Zymomonas mobilis* squalene-hopene cyclase gene (*shc*): cloning, DNA sequence analysis, and expression in *Escherichia coli

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Using a DNA probe from the gene encoding squalene-hopene cyclase (SHC, EC 5.4.99.-) from the Gram-positive bacterium *Alicyclobacillus acidocaldarius*, we have cloned a 4.3 kb *HindIII* fragment of chromosomal DNA from *Zymomonas mobilis*. An open reading frame of 1977 bp was detected that could encode a protein of 658 amino acids with a calculated molecular mass of 74077 Da. Under the control of *lac* or *tac* promoters, this gene, *shc*, was expressed in *Escherichia coli* K12 strains and its product had squalene-hopene cyclase activity. Sequence alignments with the *A. acidocaldarius* SHC, the lanosterol cyclase of the yeast *Candida albicans*, and the cycloartenol synthase of the plant *Arabidopsis thaliana* revealed six highly conserved regions (mainly in the C-terminal part) of the proteins. These regions contained the core motif Gln-X-X-X-Gly-X-Trp.

Keywords: hopanoids, squalene, squalene-hopene cyclase, lanosterol cyclase, *Zymomonas mobilis*

INTRODUCTION

In contrast to eukaryotes, bacteria do not produce sterols as membrane constituents. Instead, some eubacteria (and protozoa) contain pentacyclic triterpenes of the hopanoid class which are regarded as structural and functional equivalents of sterols in these organisms (Ourisson *et al.*, 1984; Poralla & Kannenberg, 1987; Sahm *et al.*, 1993). The intermediate squalene is at the branch point of the biosynthetic pathways for sterols and hopanoids (Taylor, 1984). Squalene-hopene cyclase (SHC, EC 5.4.99.-) is the first specific enzyme of hopanoid biosynthesis. While the cyclization of squalene to diploptene (hop-22(29)-ene) proceeds without the involvement of molecular oxygen, sterols are formed by O₂-dependent oxidation of squalene to epoxysqualene, followed by the cyclization catalysed by, for example, lanosterol cyclase in yeasts or cycloartenol synthase in plants (Abe *et al.*, 1993).

SHC from the thermophilic and acidophilic bacterium *Alicyclobacillus acidocaldarius* (formerly *Bacillus acidocaldarius*) has been purified (Neumann & Simon, 1986; Seckler & Poralla, 1986; Ochs *et al.*, 1990), and the gene

cloned, sequenced and expressed in *Escherichia coli* (Ochs *et al.*, 1992). The purified enzyme showed a molecular mass of 75 kDa on SDS-PAGE. The DNA-derived amino acid sequence comprised 627 amino acids with a molecular mass of 69.5 kDa. The Gram-negative, ethanol-producing bacterium *Zymomonas mobilis* also contains a variety of hopanoids with different polar side chains which are thought to be involved in ethanol tolerance (Bringer *et al.*, 1985; Ingram, 1986; Horbach *et al.*, 1991). The membrane fraction contains up to 30 mg hopanoids (g dry weight)⁻¹, making *Z. mobilis* a rich source of biohopanoids (Hermans *et al.*, 1991). This content is high in comparison to other bacteria, and it is independent of ethanol concentration and temperature (Schulenberg-Schell *et al.*, 1989; Sahm *et al.*, 1993). We undertook to clone and analyse the gene for SHC from *Z. mobilis* using a DNA probe from the Gram-positive bacterium *A. acidocaldarius*.

METHODS

Bacterial strains, plasmids and culture conditions. *Zymomonas mobilis* strain CP4 (ATCC 31821) (Goncalves de Lima *et al.*, 1970) was used. For cloning, the *Escherichia coli* K12 mutants DH5 (Low, 1968) and NM522 (Gough & Murray, 1983) were used. Plasmids used were pUC18 and pUC19 (Vieira & Messing, 1982), pGEM-7-Zf(+) (Promega), pUCBM20 (Boehringer Mannheim), and pZY507 (*lacI^q*, *tacP*, *cat*), a mobilizable plasmid constructed by our group (G. A. Sprenger, S. Schilz & H. Sahm, unpublished results). Plasmids pZY601 and pZY602

Abbreviation: SHC, squalene-hopene cyclase.

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contain a 2.9 kb *DraI* fragment including the *Z. mobilis shc* gene, ligated into vector pUC19 (*SmaI*-digested) representing both orientations. Plasmid pZY603 contains a 4.3 kb *HindIII* fragment covering the 2.9 kb *DraI* fragment completely in vector pZY507. In plasmid pZY603 the *shc* gene is inserted in appropriate orientation to the *tac* promoter. Plasmid pP2 contains the cloned *shc* gene from *A. acidocaldarius* DSM 406 (ATCC 27009) (Ochs *et al.*, 1992) and was used to prepare the DNA probe. *Z. mobilis* CP4 and its derivatives were grown anaerobically in complex medium (VM) with 2% (w/v) glucose (Uhlenbusch *et al.*, 1991) using glass tubes (Bellco) stoppered with butyl rubber. Agar plates were incubated in jars (Oxoid) with a gas-generating kit to provide an anaerobic (H_2/CO_2) environment. *E. coli* strains were grown in LB medium (Sambrook *et al.*, 1989) with appropriate antibiotics. Plasmids pZY507 and pZY603 were transferred by conjugation from *E. coli* donors to *Z. mobilis* CP4 as described previously (Uhlenbusch *et al.*, 1991).

Oligonucleotide preparation, labelling and DNA hybridization. DNA oligonucleotides were synthesized using a Gene Assembler (Pharmacia). The oligomers used to amplify the DNA fragment from *A. acidocaldarius* consisted of a 20-mer (TCCGCGCCTTGGACTGGTTG) and a 19-mer (GTGCCGAAACACTCGAGCA). Oligonucleotides were purified by passage through NAP 10 columns (Pharmacia). For *Taq* DNA polymerase-dependent cyclic DNA amplification (Mullis & Faloona, 1987), oligonucleotides were used as primers (50–100 ng each) and an internal fragment of the cloned *A. acidocaldarius* gene was used as the template (100 ng). DNA amplification was done in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl, 0.1 mg gelatin ml^{-1} , pH 8.3 (20 °C) with *Taq* DNA polymerase (Boehringer Mannheim) for 30 cycles of 94 °C (1.2 min), 45 °C (1.2 min), 70 °C (2.2 min). The amplified fragment was 643 bp long (bp 987 to 1629 of the published sequence) (Ochs *et al.*, 1992), and was labelled using random hexamer primers in conjunction with the digoxigenin-dUTP labelling kit of Boehringer Mannheim according to the manufacturer's instructions. The labelled fragment was used without further purification. *Z. mobilis* CP4 chromosomal DNA was cleaved with restriction endonucleases and 5 µg samples were electrophoresed (0.8% agarose) in Tris/borate/EDTA buffer (pH 8). Afterwards, DNA fragments were denatured and neutralized (but not depurinated) and Southern blotted (Southern, 1975) to positively charged nylon membranes (Boehringer Mannheim) overnight in $10\times$ SSC buffer. Pre-hybridization and hybridization were performed as described previously (Kessler *et al.*, 1990). Hybridization temperatures and formamide concentrations were varied in the range 35–42 °C and 30–40% (v/v), respectively. The filters were washed once with $0.5\times$ SSC buffer and then with $0.1\times$ SSC (both containing 0.1% SDS) at the hybridization temperatures. Detection was by light emission with AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioxetane) as substrate for alkaline phosphatase, on a Kodak X-ray film (type X-OMAT AR; 20–60 min exposure).

Cloning, restriction analysis, and DNA transformation. Chromosomal DNA from *Z. mobilis* was prepared by the lysozyme/freeze-thaw method (Eddy *et al.*, 1988). Plasmid DNA was prepared by alkaline lysis (Sambrook *et al.*, 1989) or via column purification (Diagen). DNA fragments were recovered by excision from agarose gels in Tris (40 mM)/acetate (20 mM) buffer at pH 8, and were purified using glassmilk/

iodine (GeneClean kit, Dianova). DNA fragments were ligated with *T4* DNA ligase (Boehringer Mannheim) with vector pUC19 (Vieira & Messing, 1982), which had been cleaved with restriction enzymes and dephosphorylated. The ligation mixture was used to transform *E. coli* strain NM522. Transformants were selected on LB agar plates containing ampicillin (100 mg l^{-1}) and X-Gal (30 mg l^{-1}). DNA from 84 pools of 10 transformants each was prepared and analysed. The presumed DNA inserts were recovered by digestion with *EcoRI* plus *HindIII*, gel electrophoresed and Southern blotted (see above). From pools which gave positive signals in the hybridization assay, individual clones were re-analysed by digestion and hybridization.

DNA sequencing. DNA fragments were subcloned into vectors pUC18/19 or pUCBM20 for sequencing. Exonuclease III treatment (Henikoff, 1984) to create successively shortened clones was performed according to the protocols of the Erase-a-base kit (Promega). The *HindIII* DNA fragment, containing the presumed *shc* gene, was sequenced by the dideoxy method (Sanger *et al.*, 1977), using *T7* DNA polymerase (Tabor & Richardson, 1987) and the AutoRead sequencing kit (Pharmacia). Fluorescent-labelled primers (M13 universal and reverse-sequencing primers) were used as specified by the protocols for automated laser fluorescent (ALF) sequencing (Pharmacia). DNA was prepared by alkaline lysis and additionally treated with lithium chloride/2-propanol to remove RNA (Sambrook *et al.*, 1989). Each sequencing reaction used 5 µg plasmid DNA. Both strands were sequenced using the ALF sequencer and the manufacturer's software. The sequence data obtained were further analysed using the MicroGenie program 7.0 (Queen & Korn, 1984).

SHC assay. Cells of *Z. mobilis* from a 200 ml culture were harvested in the late-exponential growth phase (OD_{600} 2); cells of *E. coli* (100 ml culture) were harvested at an OD_{600} of 1.5. After centrifugation, the cells were washed with potassium phosphate buffer (50 mM, pH 7.5, sparged with argon to remove oxygen; plus 14 mM mercaptoethanol). After resuspension in 1 ml phosphate buffer, cells were packed into a precooled ($-20\text{ }^{\circ}\text{C}$) X-press chamber (type X-5; Bachofer) and kept overnight at $-20\text{ }^{\circ}\text{C}$. The cell paste was ruptured using an X-press (Enerpac RD2510) by two strokes from each side of the chamber. The frozen cell paste was thawed and treated with DNase I to reduce viscosity. Protein was determined by a dye-binding method (Bradford, 1976) with BSA as a standard.

SHC activity was determined according to the method of Ochs *et al.* (1990) with modifications (Tappe, 1993). In a total volume of 250 µl, about 200 nCi [^3H]squalene [50 µM; sp act. 16.2 Ci mmol^{-1} ($6\times 10^{11}\text{ Bq mmol}^{-1}$), $4.4\times 10^5\text{ d.p.m. per assay}$] was incubated at 30 °C for 1 to 3 h with crude extract (3 mg protein ml^{-1}) in potassium phosphate buffer (100 mM, pH 6.0) and 0.5% (w/v) CHAPS [3-((3-cholamidopropyl)-dimethylammonio)-1-propane-sulfonate]. The incubation was stopped by extraction with 2 vols hexane/2-propanol (3:2, v/v). The organic phase was applied to a thin-layer silica gel plate (Merck no 5715). The sample was air-dried and developed in a vertical tank, first with chloroform (to 7 cm) then with hexane (to 16 cm). The dried TLC plate was analysed in a TLC scanner (LB 2821; Berthold). Authentic, non-labelled standards of hopene (gift from Dr M. Rohmer, Université de Haute Alsace, Mulhouse, France) and squalene (Sigma) were visualized with iodine vapour. The isotope peaks were integrated and calculated to give the amount of hopene being formed.

Preparation of hopene for GC/MS analysis. *E. coli* DH5(pZY601) was grown under aerobic conditions at 30 °C in

Fig. 1. Restriction map and sequencing strategy of the 4.3 kb *HindIII* DNA fragment containing the *shc* gene of *Z. mobilis*. ORF1 designates the ORF upstream of *shc*. The two vertical arrows indicate the borders of the *DraI* fragment cloned on pZY601 and pZY602. The horizontal arrows indicate the orientation and length of sequenced fragments created by subcloning or by exonuclease III treatment.

eight 200 ml volumes of LB with ampicillin to OD₆₀₀ 1.45; 1 mM IPTG was then added and growth was continued for 4 h to OD 1.75. The cells were washed, resuspended in 9 ml 100 mM potassium phosphate, pH 6.5, 0.5 % CHAPS. Squalene (1 mg) in 1 ml of the same buffer was sonicated and added to the suspension to stabilize the cyclase. This suspension was ruptured by X-press treatment as above. Nine millilitres of 100 mM potassium phosphate pH 6.5, 20 % (v/v) glycerol, 1 % CHAPS plus an ultrasonicate of 10 mg squalene in 1 ml of the same buffer were added to the total cell homogenate. Incubation was for 4 h at 30 °C. The reaction mixture was extracted twice with 10 ml hexane/2-propanol (3:2, v/v) and centrifuged to increase phase separation. The extract was evaporated and purified by preparative TLC on silica gel (solvent system: to 6 cm with dichloromethane, and after drying, to 18 cm with hexane). The region with the same *R_F* value as hopene was scraped out, extracted with dichloromethane and filtered. After evaporation the residue was redissolved and analysed by GC/MS, as follows. The gas-liquid chromatograph was a Carlo Erba Fractovap 2009 connected to a Varian mass spectrometer MAT 112S (ionization –80 eV). The column was a CP-Sil-5 (10 m × 0.25 mm, film thickness 0.12 µm) and was run at a temperature gradient of 5 °C min⁻¹ from 220–300 °C.

RESULTS AND DISCUSSION

Detection of the *shc* gene with a heterologous DNA probe

In order to clone the *shc* gene of *Z. mobilis*, we prepared a DNA probe from the gene of the Gram-positive bacterium *A. acidocaldarius*. The gene was cloned and sequenced recently (Ochs *et al.*, 1992). From plasmid pP2, an internal 643 bp *XhoI* fragment was amplified using *Taq* DNA polymerase and two oligonucleotide primers, and was digoxigenin-labelled to produce a non-radioactive probe for *shc*. Chromosomal DNA from *Z. mobilis* was prepared and digested in parallel with different restriction enzymes. The digests were agarose gel electrophoresed and blotted onto nylon membranes, and further processed for DNA hybridization with the digoxigenin-labelled DNA probe. Positive signals could be best visualized under low-stringency conditions: 30 % (v/v) formamide at 40 °C. Hybridization signals were found with DNA digested with *HindIII* in the range of 4.3 kb, and with *DraI* in the range of 3.0 kb. To clone the respective DNA

fragments, appropriately digested DNA (size range 3.5–4.5 kb for *HindIII*, or 2.8–3.3 kb for *DraI*) was recovered from agarose gels and ligated to pUC19 vectors cleaved with *HindIII* and *SmaI*, respectively.

Cloning and nucleotide sequence determination

Of 840 *HindIII*-derived clones, two contained an insert of 4.3 kb (plasmids pZY611 and pZY612 representing both orientations, data not shown). By restriction analysis, we found that a 2.9 kb *DraI* fragment which had also given a positive hybridization signal (data not shown) was present completely in the 4.3 kb *HindIII* fragment. The sequence of the 4.3 kb *HindIII* fragment was determined from both strands. The sequencing strategy and the derived restriction map are given in Fig. 1. Sequence analysis revealed that an open reading frame (ORF) of 1977 bp was present on the *DraI* subfragment (Fig. 1). The complete sequence of the 4.3 kb *HindIII* DNA fragment with the delineated amino acid sequences is given in Fig. 2. Five base pairs upstream of putative start codon for the *shc* ORF there was a canonical Shine–Dalgarno sequence (Gold *et al.*, 1981). A possible stem–loop structure with a ΔG^0 of –32 kcal mol⁻¹ (–134 kJ mol⁻¹) (Tinoco *et al.*, 1973) could form immediately after the stop codon and resembles a rho-independent terminator. Upstream of the putative *shc* gene, another ORF (ORF1) was detected that ends just 2 bp ahead of the RBS from *shc*. The nature of this ORF is unclear. A databank search (data not shown) revealed that ORF1 shares stretches of amino acid residues identical to conserved sequences in phytoene desaturase enzymes of bacterial or plant origin (Armstrong *et al.*, 1993).

However, this peculiar organization hints at a putative operon structure (unpublished results) and should allow read-through from a given external promoter into the putative *shc* sequence. The 1977 bp *shc* ORF could encode a protein of 658 amino acids with a calculated molecular mass of 74077 Da. The amino acid composition is apparently hydrophobic, as was found for the SHC from *A. acidocaldarius* (Ochs *et al.*, 1992). An alignment of the SHC sequences from *Z. mobilis* and *A. acidocaldarius* with the oxidosqualene (lanosterol) cyclase from *Candida albicans* (Buntel & Griffin, 1992; Roessner *et al.*, 1993) and cycloartenol synthase of the plant *Arabidopsis thaliana*

ORF1	
AAGCTTGCCCTGTTATGGACTTTGCTCAGATTGAAGTGACTTCATGAGTGAACGATATTATCGGGG	70
G L A G L S A A V A I T H A G G R V K I Y E	
GCGGCTGGCTGGATTATCGCCGGTAGCGATTACCCATGCTGGAGGACGGTCAAAATTTATGAAGCC	140
S A H A G G R A R S Y H D K K L G I E I D N G N	
TCGGCATGGCTGGGGACGGCGCTTCTATCATGATAAAAGCTGGGGATTGAATGACATGGCA	210
H N L L S G N H S A K T Y L K R I G A E N R F	
ATCATATGCTGCTGAGCAATCATTTGCGAAGACCTATTGAAAGAATAGGGGACAGCATCGTT	280
K S P K E A A F S F C D L S D K E R F T I K L	
TAAAGTCCAAAGAGGCGCTTTCTTTTGTGTATCAAGGACAAAGACGCTTTACCATTAAGTTA	350
S N G P L P H W V L C A K S R V P H S K A K D Y	
AGTAATGGCCCTTACCTTGGTGGGTTTATGCGCCAAAAGCGCGCTCCATACGAAAGCAAGGATT	420
L A L L S L L L A D N H T K I G D L V P D N T	
ATTTAGCGTTATGCTGCTGCTATGAGCATATAATACGAAATAGGTGACCTTGTTCGGGAATAC	490
A L W R K L L D P F F V S V L N T P A R E G A	
GGCTTTATGGCTAAATGCTGTGCTTTTGTATCGTCTCAATACCGGCAAGGAAGGGGCT	560
A C L A A A V I R E T L N K G G K A C I P R I A	
GCCTGTCTGGCGGACGGTATTTCGGGAACCTTGTGAAGAGGGGTAAAGCTGTATCCCGGTATCG	630
Y P N L A S S F I D P A L D Y L K A R G V E V	
CTATCCCAATTTGGCAAGCAGTTTATGACCTTGAAGTGTACTTTAAAGCCAGAGGTCGAAGT	700
D F R N R L R Q I H F S G Q D V A S L E F A H	
TGATTTCGTAACCGCTCCGTAATCATTTTCAGGTCAGGATGCGCTCTCTGGAATTTGCTCAT	770
Q D V K L G K G D K V I A L P A W V V Q S L I	
CAGGATGTAAGCTGGGAAAGCGGATAAAGTATTGCTTGGCTGGCTGGCTGCTCAATCTTGA	840
P D I E T P D K Y Q A I I N A H F L N K P T A	
TACGAGATATGAAAGCGCGGATAAATACAGGCGATTATCAAGCTCATTCTGATGAAGCTACTGC	910
A M P H I N G V V G G T A D W I F T F E N R I	
GGCTATGCTCATATTATGGGTGTGTCGGAGAACCGCTGATGGATTTCACTTTGAAATCGGATA	980
S Y T U S A A N H L L A L E K E E L V K R I W D	
TCGGTACGATCAGCGCGCTAATCATTTATGGCGCTTGAAGAGGAAGCTGTTAAGCTATCTGG	1050
D I Q T V Y A F K Q D N P K W Q V V T E K R A	
ATGATATTGACCGCTTTATGCTTCAACAGGATATGCTTAAATGGCAGGTCGTGACTGAGAAAGAGC	1120
T F E A T V E Q N N R R P P A Y T A W N N L F	
CACCTTTGAAGCGACTGTGAGCAGATAAATCGTCGCCACCGCGGTTACAGCGTGAATAACCTGTT	1190
L A G N W V R T G L P A T I E S A I R S G Q T A	
TTAGCCGAAATGGGTGAGAACGCGCTGCCGCGACTATTGAAGCGCTATACGATCTGGCCAGACTG	1260
SHC	
A D L A L S H S *	
CGGCTGATCTGCTTTATCCCATTCATAATTAGAAGCCCATATGACTGATGACTTCTCGCGCTTT	1330
H N S P L S D D V E P I I Q K A T R A L L E K Q	
CATCATAGCCGTTGTGATGATGTTGAGCGGATTTACAAAGGCCACCGTGCTTGTGTAAGAAGC	1400
Q Q D G H W V F E L E A D A T I P A E Y I L L	
AGCAGCAGGATGGCCATTGGGTTTGAATGGGAAGCGGATGCAACCATTCGCGTGAATACATCTGTT	1470
K N Y L G E P E D L E I E A K I G R Y L R R I	
AAAGCATATTGGGTGAACCGAAGATTAGAAATGAGGCGAAGATAGTTCGCTATTGCGTCGTATT	1540
Q G E H G G W S L F T G G G D L D L S A T V K A Y	
CAGGCGGAGCATGGCGGATGCTTTGTTTATGGTGGTGTCTGATTTGATGCGCCACGCTCAAGCCT	1610
F A L K N I G D S P D A P N M L R A R N E I L	
ATTGCTGTGAAATGATCGGAGATTCTCTGATGCGCTCATATGCTTCGAGCCAGAAATGAAATTT	1680
A R G G C A N R A N V T F T R I Q L A L F G A S	
GGCAGGGGTGGGCGATGCGTCAATGCTTTACAGTATTCATGCTTCGTGCGGCAAGTGCA	1750
W E H V P Q N P V E L N L N P E W F P V H I N K	
TGGGAGCATGCTCAATGCCGTAGAGTTGATGTGATGCGGGAATGTTCCGGTTCACATCAATA	1820
M A Y W A R T V L V P L L V L Q A L K P V A R	
AAATGGCTATTGGGCAAGAACCGTTTATGCCGCTATTGGTTTTACAGCGCTTAAAGCCTGTGCGCC	1890
H R R G I L V D E L F V P D V L P T L Q E S G	
TAATCGGCGGGTATCTGGTGTATGAATATTGTGCGCGATGTTTACGACCTTCAGGAAGCGGT	1960
D P I W R R F F S A L D K V L N K V E P Y W P K	
GACCTATATGGCGTCGTTTTTTTCGGCACTTGATAAGATTGCATAAGTAGAACCTTAITGGCGGA	2030
N N R A K A I H S C V H F V T E R L N G E D G	
AAATATGCGCGCAAGGCTATTCATAGCTGTGTCCATTTGTGACGACGCTTTGATGGTGAAGACGG	2100
L G A I Y P A I A N S V M N Y D A L G Y P E N	
GTTGGGTGCTATTATCGGCGATTGCCAATAGCTCATGATGATGATGCTTGGGATATCCCGAAAC	2170
H P E R A I A R R A V E K L M V L D G T E D Q G	
CATCCAGAAAGACCATTCGCCGTCGGGCTGTGAAAAATGATGGTGTATGATGGCAGGAGATCAGG	2240
D K E V Y C Q P C L S P I W D T A L V A H A M	
GTGATAAAGAGTCTACTGTGACCTTGTATCCCGCTTGGGATATGCTTGGTGTCCCATGCCAI	2310
L E V G G D E A E K S A I S A L S W L K P Q Q	
GTTGGAAGTCGAGGCGATGAGGCTGAAAAATCGGCTATTCTGCTTGAGCTGGTAAAGCCGACAA	2380
I L D V K G D W A H R R P D L R P G G W A F Q Y	
ATTTGGATGTAAAGGCGATGGGCTATGGCGCGCTGATCTAGACCCGGGGGATGGGCTTTCAAT	2450
R N D Y Y P D V D D T A V V T H A M D R A A K	
ATAGATAAGTACTATTTCGCGATGCTGATGATGCTGTTGTGATGATGCTTGGGATGAGGACCGCA	2520
L S D L N D D F E E S K A R A M E W T I G M Q	
ATTGCGGATCTTCAGGATGATTTGAGGATCTAAAGCGCGCTGCCATGGAATGGACATGGGATGCA	2590
S D N G G W G A F D A N N S Y T Y L N N I P F A	
AGCGATAAGCGGCTGGGCGCTTCGATGCCAATAACAGCTACTATTCTGAATAATATCCCTTTG	2660
D N G A L L O P P T V D V S A R C V S N H A Q	
CTGATCATGGCGCTTACTGTGATCGGCAACGGTGTGATGCTGCGGACGCTGCTTCAATGATGGCGCA	2730
A G I S I T D P K N K A A V D Y L L K E Q E E	
AGCGGATCTCGATTACAGATCCCAAAATGAAAGCGGCGATTGATATCTCTGAAGAGCAAGAAGAG	2800
D G S W G R W G V N Y I Y G T W S A L C A L N	
DAGTGATGGTGTGGGCGTGGGCTCAATTACATATATGACACATGCTGGGCTTATGCTGATTGA	2870
V A A L P H D N L A V Q K A V A W L K T I Q N	
ATGTCGCGCTTACCCCATGATCATTTAGCTGTTCAGAAAGCTGTGGCTTGGCTGAAACTATTCAAAA	2940
E D G G W G E N C D S Y A L D Y S G Y E P M D	
TGAAGATGGTGTGGGTTGAAATTCGCGATGATGCTTGTATATAGCGGATACGAGCGATGGAT	3010
S T A S Q T A W A L L G L M A V G E A N S E A V	
TCAGCGCTTCCCAACAGCATGGGCTTTATGGGCTGTATGCGCTGTGGGAGCTAATTCGAGGCGC	3080
T K G I N W L A Q N Q D E E G L W K E D Y Y S	
TGACAAAGGTTAAACTGGTGGCACAAATCAGGATGAAGAAGGATGTGGAAGAAGATATTATAG	3150
G G G F P R V F Y L R Y H G Y S K Y F P L W A	
TGGCGTGGTTTTCCCGTGTTTTTATCTCGGTATACGGTATTCCCAATATTTCTCTTTGGGCT	3220
L A R Y R N L K K A N Q P I V H Y G N *	
TTAGCGGCTATCGCAATTTGAAAAAGCAATACGCGGATGTTTCATTATGGGATGAACAGGCTGATT	3290
T T A T A C C T T G T A T A T G A A T A C G T C A A T C A A A T A C G G C G A T T T T T T G T A	3360
T T A T C A T A T G T T T T A T A A A T A T T G T G A T A T G C G G G T A T T A T G A A G A A A A T A T G C T G A A C C	3430
A T A T C C T G C T G T G A C C G G A A T A G A T C G T G A A G C A G G G C G G C C A A T G A A A A G T T T G T C T C T	3500
G G C A A G G G A G G G A T A T T C G T C T C A A A A T C T T T G C G C G A A T T A T G A A G A A A A C A G A T T G C C	3570
G G T T A G T G A T T T G G T T T G C G T G C G C T T G G A T T C G A T T T A A A A G T A G G C G A T T G G G T G T C G G A A	3640
G C C A T T A A C A G G G A A T A T T A T T C A G A T G C C G A T T G C G A T G A A G A C T C G C T T G A G A A A T T T T C C C	3710
T A A A T A A A A A G G A A T A T T A T T C A G A T G C C A G T T G T T T C C G A T A C G C C A G A A A C A A G A C A T T	3780
C A G A A A A A C A A A G C T A T C G C G T G A T A T G A A T C C C A T C G T T G C C C A A T A G C C A A G A A T A T A A	3850
T T G T C A T T A T T A T T A G A G T A T T C A G A T C A G G C G A T C A G C T C G C C T C C C G T T T T T C G G T G	3920
G C G A T C A C C T G A T G C C A G C T G C G A T A C C A C C C T A C T G A A A T C A T T G T A T T A A A C C G A C C A A A	3990
T A C C G C T T T G C T C A G C A C A A A G G C G C A A G A A G C C T G A A A G A G T A G G C C G C T C G T T T C C T	4060
T T T T G C A G C A C C T C G C T T C C G A T T C G C T A A C G C T G T G A A G C T G A C G G A A C A C G T C C T T	4130
G G C G C A C G C T G A T T G C C A A G G C T A T C C G G T G C C A T A G C A C C T C T G T T T T G A T G C C T T T A A A G C A	4200
A C C A T A G C A G C T T C A A C G A T G G C A A C G G T G C A T A A C C C C G T G C T A A A A C C A G A G T A A A C C A	4270
T A C A G T C T T G G C A T T T T C A A T T T G C A A C G C G T A T C A T C C A A G C G T A T T T C C A T A G G T C T T	4340
G A A G C T T	4347

Fig. 2. Nucleotide sequence and derived amino acid sequence (single-letter code in bold face) of a 4347 bp DNA fragment (*Hind*III) containing the putative *shc* gene from *Z. mobilis* and an unknown ORF1. The putative ribosome-binding sites (RBS) are underlined, the stop codons are marked by asterisks and a possible stem-loop structure is marked by double lines 3' to the *shc* sequence.

(Corey *et al.*, 1993) is given in Fig. 3. The four proteins range from 627 to 759 residues and display significant sequence identities, especially in their C-terminal regions (up to 44%), whereas the respective N-termini had less identity. The derived SHC sequence from *Z. mobilis* has 34% identical amino acid residues with the *A. acido-*

caldarius sequence. With the lanosterol cyclase and the cycloartenol synthase the identities are 29% and 27% respectively. A multiple motif was detected in all four sequences (Poralla *et al.*, 1994) and consists of the core sequence Gln-X-X-X-Gly-X-Trp, which is found six times, in the SHC sequences of both *Z. mobilis* and *A.*

Fig. 3. Alignment of the derived amino acid sequences of SHC from *Z. mobilis* (ShcZ) with SHC from *A. acidocaldarius* (ShcA) (Ochs et al., 1992), lanosterol cyclase from *Candida albicans* (Erg7) (Buntel & Griffin, 1992; Roessner et al., 1993) and cycloartenol synthase (CasA) from *Arabidopsis thaliana* (Corey et al., 1993). The alignment was done by the MicroGenie program 7.0 (Queen & Korn, 1984) and fitted afterwards visually. Amino acid residues that are identical in at least three sequences are given in bold, and identical residues in all four proteins are additionally marked with an asterisk below the sequence. Residues are numbered according to the *Z. mobilis* sequence.

acidocaldarius (see Fig. 3). While a possible function of this motif in electron shift reactions during the cyclization of squalene to hopene (involving six electron pair shifts) is still speculative, it is nonetheless remarkable, especially as Trp residues are involved in several cation- π -electron interactions (Poralla et al., 1994).

Expression of *shc* in *Z. mobilis* and *E. coli* K12

That the presumed *shc* ORF indeed encoded a squalene-hopene cyclase enzyme was shown by expressing the cloned DNA fragment in an *E. coli* host. *E. coli* DH5 with vector pZY507 and recombinant derivatives with the putative *shc* gene in the correct orientation to the *tac* promoter on plasmid pZY603 and to the *lacZ* promoter on plasmid pZY601, and in reverse orientation to the *lacZ* promoter on plasmid pZY602, were tested in a radioactive SHC (^3H -labelled squalene) assay. In parallel, extracts from *Z. mobilis* CP4 and its derivative CP4(pZY603) were also assayed. Labelled products were detected by radio-scanning TLC plates. As can be seen from Fig. 4, an additional peak that was more hydrophobic than squalene was only detected in recombinant *E. coli* strains containing the *shc* gene in the correct orientation to the *lacZ* or *tac* promoter on plasmid pZY601 and pZY603, respectively.

Using unlabelled standards for squalene and hopene, this new peak was identified as hopene. It also appeared when cell extracts from *Z. mobilis* were incubated with squalene. This hopene peak was enlarged about five times with the recombinant *Z. mobilis* extracts (Fig. 4). A small, but visible, peak in the more hydrophilic region (possibly diplopterol) was also present in the TLC scans using *Z. mobilis* or recombinant *E. coli* extracts (see Fig. 4). Diplopterol is a known by-product of SHC activity in *A. acidocaldarius* (Seckler & Poralla, 1986). We suggest that the 1977 bp ORF encodes squalene-hopene cyclase.

Identification of hopene

As a further proof of the expression of the gene for SHC of *Z. mobilis* in *E. coli*, the squalene conversion product of the recombinant *E. coli* strain DH5(pZY601), containing solely the putative *shc* gene in the correct orientation to the *lacZ* promoter, was identified by GC/MS analysis. From a preparative enzyme assay of this strain, 1 mg of a conversion product was isolated. Since *E. coli* does not produce squalene, a conversion product will not appear during growth. The isolated conversion product had the same properties in TLC, GC and MS as compared to hop-21-ene (Rohmer et al., 1980). The most indicative fragments of the mass spectrum were: 410.3 (M^+ , 21%), 367.3 ($M^+ - \text{C}_3\text{H}_8$, 9%), 341 (below 5%), 191.2 (ring C

Fig. 4. Radioscans of thin-layer chromatograms showing conversion of [^3H]squalene to [^3H]hopene in cell-free X-press extracts from *E. coli* DH5(pZY601) (a), DH5(pZY602) (b), DH5(pZY603) (c), DH5(pZY507) (d), *Z. mobilis* CP4 (e) and CP4(pZY603) (f). A control incubation without cell-free extracts is given in (g). The numbers above the prominent peaks are: 1, squalene; 2, hopene; 3, diplopterol (putative). Other peaks were not identified. Total measuring time was 120 min. Note the differences in the detectable counts, due to differing extraction efficiencies.

cleavage, 100 %), 189.2 (ring C cleavage, 79 %), m/z (%). The molecular peak at m/z 410.3 and the main fragment at m/z 191.2 are very indicative of hopene. Therefore, it is evident that the expression of the *sbc* gene was achieved and that the enzyme activity is caused by the *sbc* gene product only.

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