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FIG. 2. Nucleotide sequence of the esterase gene, predicted amino acid sequence of the open reading frame, and sequences of 5′ and 3′ untranslated regions. Sequences were determined from overlapping segments of the DNA and confirmed by sequencing both strands of the DNA as outlined in Fig. 1. The proposed start point for transcription of the esterase mRNA, determined by nuclease S1 mapping (Fig. 3), is indicated by the asterisk above the G residue at position 264. The two boxed Met residues are two potential start sites for translation of the esterase mRNA. The underlined sequence GAAAGG at positions 301 to 306 is the proposed Shine-Dalgarno sequence for translation initiation. The amino acids underlined from positions 432 to 521 correspond to those determined by Edman degradation sequencing of the purified extracellular esterase protein, and those underlined with the dotted line (483 to 515) correspond to the sequence of the mixed synthetic oligonucleotide used as the probe for cloning the esterase gene. The arrow indicates the proposed site for cleavage between the signal sequence and the secreted form of the esterase.

esterase mRNA was estimated to be about 1,440 nucleotides in length, which is sufficient to encode the 36-kDa protein. Two additional bands that were detected in the hybridization most likely arose from nonspecific hybridization of the *Bgl*I fragment with the 16S and 23S rRNAs. These results suggest that zinc is involved in regulating the level of esterase gene transcription or possibly affects the stability of the esterase message.

DISCUSSION

The extracellular esterase produced by *S. scabies* FL1 appears to be different from other known esterases. The nucleotide sequence of the esterase gene was compared with the sequences of other known genes. In addition, the deduced amino acid sequence was compared with sequences of other available proteins. For either the nucleotide sequence or the amino acid sequence, no significant similarity was found to the *S. scabies* esterase. There was not even significant similarity between the *S. scabies* FL1 esterase and the cutinases reported by Ettinger and co-workers (11). This may not be surprising since there was only 47% homology found when the fungal cutinases from *Fusarium solani* f. sp. *pisi* and *Colletotrichum* species were compared with each other (11). The results of these comparisons suggest that the *S. scabies* esterase is different from other proteins that have been analyzed.

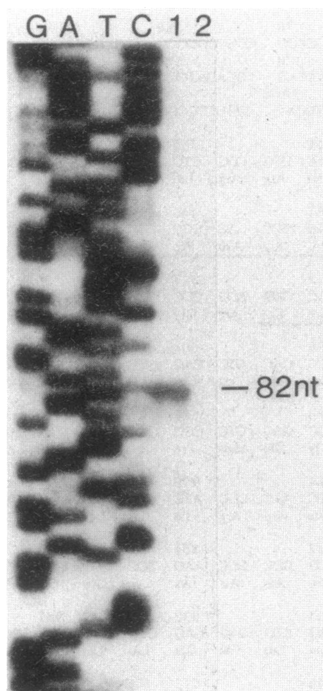

FIG. 3. Nuclease S1 mapping of the 5′ end of the esterase mRNA. The 5′-end-labeled probe was incubated either with total cellular RNA isolated from *S. scabies* FLI grown in minimal medium with added zinc (lane 1) or without added RNA (lane 2). The resulting hybrids were treated with nuclease S1, and the products were analyzed by electrophoresis in a 6% polyacrylamide–8 M urea gel. The length of the protected fragment (lane 1) is indicated and was determined by comparison with a sequencing ladder of M13mp18. The four separate sequencing reactions are labeled G, A, T, and C. nt, Nucleotides.

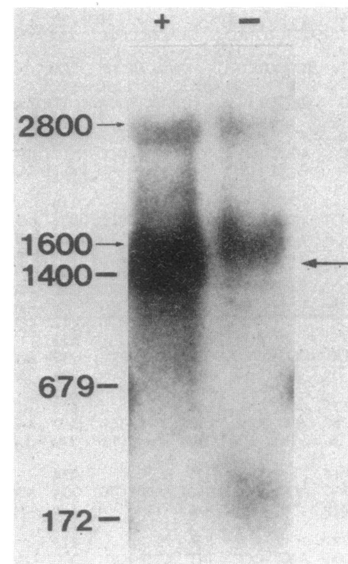

FIG. 4. Analysis of esterase-specific mRNA by Northern hybridization. Total RNA (30 μg) extracted from cells grown with (+) or without (−) 2 μM zinc was probed with a DNA fragment contained within the coding sequence of the esterase gene. Numbers on the left indicate the migration (in nucleotides) of labeled RNA transcripts that were synthesized in vitro and used as molecular length markers; arrows on the left indicate the migration of 23S and 16S rRNAs; the arrow on the right indicates the migration of the esterase-specific mRNA extracted from the cells grown in the presence of zinc.

One similarity of the esterase amino acid sequence to sequences of other secreted proteins lies in the putative signal sequence. This sequence has many of the characteristics common to the signal sequences of proteins processed by the signal peptidase I type of pathway (55). The esterase signal sequence has a positively charged n region and a long central hydrophobic h region. It also has one of the most common primary sequences at the processing site, with alanine at both the −3 and −1 positions. One of the striking differences between the *S. scabies* esterase signal peptide sequence and those identified for secreted proteins from organisms other than the streptomycetes (14) is its 39-amino-acid length. These features of the signal peptide have been recognized for other secreted streptomycete proteins (5, 10, 22, 29, 34, 43, 47), even though the components of this sequence essential for efficient secretion have not been identified.

The nucleotide sequence 5′ to the proposed transcriptional start site for the esterase gene has been analyzed for an RNA polymerase-binding site by comparison with other known streptomycete gene promoters. There does not appear to be a consensus *E. coli*-like promoter sequence as outlined by Bibb (1) and Hopwood et al. (19). Supportive of this conclusion is the finding that the esterase gene is not expressed in *E. coli* unless a promoter, such as the *lac* promoter, is placed 5′ to the esterase-coding sequence (unpublished results).

Sequence comparison of the esterase 5′ region with other streptomycete gene promoters that are not *E. coli*-like has revealed some similarities in both the −10 and −40 regions. A sequence centered 10 nucleotides from the esterase gene transcriptional start site (CTGAATG) shares homology with a *dagA* P3 (5) sequence centered at −12 (GAATG), with a *gal* P2 (15) sequence centered at −10 (CTGAA), and with a *tsr* P2 (23) sequence centered at −10 (GAAT). There is also similarity between the esterase −10 sequence and an *actIII* P1 (16) sequence (CTGAgcG); however, this sequence is centered at −4 relative to the transcriptional start site and may not be relevant to RNA polymerase binding. Similarities have also been found between the esterase gene sequence centered at −41 (CTCCA) and other streptomycete gene sequences. The β-galactosidase gene P1 promoter (10) has a similar sequence positioned at −46 (CTCCg), as do the promoters pARC1 (CTCCc) at −39 (21), *actIII* P1 (CTCCc) at −41 (16), *dagA* P3 (CTCCt) at −42 (5), *aml* P1 (gTCCA) at −44 (34), and *gal* P2 (CTCCA) at −51 (15).

Homology between the *dagA* P3 promoter sequence and the esterase gene sequence is of particular interest. *dagA* P3 is apparently recognized by a form of *Streptomyces coelicolor* RNA polymerase designated $E\sigma^{49}$, whose activity predominates in stationary-phase cultures (6). The esterase activity in *S. scabies* is not detected until the culture has reached the late exponential to early stationary phases of growth (unpublished results), suggesting the possibility that transcription depends on a form of RNA polymerase that is active during the later stages of growth. In the streptomycetes, heterogeneity in sigma factors associated with RNA polymerase that may mediate specific regulation of gene expression at the level of transcription has been recognized (4). These homologies between the 5′ esterase gene sequence and those of other streptomycete gene promoters

may indicate similarities in the form of RNA polymerase utilized for transcription. Further studies are needed to precisely identify the RNA polymerase-binding site for the esterase gene and to characterize the species of RNA polymerase responsible for transcription of this gene.

Another unique feature of the *S. scabies* esterase is the effect of zinc on esterase mRNA levels. The mechanism of zinc action is not yet understood and could involve either the stability of the esterase mRNA or esterase gene transcription. In procaryotic systems, metals have been shown to play a role in gene expression. The synthesis of iron uptake systems is responsive to iron concentrations (44). The repressor of the *mer* operon acts as both a negative and a positive regulator of transcription of the *mer* genes, depending on the presence of mercuric ions. In the absence of mercuric ion as an inducer, the repressor binds to the DNA and inhibits transcription. When inorganic mercury is present, it is thought to bind to the repressor and the Hg-repressor complex stimulates transcription (45). Studies in eucaryotic systems demonstrating the effects of metals in enhancing transcription of particular genes have been reported. For example, cadmium induces transcription from the metallothionein promoter (25, 51). Zinc has also been recognized as a metal which modulates transcriptional activation of certain genes (12, 41). For the cases in which zinc is involved, a transcriptional activation factor is a zinc-binding protein. The TFIIIA protein of *Xenopus laevis*, which is an RNA polymerase III transcription factor, contains nine zinc finger domains (39). The mammalian SP1 transcription factor contains three of these repeated structures (24). Zinc is necessary for the regulatory protein to bind to the DNA and stimulate transcription.

These examples suggest a likely model for regulation of *S. scabies* esterase gene expression in which a transcription activation factor required for esterase gene expression is a zinc finger protein. Zinc may be required for efficient binding of the factor to the DNA in order to enhance transcription. Studies are currently focused on identifying the nucleotide sequence of the esterase gene that is involved in zinc-regulated expression and on isolation of the putative regulatory protein required for esterase gene transcription.

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