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Screening of Environmental DNA Libraries for the Presence of Genes Conferring Lipolytic Activity on *Escherichia coli*

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Environmental DNA libraries prepared from three different soil samples were screened for genes conferring lipolytic activity on *Escherichia coli* clones. Screening on triolein agar revealed 1 positive clone out of 730,000 clones, and screening on tributyrin agar revealed 3 positive clones out of 286,000 *E. coli* clones. Substrate specificity analysis revealed that one recombinant strain harbored a lipase and the other three contained esterases. The genes responsible for the lipolytic activity were identified and characterized.

Lipases catalyze both the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. These enzymes usually exhibit broad substrate specificity and degrade acyl *p*-nitrophenyl esters, Tweens, and phospholipids, often with positional selectivity, stereoselectivity, and chain length selectivity (16). Lipases resemble esterases but differ markedly from them in their ability to act on water-insoluble esters (7). Both types of enzymes have been recognized as very useful biocatalysts because of their wide-ranging versatility in industrial applications. The term "lipolytic enzymes" used throughout this report comprises lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1).

The classical and cumbersome approach to isolate new lipolytic proteins is to screen a wide variety of microorganisms for the desired lipolytic activity. The enzymes and the corresponding genes are then recovered from the identified organisms. In this method, a large fraction of the microbial diversity in an environment is lost due to difficulties in enriching and isolating microorganisms in pure culture. It has been estimated that >99% of microorganisms observable in nature typically cannot be cultivated using standard techniques (3). An alternative approach is to use the genetic diversity of the microorganisms in a certain environment as a whole to encounter new or improved genes and gene products for biotechnological purposes. One way to exploit the genetic diversity of various environments is to isolate DNA without culturing the organisms present. Subsequently, the desired genes are amplified by PCR and cloned. The sequences of the primers used are derived from conserved regions of already known genes or protein families (22). Thus, the identification of entirely new genes or gene products by PCR-based methods is limited. Another way is to use the DNA for the construction of DNA libraries and to clone directly functional genes from environmental samples. The knowledge of sequence information prior to cloning is not required. Another advantage is that the already prepared environmental libraries can be employed for the screening of various targets. This approach shows an alternative way to access and exploit the immense pool of genes from microorganisms that have not been cultivated so far. We and other authors applied this method successfully, i.e., for the direct cloning of genes encoding 4-hydroxybutyrate dehydrogenases (14) and chitinases (9).

In this study, three different environmental DNA libraries were screened for the presence of genes conferring lipolytic activity. The genes encoding lipolytic activity were recovered from the obtained positive *Escherichia coli* strains and then sequenced. Subsequently, the corresponding gene products were analyzed.

Screening for genes conferring lipolytic activity. The environmental DNA libraries were constructed from soil samples using *E. coli* DH5α as a host and pBluescript SK(+) [pSK(+)] as a vector (14). The samples were collected in Germany from a meadow near Northeim (library I), a sugar beet field near Göttingen (library II), and the valley of the Nieme River (library III) as described previously (14). The DNA was isolated from the samples by direct lysis of the microorganisms in the soil. The three libraries revealed average insert sizes of 5 to 8 kb. The percentage of plasmids containing inserts was approximately 80% (14).

For the detection of *E. coli* clones exhibiting lipolytic activity, two types of indicator plates were employed. Lipase activity was detected on Luria-Bertani agar containing triolein (1%, vol/vol) and the fluorescent dye rhodamine B (0.001%, wt/vol). Orange fluorescent halos around lipase-positive *E. coli* strains could be seen when these plates were exposed to UV light of 254 nm (18). Lipolytic activity was detected on Luria-Bertani agar containing tributyrin (1%, vol/vol). For determination of substrate specificity tributyrin was replaced by other triglycerides (see below). Positive *E. coli* clones were detected by zones of clearance around the colonies.

One out of approximately 730,000 and 3 out of approximately 286,000 *E. coli* clones were positive during the initial screens on triolein- and tributyrin-containing agar plates, respectively (Table 1). In order to confirm that the lipolytic phenotype of the clones is plasmid encoded, the recombinant plasmids were isolated and retransformed into *E. coli*, and the resulting *E. coli* strains were screened again on indicator plates (see above). All four different recombinant plasmids, designated pAH110 to pAH113, conferred a stable lipolytic phenotype on the resulting recombinant *E. coli* strains. One was obtained from library I, one was from library II, and two were from library III (Table 1). The insert sizes of pAH110 to pAH113 were in the range of 2,185 to 6,661 bp (Fig. 1).

Characterization of the lipolytic *E. coli* clones. We investigated the substrate specificity of the lipolytic enzymes of *E.*

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TABLE 1. Screening of three environmental libraries for genes conferring lipolytic activity

Test substrate	Library	Sample site	No. of *E. coli* clones tested	No. of lipolytic *E. coli* clones after the initial screen	No. of lipolytic *E. coli* clones with a stable phenotype (designation)
Triolein	I	Meadow	430,000	0	
	II	Sugar beet field	180,000	1	1 (pAH110)
	III	Nieme River valley	120,000	0	
Tributyrin	I	Meadow	73,000	1	1 (pAH111)
	II	Sugar beet field	115,000	0	
	III	Nieme River valley	98,000	2	2 (pAH112; pAH113)

coli/pAH110 to *E. coli*/pAH113 by using various substrates. The following triglycerides were tested in the plate assay: tributyrin, tricaproin, tricaprylin, tricaprin, trilaurin, and triolein. Since lipases are, by definition, carboxylesterases that have the ability to hydrolyze long-chain acylglycerols ($\geq C_{10}$), whereas esterases hydrolyze ester substrates with short-chain fatty acids ($\leq C_{10}$) (25), this assay allowed us to differentiate between both types of lipolytic enzymes. *E. coli*/pAH110 behaved like the lipase-producing control strain *Acinetobacter* sp. strain BD413 (17) with all substrates. Lipolytic activity of *E. coli*/pAH111 to *E. coli*/pAH113 was recorded only with tributyrin as a substrate. This is typical for esterases (18, 19).

A direct quantification of hydrolytic activity in crude extracts of *E. coli*/pAH110 to *E. coli*/pAH113 was done spectrophotometrically at 410 nm and 37°C with emulsified *p*-nitrophenyl esters of different fatty acids as substrates (26). One enzyme unit corresponded to the hydrolysis of 1 μmol of substrate per min. The preparation of crude extracts was performed by sonication as described previously (14). Protein concentrations were measured by the method of Bradford (6) with bovine serum albumin as a standard. With *p*-nitrophenyl esters of fatty acids as substrates, activity was observed only in crude extracts of *E. coli*/pAH110. The rates of hydrolysis for the following fatty acids were as indicated (in units per milligram): butyrate, 68.8; caprylate, 38.7; laurate, 16.5; and palmitate, 16.4. The enzyme showed higher activity towards esters of short- to medium-chain (C_4 and C_8) fatty acids. A similar substrate specificity was found for the crude preparation of lipase 2 from *Moraxella* sp. strain TA144 (13) and the cold-adapted lipase (LipP) from *Pseudomonas* sp. strain B11-1 (8). The lack of activity with *p*-nitrophenyl esters of fatty acids as found in crude extracts of *E. coli*/pAH111 to *E. coli*/pAH113 was also reported for the lipolytic enzyme of *Streptomyces cinnamoneus* (23). Thus, the results of both assays indicated that the enzyme produced by *E. coli*/pAH110 is a lipase, whereas the other three enzymes are esterases.

Because of its broad substrate specificity, the lipase was studied further. In order to determine the molecular mass of the enzyme, the crude extract of *E. coli*/pAH110 was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (21) and activity staining. For activity staining the sample applied to the gel was not boiled, and SDS was removed after electrophoresis by gentle shaking of the gel for 20 min in 20% (vol/vol) isopropanol and for 10 min in distilled water. Subsequently, the gel was transferred for detection of lipase activity to an agar plate containing 1% tributyrin or tricaprylin, 25 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, and 1.3% agar (23). Afterward, the gel was incubated for 12 h at room temperature. As shown in Fig. 2, the crude extract of *E. coli*/pAH110 revealed a band with lipolytic activity at 29,000 Da. No reaction was observed with the crude extract of the negative control *E. coli*/pSK(+), which contained only the cloning vector. Identical results were obtained when tributyrin agar was used instead of tricaprylin agar (data not shown). In addition, the temperature dependence of the lipase with *p*-nitrophenyl palmitate as a substrate was determined. The enzyme was active at temperatures from 12 to 60°C. The maximal activity was recorded at 42°C (data not shown).

Molecular analysis of pAH110 to pAH113. The inserts of all recombinant plasmids recovered from *E. coli*/pAH110 to *E. coli*/pAH113 were sequenced by the Göttingen Genomics Laboratory (Göttingen, Germany). The sequences were analyzed with the Genetics Computer Group program package (11) and compared to the sequences in the National Center for Biotechnology Information databases (2). In order to identify the genes on pAH110 to pAH113, which are responsible for the lipolytic activity of the corresponding recombinant *E. coli* strains, the inserts were partially subcloned by restriction digestion with various enzymes and subsequent ligation into pSK(+). The resulting constructs were transformed into *E. coli*. The recombinant *E. coli* strains were screened again on indicator plates containing tributyrin or triolein as a substrate. This strategy was successful for all four plasmids. The four corresponding positive *E. coli* strains (*E. coli*/pAH114 to *E. coli*/pAH117) were indistinguishable from the original clones with respect to substrate specificity. The restriction maps, the designation of the recombinant plasmids derived from pAH110 to pAH113, and the localization of the identified genes are given in Fig. 1. All four of the below-mentioned genes encoding lipolytic enzymes were preceded by a potential ribosome binding site, appropriately spaced from the start codon.

The 1,232-bp insert of pAH114 revealed an open reading frame (846 bp), which is similar to the *lip* gene of *Streptomyces albus* (10). Therefore, the open reading frame was designated *lip* accordingly. The predicted molecular mass of 28,683 Da for the gene product is in good accordance with that obtained during activity staining of crude extract of *E. coli*/pAH110 (see above). The deduced gene product of *lip* (281 amino acids) is 30% identical (35.9% similar) to the lipase (Lip) of *S. albus*. Similar amino acid identities were obtained for the lipases of *Streptomyces coelicolor* (24) and a *Moraxella* sp. (12). The enzymes of the latter two organisms revealed the same broad substrate spectrum as Lip from *E. coli*/pAH110 (12, 13, 24). A comparison of the lipase from *E. coli*/pAH110 with other known lipases showed high similarity with regard to the conserved motif [LIV]-X-[LIVFY]-[LIVMST]-G-[HYWV]-S-X-G-[GSTAC], commonly found within the sequences of lipases and esterases (5). This motif carries the active-site serine of hydrolytic enzymes. This signature pattern was also more or less conserved in the deduced amino acid sequences of the other three genes identified in this study, which encode putative esterases (Fig. 3).

The sequence of the 2,136-bp insert of pAH115 harbored a single large open reading frame (1,248 bp), designated *orf111* (Fig. 1). The deduced gene product (415 amino acids), with a

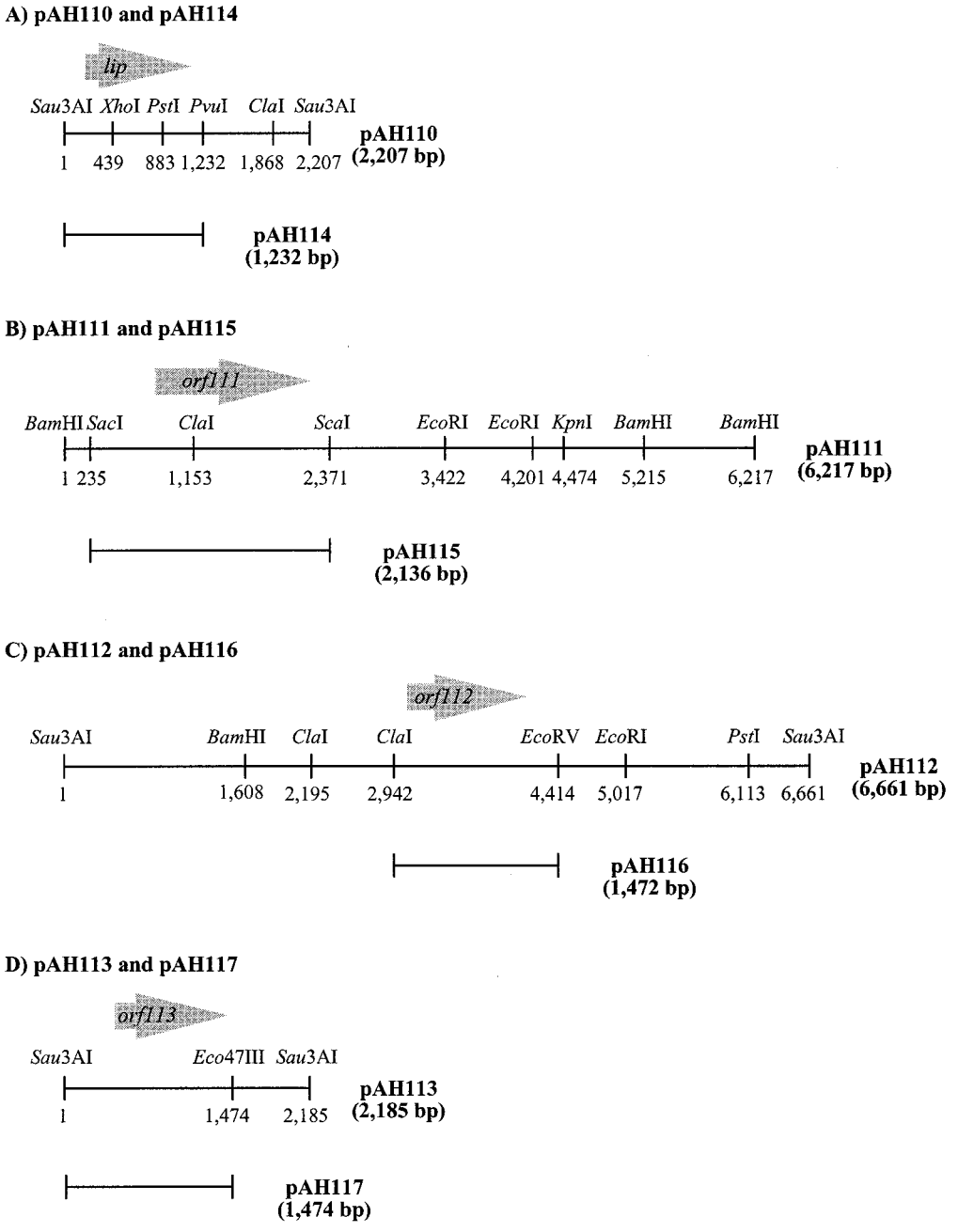

FIG. 1. Restriction maps of the inserts of pAH110 to pAH113 and of the corresponding subcloned inserts of pAH114 to pAH117. The arrows represent length, location, and orientation of the genes *lip*, *orf111*, *orf112*, and *orf113*.

predicted molecular mass of 44,628 Da, is 34.3% identical (40.0% similar) to one hypothetical lipase/esterase of *S. coelicolor* (SCH10.22c) (20).

DNA sequence analysis of the insert of pAH116 (1,472 bp) revealed one potential gene, designated *orf112*, within the sequence. The *orf112* gene (927 bp) codes for 308 amino acids with a predicted molecular mass of 34,161 Da. Database searches showed similarities (47.8 and 34.6% identity and 53.5 and 47.2% similarity) to the hypothetical esterase from *S. coelicolor* (SCE9.22; 27,211 Da) (20) and the esterase from *Acinetobacter lwoffii* (Est; 33,974 Da) (1, 19). Both esterases belong to the G-D-X-G family of lipolytic enzymes. For the detection of members of this protein family two specific fingerprint patterns are available in the PROSITE database (15); the first one, including the putative active-site histidine, is [LIVMF]-X(2)-[LIVMF]-H-G-G-[SAG]-[FY]-X(3)-[STDN]-X(2)-[ST]-H, and the second one harboring the putative active-site serine, is [LIVM]-X-[LIVMF]-[SA]-G-D-S-[CA]-G-[GA]-X-L-[CA]. The *orf112* gene product showed both fingerprint patterns.

The plasmid pAH117 (1,474 bp) contained a single open reading frame of 927 bp designated *orf113* (Fig. 1). The deduced gene product (307 amino acids), with a predicted molecular mass of 32,484 Da, exhibited 43.8% identity (53.6%

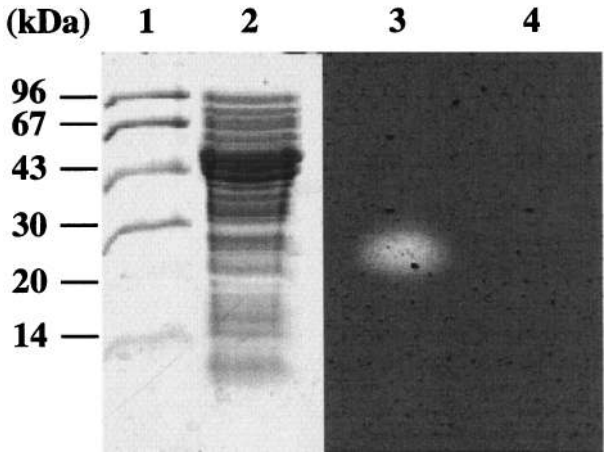

FIG. 2. Lipase activity staining after SDS-polyacrylamide gel electrophoresis of *E. coli*/pAH110 crude extract. Lanes: 1, marker proteins; 2, Coomassie blue-stained crude extract of *E. coli*/pAH110; 3, activity-stained crude extract of *E. coli*/pAH110; 4, activity-stained crude extract of *E. coli*/pSK(+). In lanes 3 and 4, tricaprylin was used as a substrate.

similarity) to the above-mentioned esterase of *A. lwoffii*, but in contrast to the *orf112* gene product, the signature pattern for members of the G-D-X-G family of proteins was not present in the amino acid sequence. In addition, the *orf113* gene product revealed 28.6% identity (36.3% similarity) to the lipolytic enzyme of *Sulfolobus acidocaldarius*, which, like the *orf113* gene product, hydrolyzes only short-chain triacylglycerols, such as tributyrin (4).

In summary, four novel lipolytic enzymes (one putative lipase and three esterases) were found during screening of environmental DNA libraries. The deduced amino acid sequences of the four proteins revealed only moderate identity (<50%) to any other sequence available in the various databases. This result indicates that the constructed DNA libraries harbor genes from a wide variety of microorganisms of which many have not been investigated or even cultivated. Thus far, characterized genes encoding lipolytic proteins from cultivated microorganisms are very different (16). Therefore, sequence information collected solely with cultivated microorganisms will not be sufficient to design universal PCR primers to retrieve the variety of genes encoding lipolytic enzymes from natural microbial communities. Molecular methods that do not rely on isolation of microorganisms into pure culture are needed to recover novel genes. The presented recombinant approach showed the feasibility of direct cloning of functional genes from environmental DNA. This method of accessing and exploiting the natural biodiversity, together with high-throughput screening systems, will have a great impact on microbial biotechnology in the future.

Lip	169	V G A S G H S Q G G	178
Orf111	109	L Y V T G Y S Q G G	118
Orf112	137	I I F C G D S A G G	149
Orf113	112	A H V V G A S M G G	121

FIG. 3. Amino acid alignment of the protein regions of the deduced *lip*, *orf111*, *orf112*, and *orf113* gene products containing the conserved motif around the proposed active-site serine of lipolytic enzymes. Matching amino acids of the consensus sequence for this region ([LIV]-X-[LIVFY]-[LIVMST]-G-[HYWV]-S-X-G-[GSTAC]) (5) are shaded.

Nucleotide sequence accession numbers. The nucleotide sequences of the inserts of pAH114, pAH115, pAH116, and pAH117 have been deposited in the GenBank database under accession numbers AF223645 to AF223648, respectively.

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