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Cloning, Expression, and Sequencing of Squalene-Hopene Cyclase, a Key Enzyme in Triterpenoid Metabolism

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The pentacyclic hopanoids, a class of eubacterial lipids, are synthesized by squalene-hopene cyclase and side chain-elongating enzymes. With the aid of DNA probes based on the amino-terminal sequence of purified squalene-hopene cyclase from *Bacillus acidocaldarius*, clones of *Escherichia coli* that express this enzyme in the cytoplasmic membrane were isolated. According to the DNA sequence, the cyclase contained 627 amino acids with a molecular mass of 69,473 Da. A high percentage of the amino acids were basic. No significant similarity to existing sequenced proteins was found.

Hopanoids are a class of pentacyclic triterpenoids that occur in all crude oils (18) and are also widespread eubacterial lipids (19, 24). They have a structure similar to that of sterols (18) and condense phospholipids in model membrane systems (2, 22) and supposedly in cellular membranes. Hopanoids are more effectively produced at higher growth temperatures by *Bacillus acidocaldarius* (21), *Zymomonas mobilis* (26), and *Rhodospirillum acidophila* (12) and at high ethanol levels by *Z. mobilis* (6). In *Mycoplasma mycoides* they are able to replace sterols (11). It was recently found that the N_2-fixing symbiont *Frankia* sp. contains high levels of hopanoids (3).

In contrast to sterol biosynthesis, the biosynthesis of hopanoids is independent of molecular oxygen as a substrate. Hopanoids are cyclized directly from squalene by squalene-hopene cyclase (28), which may be homologous to sterol cyclases and other triterpenoid cyclases from eucaryotic cells, synthesizing by a similar reaction a similar product with analogous membrane properties. It would therefore be of interest to compare the amino acid sequence of hopene cyclase with those of sterol cyclases, thereby increasing our understanding of the recruitment of these enzymes. If homologies are found, in vitro mutagenesis studies would be of value in understanding the evolution of sterol cyclases. So far no triterpenoid cyclase has been sequenced. As a first step in this direction, the squalene-hopene cyclase gene from *B. acidocaldarius* was cloned, expressed, and sequenced; this enzyme appears to represent a new gene family.

MATERIALS AND METHODS

Bacterial strains and plasmids. The source of squalene-hopene cyclase and genomic DNA was *B. acidocaldarius* ATCC 27009. *Escherichia coli* BMH71-18 (14) was used for transformation. Plasmid pUC19 was described previously (31).

Culture conditions. *B. acidocaldarius* was grown on sporulation medium at pH 3 and 60°C (8). *E. coli* was grown at 37°C in LB medium (GIBCO, Neuisenburg, Germany) or on LB plates, both containing 30 mg of ampicillin per liter, when plasmid-containing cells were selected.

DNA preparation. *B. acidocaldarius* grown to the early exponential phase was harvested by centrifugation and washed with 150 mM NaCl–10 mM EDTA (pH 8.0) (SE). The wet cells (1 g) were resuspended in 10 ml of SE. After lysozyme (1 mg/ml) was added, the suspension was incubated at 37°C. After 1 h, the cells were lysed by the addition of 200 μl of 20% (wt/vol) sodium dodecyl sulfate (SDS) and 100 μl of pronase E (0.1 g/ml). The mixture was shaken carefully at 60°C for another 2 h. The lysate was extracted three times with 1 volume of SE-saturated phenol and twice with chloroform-isoamyl alcohol (24:1). The solution was then treated with 50 μg of DNase-free RNase at 37°C for 30 min. After three extractions with 1 volume of chloroform-isoamyl alcohol (24:1), ice-cold ethanol (3 volumes) was layered on top of the aqueous solution. The DNA was spooled onto a glass rod, washed with 70% ice-cold ethanol, dried, and finally resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA.

E. coli plasmid DNA was isolated by the alkaline SDS method (4, 14) and purified by CsCl-ethidium bromide density gradient centrifugation. Small-scale plasmid preparations were made by the boiling method (9).

Oligonucleotide synthesis and labeling. Mixed-sequence oligonucleotides corresponding to several regions of the amino terminus of the cyclase (17) were synthesized on an Applied Biosystems model 380A DNA synthesizer (16). Most suitable were probe A (13-mer) and probes B through E (17-mers); each consisted of an oligonucleotide pool representing a 64-fold degeneracy.

The oligonucleotides were 5′ end labeled with ^{32}P as follows. A 50-pmol sample of each oligonucleotide pool was incubated with 1 U of T4 polynucleotide kinase (Boehringer, Mannheim, Germany) and 1.5 MBq of [γ-^{32}P]ATP in a buffer containing 50 mM Tris-HCl (pH 9.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, and 0.1 mM EDTA. The solution was incubated at 37°C for 2 h, and the labeled oligonucleotides were purified on DEAE-Servacel 23SH.

DNA hybridization. *B. acidocaldarius* chromosomal DNA was cleaved with restriction endonucleases according to the specifications of the manufacturer (Boehringer Mannheim). A 10-μg sample of the digest was electrophoresed in an agarose gel in Tris-borate buffer. DNA fragments were denatured and neutralized but not depurinated and then

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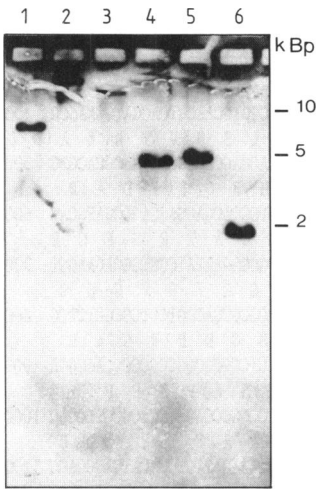

FIG. 1. Southern blot of *B. acidocaldarius* genomic DNA and the digoxigenated *Sma*I-*Bam*HI fragment of the squalene-hopene cyclase structural gene. Size markers are provided (in kilobases) on the right. DNA was digested with the following (lanes): 1, *Eco*RI; 2, *Hin*dIII; 3, *Kpn*I; 4, *Pst*I; 5, *Sma*I; 6, *Bam*HI.

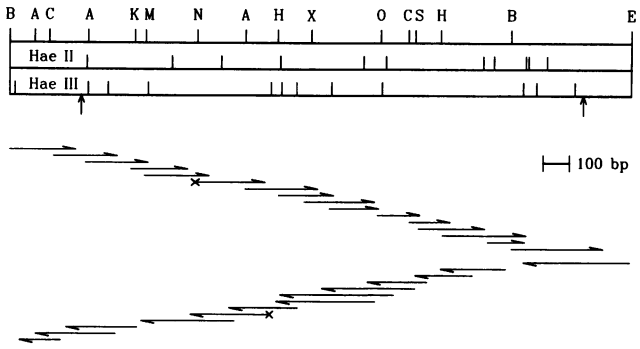

FIG. 2. Restriction map and sequencing strategy for the squalene-hopene cyclase gene. Each arrow represents the length of a clone used for sequencing and the direction of reading of the nucleotide sequence. At some positions (×), synthetic primers were used for sequencing. Vertical arrows indicate the location of the cyclase gene. Restriction enzyme site abbreviations: A, *Nae*I; B, *Bam*HI; C, *Sac*I; E, *Eco*RI; H, *Xho*I; K, *Kpn*I; M, *Sma*I; N, *Nco*I; O, *Ecl*XI; S, *Sal*I; X, *Xba*I.

transferred to nitrocellulose (Schleicher & Schuell, Germany) with 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 16 to 20 h (14). Prehybridization and hybridization were performed as described by Martin and Savage (15). Hybridization temperatures were 30°C for probe A and 40°C for probe B. The filters were washed in 1× SSC–1% SDS at the hybridization temperatures. The hybridization probes were digoxigenated as described previously (11b), and performed as specified by the manufacturer (Boehringer).

Cloning. The fragments of interest, identified by hybridization, were recovered from agarose gels by a freeze-squeeze method (29) and ligated with T4 ligase (Boehringer) into the appropriate dephosphorylated site of pUC19. The ligation mixture was used to transform *E. coli* BMH71-18. Transformants were selected on LB agar containing ampicillin (30 μg/ml), isopropyl-β-D-thiogalactopyranoside (40 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 μg/ml). The transformants were screened by colony hybridization (14, 15) with probes A and B.

DNA sequencing. The gene encoding squalene-hopene cyclase was sequenced by the dideoxy method (25) with a T7 sequencing kit (Pharmacia, Germany) and ^{35}S-ATP (Amersham) as specified by Pharmacia. The DNA fragments were subcloned into pUC19 for sequencing. Sequencing was performed on both strands. The sequence data obtained were analyzed by using the Microgenie program (23).

Purification of the recombinant cyclase. All purification steps were carried out at room temperature.

For the crude extract, wet packed cells (3 g) were suspended in 8 ml of 0.2 M Tris-HCl (pH 8.0), 16 ml of saccharose buffer (0.2 M Tris-HCl [pH 8.0], 1 M saccharose), 2 ml of lysozyme (10 mg/ml), and 0.12 ml of EDTA (0.5 M). The suspension was frozen at −80°C for at least 1 h. The cells were thawed at room temperature and then lysed by the addition of 130 ml of water, and the lysate was centrifuged at 6,000 × *g* for 10 min.

Outer and inner membranes were prepared as described previously (27). The membranes were pelleted by centrifugation at 40,000 × *g* for 1 h. The pellet was resuspended in 16 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, and 10 mM $MgCl_2$, resulting in the solubilization of the cytoplasmic membrane proteins. The mixture was incubated at room temperature for 30 min and then centrifuged at 10,000 × *g* for 10 min. Most of the *E. coli* proteins in the supernatant were precipitated by heating for 10 min at 60°C. The mixture was cooled on ice and then centrifuged at 10,000 × *g* for 10 min. The resulting supernatant was purified as described previously (17).

Protein was determined as described previously (20) with bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was performed as described previously (13), and proteins were visualized by using silver staining (5). Squalene-hopene cyclase activity was measured as described previously (17).

Immunological methods. Antibodies against the *B. acidocaldarius* cylase and the 13-mer oligopeptide representing the carboxy terminus of the enzyme were raised in BALB/c mice. Before immunization, the oligopeptide was conjugated to bovine serum albumin (1). Then 50 μg of the purified protein or conjugated oligopeptide was emulsified with complete Freund adjuvant and injected intraperitoneally. Two weeks later, a new injection was performed with 50 μg of the antigen emulsified with incomplete Freund adjuvant. After another 2 weeks, blood was removed and the serum was aliquoted.

Western blotting (immunoblotting) was performed essentially as described previously (7), except that nitrocellulose (BA 85, 0.45-μm pore size; Schleicher & Schuell) was saturated with 0.5% hemoglobin from swine and washing buffers contained 0.05% Triton X-100.

Double immunodiffusion assays were performed as described previously (10). The plates, which contained 1% Triton X-100 to enhance solubility of the antigen, were stained with Coomassie blue.

Nucleotide sequence accession number. The nucleotide and amino acid sequence reported has been assigned GenBank accession number M73834.

RESULTS AND DISCUSSION

Oligonucleotide synthesis. The first 24 amino acid residues of the cyclase amino terminus were sequenced previously

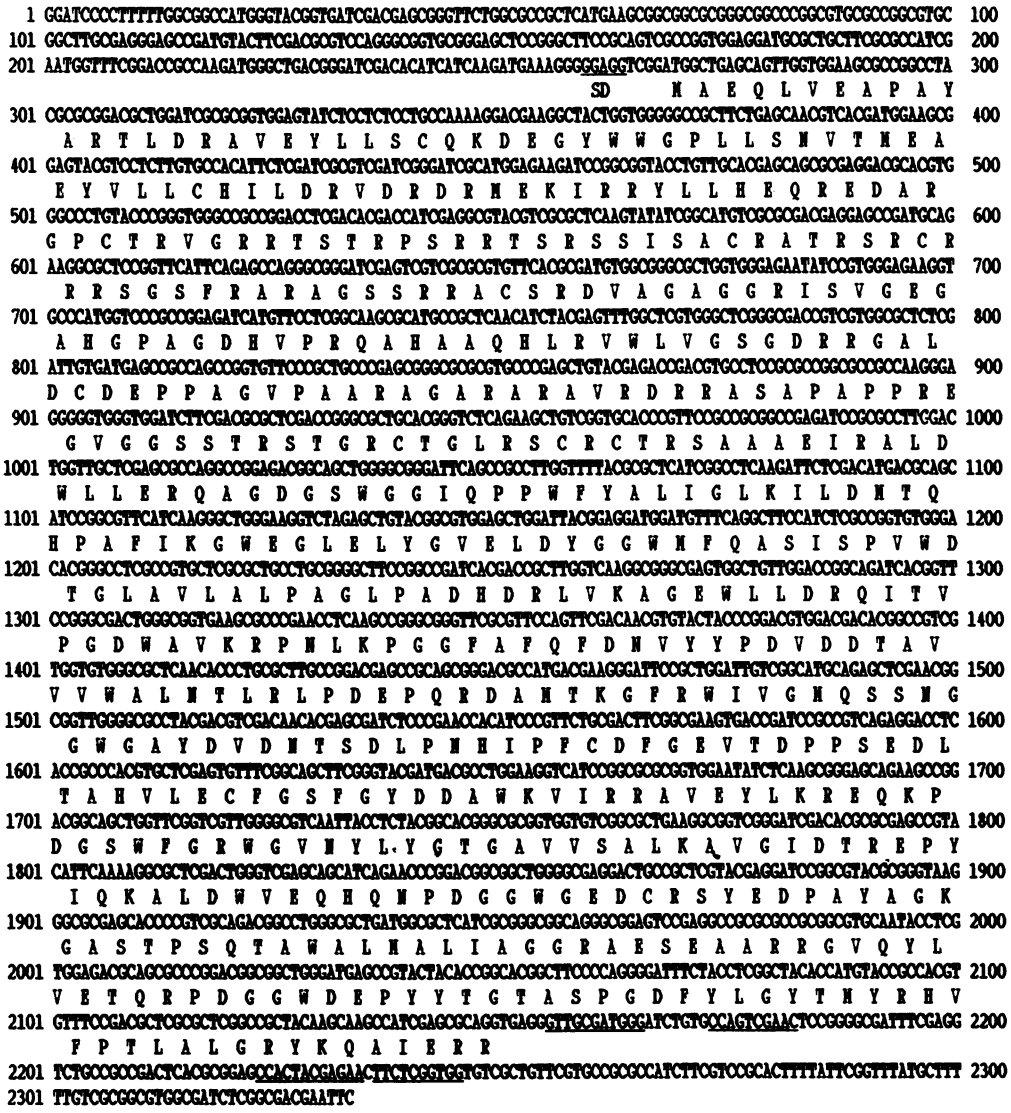

FIG. 3. Nucleotide sequence of the squalene-hopene cyclase gene and the deduced amino acid sequence. The underlined sequence upstream of the start codon is the ribosomal binding site (SD). Amino acid residues 2 through 24 were found by Edman sequencing. Two possible stem-and-loop terminator structures downstream of the stop codon are underlined.

(17). The corresponding nucleic acid sequence was inferred from the amino acid sequence. We synthesized a 13-mer oligonucleotide (probe A) corresponding to the DNA coding strand starting at the second nucleotide of the putative codon for formyl-Met and four 17-mer oligonucleotides (probes B, C, D, and E) corresponding to the DNA coding strand starting at residue 2. Each nucleotide probe represented a 64-fold degeneracy. The theoretical melting temperature ranges were 38 to 48°C for probe A, 48 to 58°C for probes B and C, and 46 to 56°C for probes D and E (30).

Identification and cloning of the cyclase gene. Blots of *B. acidocaldarius* chromosomal DNA digested with *Bam*HI and hybridized with probes A (TGGCNGA$^{A}_{G}$CA$^{AC}_{GT}$T) and B (GA$^{A}_{G}$CA$^{A}_{G}$TT$^{A}_{G}$GTNGA$^{A}_{G}$GC) showed a hybridization signal with a 1,900-bp fragment. The corresponding restriction fragments were cloned into the respective site of pUC19. Transformants were screened by colony hybridization with probes A and B. Sequencing of the respective DNA fragment of a positive clone showed that the fragment contained the amino terminus of the cyclase structural gene. With a digoxigenated *Sma*I-*Bam*HI fragment (1,000 bp) of the insert, a 4.8-kb *Pst*I fragment and a 9-kb *Eco*RI fragment could be identified by hybridization experiments with *B. acidocaldarius* chromosomal DNA (Fig. 1). The 4.8-kb *Pst*I fragment and the 9-kb *Eco*RI fragments were cloned into the respective sites of pUC19, and transformants were screened by colony hybridization with the digoxigenated *Sma*I-*Bam*HI fragment. In contrast to the clones containing the 1.9-kb *Bam*HI fragment, clones containing the *Pst*I or *Eco*RI fragments had cyclase activity, indicating that these fragments contained the entire cyclase gene. One clone containing the 4.8-kb *Pst*I fragment (strain P2) was used for purification of the recombinant protein.

Nucleotide sequence of the cyclase gene. The sequencing strategy is shown in Fig. 2, and the nucleotide sequence of the 2,336 bp containing the cyclase structural gene is shown in Fig. 3. A putative ribosome binding site (-GGAGG-) was identified; it was followed by a initiation codon (ATG) 5 bp

downstream and a 1,881-bp-long open reading frame encoding a polypeptide of 627 amino acids. A single amber codon was followed by two inverted repeats, presumably involved in rho-independent transcription termination. The experimentally determined DNA sequence agrees with that deduced from the amino-terminal sequence of the purified protein. The primarily produced polypeptide is obviously processed by removing the amino-terminal Met. The calculated molecular mass is 69,473 Da. The protein shows a rather high Arg content (66 Arg residues), resulting in a basic character; 81 of the amino acids are basic, whereas 77 are acidic. The G+C content of the open reading frame (66%) is slightly higher than that of the chromosomal DNA (61%) (8).

To verify the identity of the sequenced gene with squalene-hopene cyclase, a synthetic peptide consisting of the 13 carboxy-terminal amino acids of the cyclase was synthesized (1); the amino acid sequence was deduced from the nucleotide sequence. This oligopeptide was conjugated to bovine serum albumin. The resulting construct was used as an antigen in a BALB/c mouse. The antiserum raised proved to be positive against squalene-hopene cyclase, as shown by Western blot experiments with the cyclase from *B. acidocaldarius* and transformed *E. coli* cells (data not shown).

Expression of squalene-hopene cyclase in *E. coli*. The cyclase was expressed with the highest yield in strain P2. In Western blot analysis the cyclase in strain P2 showed the same molecular weight as that in *B. acidocaldarius*. Activity was found only in the cytoplasmic membrane. No enzyme activity was detectable in vivo, since *E. coli* does not synthesize squalene. Even when high concentrations of squalene were added to the growth medium, no conversion to hopene could be detected. Cyclase activity was observed only after enzymatic lysis or sonication of cells, indicating that the outer membrane is an efficient barrier for the emulsified squalene.

Cell extracts of strain P2 had a 4.5-fold higher specific activity than did those of *B. acidocaldarius*; the specific activities were 1.8 and 0.4 U/mg, respectively. This value is less than that expected based on the copy number of pUC19 and can be explained by reduced expression rates of the cyclase gene in *E. coli*. Apart from this, we assume that the membrane-bound cyclase could be toxic in higher concentrations, resulting in a low gene dosage effect. Induction with isopropyl-β-D-thiogalactopyranoside resulted in no increase in cyclase activity, indicating that the cyclase gene was not under the control of the *lac* promoter.

Purification of the recombinant cyclase. Since P2 showed the highest specific activity, it was used for further analysis.

The recombinant enzyme was solubilized from *E. coli* cytoplasmic membranes with Triton X-100. A heat treatment of the solubilized proteins at 60°C for 10 min and subsequent chromatography on DEAE-cellulose resulted in an electrophoretically homogenous protein (Fig. 4). The cyclase was purified 68-fold with a 29.7% yield.

The identity of the recombinant cyclase as the *B. acidocaldarius* cyclase was shown by Western blot experiments with antiserum raised against the *B. acidocaldarius* enzyme (Fig. 5) and by douple immunodiffusion (data not shown). No reaction of the cyclase antigen with preimmune serum was detected.

In addition, in Western blot experiments the antiserum reacted with 60-kDa proteins from several *Streptomyces* species; these proteins probably represent squalene-hopene cyclase, since these *Streptomyces* strains contain hopanoids (12).

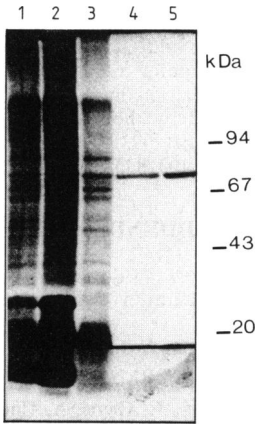

FIG. 4. SDS–10% polyacrylamide gel electrophoresis of the fractions obtained during purification of recombinant squalene-hopene cyclase from *E. coli* P2. Lanes: 1, 4 μg of the membrane fraction; 2, 4 μg of the outer membrane fraction; 3, 2 μg of the cytoplasmic membrane fraction; 4, 0.75 μg of partially purified squalene-hopene cyclase from DEAE-cellulose; 5, 0.75 μg of purified enzyme from Sephacryl S-300. The continuous band below 20 kDa is an artifact that is not caused by the protein samples in the slot. Size markers are provided on the right.

Properties of the squalene-hopene cyclase. In a hydropathy plot determined by the Hycon program, no hydrophobic transmembrane sequence could be detected. We therefore suppose, in agreement with previous results (17, 28), that the protein is anchored in the membrane of *B. acidocaldarius* by hydrophobic interactions and is not an integral membrane protein.

A homology search was made through the SWISS-PROT data bank. The sequence showed no significant homology with other known proteins.

To our knowledge, only one similar gene, the epoxysqualene-lanosterol cyclase gene of *Candida albicans*, has been cloned but not sequenced (11a). Therefore, the squalene-hopene cyclase gene represents a new gene family.

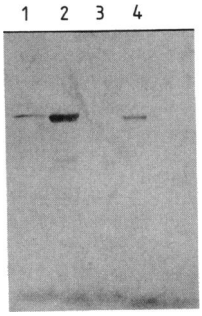

FIG. 5. Western blot analysis of the recombinant squalene-hopene cyclase. Proteins were separated on a SDS–10% polyacrylamide gel and then transferred to nitrocellulose filter. After incubation with the antiserum, staining was carried out with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate. Lanes: 1, homogenate of *E. coli* bearing pUC19 with the 9-kb *Eco*RI fragment; 2, homogenate of *E. coli* bearing pUC19 with the 4.8-kb *Pst*I fragment; 3, homogenate of *E. coli* BMH71-18; 4, homogenate of *B. acidocaldarius*.

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ADDENDUM

Recently, the content of single hopanoids in *Z. mobilis* was found to be constant under different growth conditions (8a).

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