

Molecular Analysis of a Gene Encoding a Cell-Bound Esterase from *Streptomyces chrysomallus*

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A gene (*estA*) encoding a 42-kDa cell-bound esterase, EstA, was found to be located 75 bp upstream of the cyclophilin A gene (*cypA*) of *Streptomyces chrysomallus*. Western blot analysis revealed the presence of EstA (42 kDa) in cell extracts of *S. chrysomallus* X2 and *Streptomyces lividans*. EstA specifically hydrolyzes short-chain *p*-nitrophenyl esters. EstA formation starts at the end of growth phase, and its activity level remains constant throughout stationary phase. Expression of *estA* from the melanin (*mel*) promoter in plasmid pIJ702 led to a substantial increase of total esterase activity in streptomycetes.

Streptomycetes are filamentous soil bacteria possessing a large repertoire of extracellular enzymes for the degradation of biopolymeric material in their natural habitats (13). The presence of these enzymatic activities is often substrate regulated but can also depend on a specific stage in the life cycle (21). The life cycle of the streptomycetes is characterized by the development of a vegetative substrate mycelium from which aerial mycelium and later spores are formed. Production of antibiotics and other secondary metabolites is a characteristic feature of streptomycetes and usually starts at the onset of the stationary growth phase (2). The regulation of formation of lipases and esterases in streptomycetes has attracted particular interest. Stationary-phase-dependent formation of lipases has been demonstrated in several cases, e.g., with *lipA* from *Streptomyces exfolians* (20). In addition, interesting regulation systems involving coexpression of activator proteins for *Streptomyces* lipase or esterase gene expression have been described previously (1, 20). Esterases hydrolyze water-soluble or emulsified esters with short-chain carboxylic acids, whereas lipases are specific for emulsified substrates with fatty acyl chains. Esterase sequences show the conserved motif G-X₁-S-X₂-G containing the active-site serine representing the nucleophilic residue in the catalytic triad of these enzymes (4, 13). This triad normally consists of an additional histidine and an additional aspartate located at specific positions in the polypeptide chains, which, however, are not obviously the same ones in the various enzymes (3–5). Interestingly, two previously identified *Streptomyces* esterases lack the classical G-X-S-X-G/G-X-S motif and may contain a novel variant of the catalytic triad (22).

Sequencing of the upstream region of *cypA* from *Streptomyces chrysomallus*. Plasmid pMS104 (17) contains a 9-kb *Xho*I fragment of the *S. chrysomallus* X2 (10) chromosome carrying the cyclophilin gene *cypA*. About 2.4 kb of the immediate upstream region of *cypA* was sequenced (EMBL accession no. Z15137), and sequence analysis revealed two open reading frames (ORFs) (*estA* and *orfA*; 1,170 and 435 bp, respectively).

The identified ORFs are separated by 268 bp and are in same orientation as that of *cypA* (Fig. 1); *estA* starts with a GTG codon, and the TGA stop codon is 74 bp in front of *cypA*. The noncoding regions upstream of *orfA* and *estA* did not conspicuously show conserved promoter structures. The G+C content of *estA* is 74.4% (of *orfA*, 74.8%), and the codon usage is typical for a streptomycete gene with an average G+C content of 93.5% (*orfA*, 87.1%) in the third codon position. From the deduced amino acid sequence, it is apparent that *estA* encodes a protein of 41,178 Da and *orfA* encodes one of 15,441 Da. Southern hybridization analyses showed that *estA* is present as a single copy on the chromosome of *S. chrysomallus*.

***estA* encodes an esterase.** Database searches with the complete protein sequence deduced from the *estA* nucleotide sequence revealed the highest similarities to two cell-bound esterases from psychrotrophic pseudomonads (11, 14) (43% identity to both) and to the ethyl chrysanthemate esterase from *Arthrobacter globiformis* (15) (38% identity) (Fig. 2). Like these proteins, EstA does not contain a signal sequence, which indicates that it is a cell-bound protein. EstA contains the sequence GGS³⁴³CG, which agrees with the consensus sequence surrounding the active-site serine, G-X₁-S-X₂-G, observed in a large number of serine esterases, including lipases and serine proteases (4). Interestingly, the N-terminal part of EstA (amino acids 30 to 100) revealed additional sequence similarity to β -lactamases, e.g., to cephalosporinase from *Escherichia coli* (8) (50% identity) and to β -lactamase from *Citrobacter freundii* (16) (44% identity), with the sequence featuring the conserved S-X-X-K motif which forms part of the catalytic center of β -lactamases (16).

Heterologous expression of *estA* in *E. coli*. *estA* was modified by PCR with oligonucleotides A (5'-AGGGAGGCCGCATGCCGAGATCCAC-3') and B (5'-AACTGCAGTCACCTCCCGCGGCCCTC-3'). The PCR fragment was cloned into the *E. coli* expression plasmid pQE32 (Qiagen), which generates pQEBOX321 encoding an N-terminal six-His-tagged EstA. In crude extracts of *E. coli* transformants, a 42-kDa protein was detectable. Only a small amount of the protein was soluble, and it was tested with *p*-nitrophenylbutyrate as the substrate. The specific esterase activity was four to five times higher than that in the extract from the control strain (data not shown). Only the denatured soluble protein (in 6 M urea or 8 M guanidine hydrochloride) binds to the Ni-chelate matrix, indicating that the six-His tag is buried in the native form. To generate antibodies, the denatured form was eluted from the

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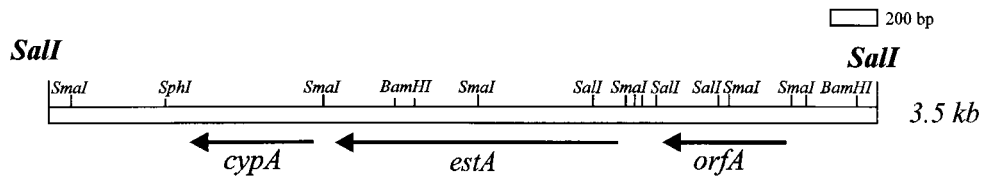


FIG. 1. Organization of the *estA* gene region of *S. chrysomallus* X2. A ca 2.4-kb fragment of genomic *S. chrysomallus* X2 DNA is shown. The ORFs are represented by arrows. *cypA* encodes cyclophilin A, *estA* encodes cell-bound esterase A, and *orfA* is of unknown function.

Ni-chelate matrix and further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Overexpression of *estA* and characterization of its gene product in *S. chrysomallus* and *Streptomyces lividans*. For overexpression of *estA* in streptomycetes, two PCR-generated *estA* derivatives were cloned as *SphI-SstI* fragments behind the melanin promoter in plasmid pIJ702 (7, 9), which generates pBOX9 and pBOX19. Primers A and B (see above) were used to generate the wild-type gene (pBOX9), primers C (5'-GGAG

GGCCGCATGCACCACCACCACCACCACCCTGCGACGACCGCTTCAG-3') and B were used to generate an *estA* derivative coding for a six-His-tagged EstA (pBOX19). Both PCR fragments were first subcloned as *SphI-PstI* fragments into pTZ18 (Pharmacia) to obtain a 3' *SstI* site, necessary for cloning into pIJ702. *S. chrysomallus* X2 and *S. lividans* transformants carrying plasmids pBOX9, pBOX19, and pIJ702 as well as the nontransformed strains were then grown in complete medium (2-day-old mycelium was used), and protein extracts

EstA	S.c.:	-----MPQIHGH CDDRFSGVYDAFEEN TERGELCAAVTVLVDGKPYDIDGCVABAAR	: 54
EstIII	P.f.:	MVFEEAAYVQIQGHYELQFPAIRZAFALDDPQERCAGLCIQIGGETVYDIAWAGTADKDG	: 61
EstA	P.s.:	-----MQIQGHYELQFPAVREAFALDDPQERCAGLCIQIGGETVYDIAWAGTADKDG	: 53
CEH	A.g.:	-----MDAQTAPGFSVAALLEGFRFLSEDEYSAQIAAYHFGVKVLDISGCPHR--R	: 50
SXXX			
EstA	S.c.:	TRPHERDILVNVWSTGKGPPTLCAHVLDADRGLDLDAVVAHWPEFPAAGKSVLVRHLLS	: 115
EstIII	P.f.:	AEAHSDAIVNLSCTKTFYAVTALQLVAEGKQLDADAVKAWPEFPAAGKSVLVRHLLC	: 122
EstA	P.s.:	TEAHSDAIVNLSCTKTFYAVTALQLVAEGKQLDADAVNVAWPEFPAAGKAVLVRHLLC	: 114
CEH	A.g.:	P-----DSVTGVSSSKGVSGLVIALLVQDGFLLDAEYVVKWPEFGAEGKATLVAQLLS	: 106
SXXX			
EstA	S.c.:	HRSFVAGPDTPLTLLELYDMETACARLATTPEPGTRSGYHASTYGFVYCEVVRITGL	: 176
EstIII	P.f.:	HOAGCPAIREMLPTLALYDHRIMVDTLAEAPWTPGQGHGYEATYGNLYCELRADCR	: 183
EstA	P.s.:	HOAGCPAIREMLPTLALYDHRIMVDTLAEAPWTPGQGHGYEATYGNLYCELRADCR	: 175
CEH	A.g.:	HOAGLLGVEGGLTLAEYNNSELAALKLAQMRPLWPGTAFGYHALTIQVFMEELCRITCS	: 167
SXXX			
EstA	S.c.:	LDGAFLRQEITGHTGIDFTFGLPESETHRLAELVQERP---DRAAQAALDRM-TFVAVAS	: 233
EstIII	P.f.:	GGFSIVARVARVPLGLDFVGLADEEFYRVAHIARSKGNMGDEAAORLLQVMMREPTAMTT	: 244
EstA	P.s.:	GGFSIVARVARVPLGLDFVGLADEEFYRVAHIARSKGNMGDEAAORLLQVMMREPTAMTT	: 236
CEH	A.g.:	TLQRIYEQIRSVTGAHDFLGLPESEEPYVATLRWAAD---PSQPWIDPASHFGLSANSVAV	: 225
SXXX			
EstA	S.c.:	--LLNPTGRAAANTPAMRAAEIDVANGCTARAVAAALYGILAGRCSDGRRILSEKAAAR	: 292
EstIII	P.f.:	RAFANPFSILTSTNKEPWRMQQPAANGCNARSLAGFYSCLLD-GSILEADMEEQLTREH	: 304
EstA	P.s.:	RAFANPFSILTSTNKEPWRMQQPAANGCNARSLAGFYSCLLD-GSILEADMEEQLTREH	: 296
CEH	A.g.:	GDILDLENLREVR--AAGLSSAAGVASAEGMARVYAAALTGLAANGDRAAVAPLSEETIQ	: 284
SXXX			
EstA	S.c.:	VGEGQGPCRDVLVLDGDFVHETEIGLGLWVSG---A-NRSYGPDFRALGHDGAGCSCGLADP	: 349
EstIII	P.f.:	S---IGFDKTLT-----QTRIGLGCMLDQ-QPMPNATFGLGFRAGHFGAGCSVGLADP	: 355
EstA	P.s.:	S---IGFDKTLT-----QTRIGLGCMLDQQPQLPNATFGLGFRAGHFGAGCSVGLADP	: 348
CEH	A.g.:	T---VTAEQVFGI-----DRVIGETSCFGTVFKSHARSPYGSYRAGHDGASASLGLADP	: 336
GXSGX			
EstA	S.c.:	DAGIALGYVMRMGSRLADDPKRTALVNAVYAAYGEAAGR--	: 389
EstIII	P.f.:	EHDVAFGFVINTLGPYVLMPPRAQKLV-----GILAGCL-	: 389
EstA	P.s.:	EHDVAFGFVINTLGPYVLMPPRAQKLV-----GILAGCL-	: 382
CEH	A.g.:	VYELAFGYVPPQQAEPGGAG-CRNLELSAAVR---KAVTELAQ	: 374

FIG. 2. Amino acid sequence alignment of esterase A from *S. chrysomallus* X2. Sequences for esterase A from *S. chrysomallus* X2 (EstA S.c.), esterase III from *Pseudomonas fluorescens* (EstIII P.f.), esterase A from *Pseudomonas* spp. (EstA P.s.), and carboxylic ester hydrolase from *Arthrobacter globiformis* (CEH A.g.) are shown.

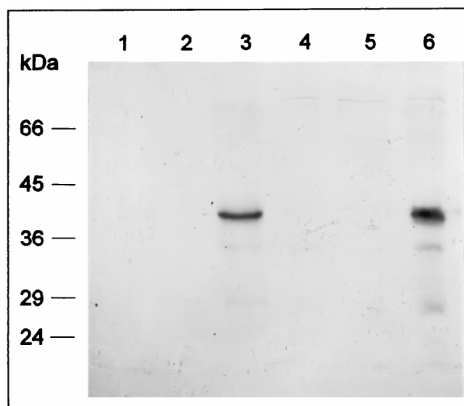


FIG. 3. Overexpression of *estA* in streptomycetes. The Western blot shows SDS-PAGE separations of total cellular protein of sonicated cells from *S. chrysomallus* X2 (lane 1), *S. chrysomallus* X2 harboring pIJ702 (lane 2), *S. chrysomallus* X2 harboring pBOX9 (lane 3), *S. lividans* (lane 4), *S. lividans* with pIJ702 (lane 5), and *S. lividans* harboring pBOX9 (lane 6). Each lane contains about 50 μ g of protein. All streptomycetes were grown for 30 h in complete medium.

derived from these strains were analyzed with respect to the presence of EstA and their lipolytic activities. In Western analyses, strains transformed with pBOX9 (Fig. 3, lanes 3 and 6) or pBOX19 (data not shown) revealed the presence of EstA. By contrast, EstA was hardly detectable in strains transformed with pIJ702 (Fig. 3, lanes 2 and 5) and nontransformed strains (Fig. 3, lanes 1 and 4). Attempts to purify six-His-tagged EstA expressed from the pBOX19 construct in *S. chrysomallus* X2 failed, as in the case of the very similar construct in *E. coli*, indicating the inaccessibility of the six-His tag in this protein. Therefore, esterase and lipase activity was determined in crude extracts by using *p*-nitrophenyl acetate (pNPAc), *p*-nitrophenyl

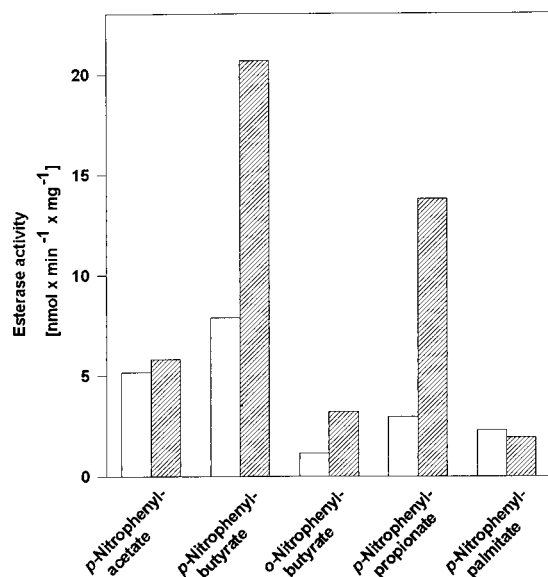


FIG. 4. Cell-bound lipolytic activity and substrate specificity of EstA. The substrate specificity of EstA encoded by pBOX9 as measured by hydrolysis of *p*-nitrophenyl carboxylic acid esters is shown. The plasmid-bearing strain (hatched bars) shows elevated levels of hydrolysis of pNPBu and pNPPr, while no increases in hydrolysis of pNPAc and pNPPa were observed. Results for Non-transformed *S. chrysomallus* X2 (open bars) are also shown. *S. chrysomallus* X2 was grown to stationary phase, and 100 μ g of total cellular protein was used in the test.

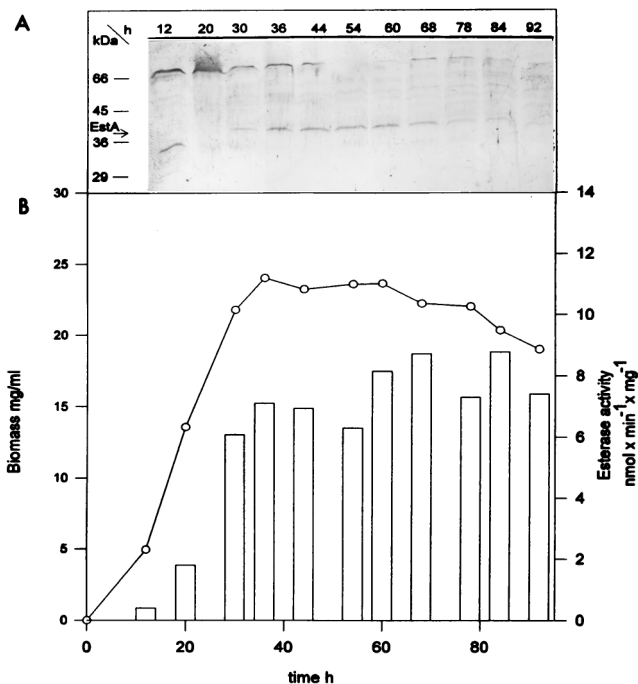


FIG. 5. Growth-phase-dependent expression of *estA* in *S. chrysomallus* X2. (A) SDS-PAGE separations of soluble protein extracts derived from sonicated cells of *S. chrysomallus* X2 from various times of cultivation in complete medium were analyzed by Western blotting. (B) Esterase activity in protein extracts derived from *S. chrysomallus* X2 grown for the indicated times was determined. Vertical bars represent esterase activity with pNPBu as the substrate; mycelial growth (wet weight) (\circ) is also shown.

propionate (pNPPr), *o*- or *p*-nitrophenyl butyrate (oNPBu or NPBu, respectively), and *p*-nitrophenyl palmitate (pNPPa) as substrates. Total esterase activity in *S. chrysomallus* X2 transformed with pBOX9 (Fig. 4) or pBOX19 (data not shown) greatly exceeds that of the untransformed strain (Fig. 4) or that harboring pIJ702. Increases in specific activities in the cleavage of pNPPr and pNPBu are observable, whereas the low basal activities for pNPPa and pNPAc hydrolysis remain unchanged. This unambiguously identifies the *estA* gene product as an esterase. Furthermore, the plasmid-encoded esterase activity was inhibited by 80% within 20 min of incubation at 37°C in the presence of 30 μ M phenylmethylsulfonyl fluoride. Phenylmethylsulfonyl fluoride covalently binds to the active-site serines of many serine proteases and lipases. Measurements of esterase activity in cell extracts of untransformed *S. lividans* (with pNPBu as the substrate) revealed a ca. 10-fold-higher basal esterase activity than that in *S. chrysomallus* X2, which indicates that *S. lividans* may contain additional esterases distinct from EstA, because in Western blot analyses (see below) the EstA levels in *S. lividans* and *S. chrysomallus* X2 are nearly the same.

***estA* is expressed in the stationary phase.** Immunoblot analyses of protein extracts from *S. chrysomallus* X2 mycelium harvested at different times of cultivation revealed that formation of EstA begins at the end of the growth phase (Fig. 5). For extracts from all stages of cultivation, there was an additional cross-reacting immunoreactive band of 66 kDa. The nature of this protein is not known. Esterase activity determined with pNPBu is correlated with the appearance of EstA. Protein level and enzymatic activity remained constant during the stationary phase of cultivation (Fig. 5). A very similar picture was obtained when *S. chrysomallus* X2 was grown in chemically

defined media (data not shown). In these media, the total esterase activity was not affected by the choice of carbon sources, such as glucose, maltose, glycerol, acetate, or the esterase substrate triacetin. Results of Western analyses with *S. lividans* mycelium obtained from the growth and stationary phases show a similar pattern to that of *S. chrysomallus* X2: the appearance of a 42-kDa protein was clearly visible in stationary-phase cultures, while it was hardly detectable in younger cultures (less than 2 days of growth).

Conclusion. Previous reports have shown that streptomycetes possess various lipolytic enzymes classified as extracellular esterases or lipases (6, 18–21). In this report, we describe for the first time a cell-bound esterase from a streptomycete. Previously described cell-bound esterases from other organisms are not essential for growth, and thus their functions are not clear. With *S. chrysomallus* X2, a detailed investigation of EstA function was not possible because this organism is not amenable to gene disruption due to insufficient frequencies of transformation and integration. The gene *estA* lies immediately 5' to the previously described cyclophilin A gene (*cypA*) of *S. chrysomallus* X2. Interestingly, the gene encoding the cell-bound esterase in *Acinetobacter calcoaceticus* BD 413 also lies upstream of a cyclophilin gene in the same orientation (12).

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