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Isolation and Physiological Characterization of *Bacillus clausii* SKAL-16 Isolated from Wastewater

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An alkaliphilic bacterium, *Bacillus clausii* SKAL-16, was isolated from soil that had been contaminated with vegetable oil. The optimal pH and general pH range for bacterial growth was 8, and 7 to 10, respectively. The bacterium could grow on tributyrin and glycerol, but could not grow on acetate and butyrate. The SKAL-16 strain excreted butyric acid during growth on tributyrin, and selectively ingested glycerol during growth on a mixture of butyric acid and glycerol. The SKAL-16 generated intracellular lipase, but did not produce esterase and extracellular lipase. The DNA fragment amplified with the chromosomal DNA of SKAL-16 and primers designed on the basis of the esterase-coding gene of *Bacillus clausii* KSM-K16 was not identical with the esterase-coding gene contained in the GenBank database. Pyruvate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase activities were detected in the cell-free extract (crude enzyme).

Keywords: Tributyrin, lipase, esterase, alkaliphile, *Bacillus clausii*

Lipid-utilizing bacteria generate extracellular or intracellular lipase or esterase in order to hydrolyze lipids into glycerol and fatty acids [5, 14, 33]. Lipases (triacylglycerol ester hydrolases) catalyze the hydrolysis of long-chain acylglycerols (≥C10), whereas esterases are, by definition, enzymes with the ability to hydrolyze ester substrates with short-chain acylglycerols (≤C10) [36]. Esterases and lipases are widely detected in a variety of organisms, ranging from bacteria to higher eukaryotes. The alkaliphilic *Bacillus* species constitute a large, heterogeneus group of microorganisms, which are currently being investigated in order to better understand their physiology and biochemistry for industrial applications such as enzyme production, biological pesticide production, xenobiotics degradation, and fertilizer production from waste oil. In particular, the lipid-utilizing *Bacillus* is quite useful for the treatment of wastewater contaminated with oils [24]. In general, lipid residues can partially be recovered from wastewater by air flotation, and the remaining residues can be treated by normal wastewater-treatment processes [18]. A variety of studies regarding the alkaliphilic *Bacillus* species have concentrated largely on the biotechnology associated with the enzyme industry [12, 15, 34, 35].

Our interest herein was the physiology of a Gram-positive, alkaliphilic, and lipid-utilizing soil bacterium, *Bacillus clausii* SKAL-16, which has been shown to have a specific metabolic function different from that seen in the *B. clausii* that was described by other researchers. Some *B. clausii* have been studied for use in oral bacteriotherapy as a probiotic microorganism [32], whereas some of them have been studied with regard to the prevention of the mobility of probiotic resistance genes [2]. Christiansen *et al.* [3] previously analyzed the metabolic network of *B. clausii* grown in typical complex medium containing glucose basal medium supplemented with thiamine, riboflavin, nicotinic acid, calcium pantothenate, pyridoxal-HCl, biotin, and folic acid. In the study, the authors demonstrated that *B. clausii* shows typical metabolic flux from glucose to carbon dioxide *via* glycolysis and the TCA cycle. In general, glucose and glycerol are oxidized *via* glycolysis, and fatty acids are converted to acetyl-CoA *via* β-oxidation [7, 22]. *Bacillus clausii* SKAL-16 is a typical heterotrophic organism that is capable of growing under conditions in which both organic carbons and organic nitrogens are present, such as in lipid and yeast extract, and the strain generates fatty acid. However, the fatty acid produced by SKAL-16 is not a metabolite, but a by-product produced by the enzymatic hydrolysis of lipids. It is a highly specific phenomenon, because SKAL-16 cannot grow on fatty acids such as acetate and butyrate but does grow on glycerol and tributyrin in the presence of yeast extract. The reason that SKAL-16 generates fatty acid from lipids and cannot utilize the fatty acids is the topic biochemically and metabolically examined herein.

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MATERIALS AND METHODS

Reproducibility of Results

All individual experiments were repeated three to five times with identical results, and all data were expressed as the mean value of triplicate experiments with results of at least 95% similarity.

Chemicals

All chemicals and coenzymes used in this research were purchased from the Korea branch of Sigma-Aldrich Co. (Yongin, Kyunggi-do, Korea), except vegetable oil and Bradford reagent.

Isolation and Cultivation

Soils around containers for storage of waste vegetable oil, which have been located in the backyard of a student's restaurant in Seokyeong University from 1993 to the present, were sampled at 50 mm of depth on May 2007. *Bacillus clausii* SKAL-16 was isolated from the soil contaminated with vegetable oil using carbonate-buffered medium (CBM), which is composed of 100 mM carbonate buffer (pH 9), 2 g of K_2HPO_4/l, 0.2 g of yeast extract/l, 20 g of agar/l, and 2 ml of trace mineral stock solution/l. The trace mineral stock solution contained 0.01 g/l $MnSO_4$, 0.01 g/l $MgSO_4$, 0.01 g/l $CaCl_2$, 0.002 g/l $NiCl_2$, 0.002 g/l $CoCl_2$, 0.002 g/l $SeSO_4$, 0.002 g/l WSO_4, 0.002 g/l $ZnSO_4$, 0.002 g/l $Al_2(SO_4)_3$, 0.0001 g/l $TiCl_3$, 0.002 g/l $MoSO_4$, and 10 mM EDTA. Soybean oil (10 g/l) was emulsified in 100 ml of the carbonate buffer *via* ultrasonication and separately added to 900 ml of CBM after autoclaving. The agar plates were incubated for 72 h at 30°C, and colonies grown on the agar plates were then transferred to broth CBM containing tributyrin (tributyryl glycerol) as the sole carbon source rather than soybean oil. Tributyrin was selected as a carbon source for effective substrate, product, or metabolite analysis. Tributyrin was submerged in the culture broth and formed a clear droplet, which did not interfere with the measurement of the optical density of the bacterial cultures. Bacterial growth was measured spectrophotometrically on the basis of optical density at 660 nm. In order to determine the optimal pH for SKAL-16, bacterial cells were cultivated in CBM (or phosphate-buffered medium) containing tributyrin at pH 7, 8, 9, and 10. pH 7 and 8 were controlled with phosphate buffer, and pH 9 and 10 were controlled with carbonate buffer. The optimum temperature was determined to be 30°C by preliminary tests.

Growth of SKAL-16 on Various Carbon Sources

SKAL-16 was cultivated in the CBM containing 100 mM carbon sources such as acetate, butyrate, palmitate, glycerol, or tributyrin at pH 8.0 for 48 h. Yeast extract 0.1 g/l or 2 g/l ammonium chloride was used as a nitrogen source. The ammonium ion was used to test growth of SKAL-16 in medium with inorganic nitrogen source instead of yeast extract.

Identification of SKAL-16

SKAL-16 was identified *via* 16S rDNA sequencing. 16S ribosomal DNA was amplified *via* direct PCR using the following universal primers: forward 5'-GAGTTGGATCCTGGCTCA G-3' and reverse 5'-AAGGAGGGGATCCAGCC-3'. The reaction mixture consisted of 300 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 20 pM each of primer, 20 mM each of dNTP, 2U *Taq* polymerase (Genenmed, U.S.A.), and 20 ng of a template. Amplification was conducted for 30 cycles of 1 min at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C, using a PCR machine (T Gradient model; Biometera, German). The PCR products were then sequenced directly with an ABI Prism 3700 Genetic analyzer upon request by a professional company (Macrogen Inc., Korea). The 16S rDNA sequences were analyzed using the GenBank database and the identification was made on the basis of the 16S rDNA sequence homology. The strain SKAL-16 was registered in the GenBank database system, from which we obtained the accession number (EF406129).

Lipase Assay

SKAL-16 was cultivated in CBM (pH 8) containing tributyrin, for 48 h. Bacterial cells were harvested and washed by 40 min of centrifugation at 5,000 ×*g* at 4°C. The washed cells were resuspended in 50 mM Tris-HCl buffer (pH 8) and disrupted by 20 min of ultrasonic treatment (400 W) at 4°C. The protein concentration was determined using Bradford reagent (BioRad) as a coloring agent and bovine serum albumin as a protein standard. The cell-free extract was acquired from the disrupted cells by centrifugation at 10,000 ×*g* and 4°C for 30 min, and the extract was then used as an intracellular crude enzyme (ICE). Cell-free culture fluid separated from bacterial cells during harvesting was then concentrated with an Amicon ultrafiltration unit (Millipore, Bedford, U.S.A.) and YM 10 membranes (exclusion limit, 10 K) at 4°C and 99.99% N_2 at a pressure of 50 psi. The concentrate was then mixed with an identical volume of 100 mM Tris-HCl buffer (pH 8.0), and was used as the extracellular crude enzyme (ECE). The colorimetric method [24, 37] was used to determine whether ICE or ECE has lipase activity or not. The reaction mixture contained 180 µl of solution A (0.062 g of *p*-nitrophenyl palmitate in 10 ml of 2-propanol, sonicated for 2 min before use), 1,620 µl of solution B (0.4% Triton X-100 and 0.1% gum arabic in 50 mM Tris-HCl, pH 8.0), and 200 µl of properly diluted enzyme sample. The product was detected at a wavelength of 410 nm after 15 min of incubation at 30°C. Under these conditions, the molar extinction coefficient (ε410) of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl palmitate (*p*NPP) was 15,000 M^{-1}. One unit of lipase activity was defined as the quantity of enzyme required to release 1 µmole of *p*NP from *p*NPP/mg protein/min under the assay condition [24].

Esterase Assay

The esterase pattern of the cell-free extract of SKAL-16 was analyzed after fractionation by polyacrylamide gel electrophoresis without SDS [29]. The electrophoresis technique utilized in the present study was adapted from the methods described by Laemmli [16]. ICE or ECE was loaded onto the electrophoresis gel without heat treatment. Migration was induced for 80 min at 100 V and 10°C. After fractionation, the electrophoresis gel was stained enzymatically, according to the method described by Harris and Hopkinson [8]. α-Naphthyl butyrate and Fast Blue B were used as a substrate and coloring agent, respectively, according to the method previously described [8]. The α-naphthol generated from α-naphthyl butyrate *via* esterase catalysis reacts with Fast Blue B, generating a colored dye (pink).

Esterase-Coding Gene

A specific DNA fragment was amplified *via* direct PCR with the chromosomal DNA of SKAL-16 as the template and two pairs of primers as follows: primer I forward 5'-TGG CTC TCA TAA CGA TGC AG-3' and primer I reverse 5'-TTA AAT CAA GGG CGC CTG-3', and primer II forward 5'-AAA TCG CTG TTG TCA TGC CG-3' and primer II reverse 5'-GTT GTC GAT GCA ACG ATC-3'. The primers were designed using the full sequence (759 bp) of the gene

encoding for the tributyrin esterase of *Bacillus clausii* KSM-K16 (GenBank Accession No. AP006627). Amplification was conducted by the same method and procedures utilized for 16S rDNA amplification, with the exception of the annealing temperature. In this experiment, annealing temperatures of 48.8, 51.2, 53.6, 55.9, and 58.1°C were applied. The PCR products were directly sequenced with an ABI Prism 3700 Genetic Analyzer by a professional company (Macrogen Inc., Korea). The product sequences were analyzed using the GenBank database.

Dehydrogenase Assay

Dehydrogenases of ICE were assayed spectrophotometrically at 340 nm by measuring the reduction of NAD^+ to NADH coupled to the oxidation of specific substrates *via* pyruvate dehydrogenase (PDH), lactate dehydrogenase (LDH), isocitrate dehydrogenase (iCDH), and malate dehydrogenase (MDH). The 2.0 mM NADH (NAD^+) and 20 mM substrates were utilized in all enzyme experiments. Coenzyme A (100 μmol) was added to the reaction mixture only for the PDH assay. The specific activity is the concentration (μM) of NADH produced coupled to the oxidation of substrates per minute and mg protein. The molar extinction coefficient (ε340) of NADH reduced from NAD^+ was 6,333 M^{-1} [11]. One unit of dehydrogenase was defined as the quantity of enzyme required to reduce 1 μmole of NADH per milliliter per minute under the assay conditions.

Analysis

Tributyrin in the bacterial cultures was analyzed by HPLC (YoungLin, Korea) with a C_{18} column (Bio-Rad) and UV detector at 210 nm. Ethanol (100%) was used as a mobile phase at a 1.0 ml/min flow rate. The samples were prepared *via* the butanol extraction of tributyrin from bacterial cultures. A 0.1 volume of butanol was mixed with the bacterial culture and the mixture was then shaken vigorously, and the butanol layer was separated by centrifugation at 12,000 ×*g* and 4°C for 20 min. Supernatant (butanol and tributyrin mixture) was used as a sample and butanol was as an internal standard. The butyrate and glycerol contained in the bacterial culture or enzyme reactant were analyzed by HPLC with an Aminex HPX-87H ion-exchange column (Bio-Rad, CA, U.S.A.) and a refractive index detector. The column and detector temperatures were adjusted to 35°C. Sulfuric acid (0.008N) was used as a mobile phase at 0.6 ml/min flow rate. The samples were prepared by centrifugation at 12,000 ×*g* and 4°C for 30 min, followed by filtration with a membrane filter with a pore size of 0.22 μm. The filtrate was injected into the HPLC injector, and the injection volume was automatically controlled with a 20 μl loop. The concentration was calculated using the peak area obtained with standard materials. All chemicals used for analysis and biochemical tests were purchased from Sigma-Aldrich (USA).

Results

Bacterial Growth on Various Substrates

In general, lipid-utilizing *Bacillus* species may catabolize carbohydrate polymers and proteins [9, 28, 30], indicating that *Bacillus* species may generate a variety of extracellular enzymes, including lipase, amylase, cellulase and protease. On the basis of common knowledge, the strain SKAL-16 was expected to grow on fatty acids (acetate, butyrate, and palmitate), glycerol, and lipid. However, the SKAL-16 grew on glycerol and lipid, but not on fatty acids. SKAL-16 does not grow without yeast extract (Difco), which is composed of amino acids and vitamin B groups [4]. This indicates that the strain SKAL-16 is a typical heterotrophic microorganism that is incapable of synthesizing organic nitrogens including amino acids and nucleic acids. When isolated from soil, the SKAL-16 grew on agar plates containing CBM and soybean oil, indicating that SKAL-16 may utilize fatty acids and glycerol generated from lipids by the catalysis of extracellular lipase. However, SKAL-16 grew on tributyrin and glycerol, but not on butyric acid. This is a physiological property that greatly is different from other *Bacillus* species.

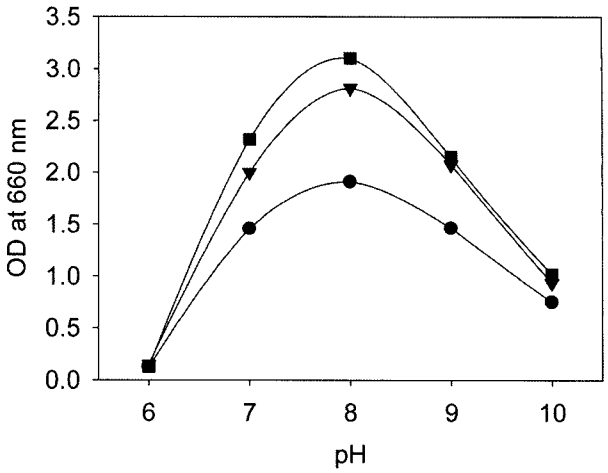

Fig. 1. Growth of *B. clausii* on medium composed of tributyrin and yeast extract at different pHs for 24 (●), 48 (▼), and 72 h (▲). The initial pH was controlled with 50 mM citrate buffer, phosphate buffer, and carbonate buffer.

Growth of SKAL-16 on Tributyrin

The effect of pH on the growth of SKAL-16 was investigated at pH values of 6–10 for 72-h cultivations in tributyrin liquid medium at 30°C and 150 rpm. Five ml of cultures was inoculated in 100 ml of medium. Fig. 1 shows that relatively higher and lower growth rates were obtained at pH 8 and pH 10, respectively, and bacterial growth was completely inhibited at pH 6. The pH for optimal growth of the SKAL-16 was pH 8, and this growth was not significantly inhibited at pH values of 7 and 9. During the cultivation of SKAL-16 on tributyrin, the specific smell of butyric acid could be detected by scent, which was the clue to analyze the bacterial culture supernatant. When SKAL-16 was cultivated at initial pH 8 and 9, the final pH of the medium was 7.7 and 8.6, respectively, after incubation for 72 h.

Production of Butyrate from Tributyrin

Tributyrin is a lipid composed of a short-chain fatty acid (butyric acid) and glycerol, in which the ester bond between

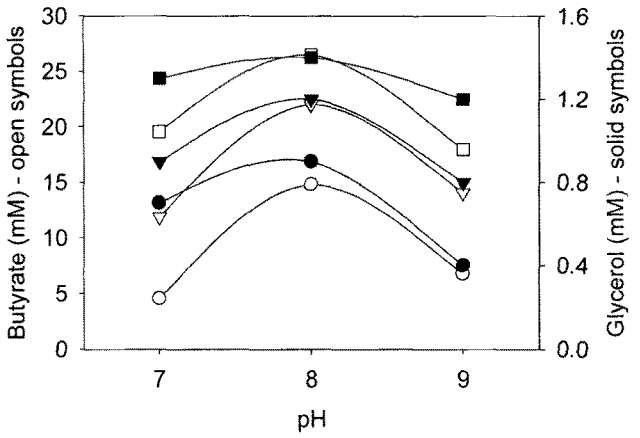

Fig. 2. Glycerol and butyrate produced by *B. clausii* SKAL-16, which was cultivated in basal medium composed of tributyrin as a sole carbon source and yeast extract, at different pHs for 16 (●, ○), 40 (▼, ▽), and 64 h (■, □).
The initial pH was controlled with 50 mM citrate buffer, phosphate buffer, and carbonate buffer.

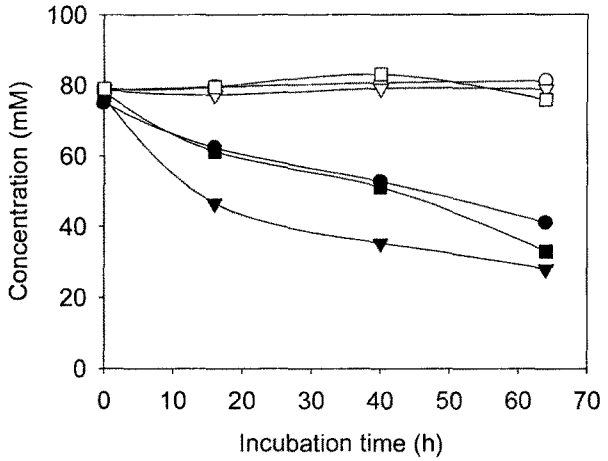

Fig. 3. Consumption of glycerol (solid symbols) and butyrate (open symbols) by resting cells of *Bacillus clausii* at pH 7 (●, ○), pH 8 (▼, ▽), and pH 9 (■, □).

the hydroxide residue of glycerol and the carboxy residue of butyric acid may be hydrolyzed by lipase [36]. As shown in Fig. 2, when the bacterial culture was periodically isolated and analyzed with HPLC, butyric acid was detected and increased in proportion to incubation time, but glycerol was not detected. In order to localize the lipase synthesized by SKAL-16, the lipase activity in ECE and ICE was estimated.

Hydrolysis of Tributyrin

Butyric acid and glycerol are products of tributyrin by lipase catalysis. Resting cells were obtained after centrifugation under aseptic conditions and incubated for 6 h at 20°C in order to induce the consumption of metabolic intermediates in the cytoplasm. As shown in Table 1, butyric acid and glycerol were generated from tributyrin by ICE, but not by ECE. The molar ratio of butyric acid and glycerol generated from tributyrin by the catalysis of ICE was nearly 3:1, which is consistent with the theoretical value. Butyric acid was generated, but glycerol was not produced when the resting cells were employed as a catalyst. On the basis of this result, the resting cells were cultivated on a mixture of glycerol and butyric acid. Fig. 3 shows that SKAL-16 selectively consumed glycerol, but not butyric acid.

Table 1. Hydrolysis of tributyrin by resting cells of *B. clausii* SKAL-16 (OD_{660}, 10) for 24 h, and intracellular crude enzyme (protein concentration, 3.4 mg/ml) and extracellular crude enzyme (protein concentration, 5.6 mg/ml) for 10 h.

Catalysts	Products (mM)	
	Butyric acid	Glycerol
Intact resting cells	26	0
Intracellular crude enzyme	33	12
Extracellular crude enzyme	0	0

pH of all reactants was adjusted to 8.0. Protein concentration was determined with Bradford reagent.

Lipase

Lipase activity was estimated, based on the production of *p*-nitrophenol from *p*-nitrophenyl palmitate. ICE and ECE were used to assay intracellular and extracellular lipase, respectively. As shown in Table 2, the intracellular lipase activity of *B. clausii* SKAL-16 was 15 μM/min/mg protein, but no extracellular lipase was detected. On the other hand, the intracellular and extracellular lipase activities of *Micrococcus* sp. (GenBank Accession No. EU569295), which was used as a positive control for the verification of lipase, was 32.8 and 19.2 μM/min/mg protein, respectively, which was higher than that of SKAL-16.

Esterase

Esterase catalyzes the hydrolysis of the ester bond of lipids composed of short-chain fatty acids [10, 17, 21]. In order to estimate esterase activity, α-naphthyl butyrate was used rather than lipids, because α-naphthol generated from α-naphthyl

Table 2. Lipase activity of *B. clausii* SKAL-16, estimated by the production of *p*-nitrophenol from *p*-nitrophenyl palmitate. *Micrococcus* sp. cell extract was used as a positive control.

Enzyme sources	Specific activity (μM *p*-nitrophenol/min mg/protein)	
	ICE (intracellular crude enzyme)	ECE (extracellular crude enzyme)
Bacillus clausii SKAL16	12.3	-
Micrococcus sp.	32.8	19.2

1 atggctctca taacgatgca gtttcattcc caaacattag gaaaacaaac aagcttggcg 61 atcattttgc ccgatgaagc gccgccgccc
ttcaaaacat tatacttgct tcatgggtgg 121 agcgacgacc attctgcctg gctgcggcaa acaagcattg agcgttatgc aattcgagag
181 caaatcgctg ttgtcatgcc g aatgtcgac ttgagctttt ataccgatat ggtttacggt 241 agcccttatt tcaccttttct agcagacgaa
ttgcctgagc ggcttgcccg gcttttccca 301 ttctcggaca aacgggaaga ccagtttgtt gctggattgt cgatgggcgg ttttggcgca
361 ttcaaatggg cgcttcacaa tactgagcgc tttcaggccg cagctagctt ttcaggcgcc 421 cttgatttaa aaagtctatt gaagcacct
atcccggaga gaatccccca tatgaaagcc 481 gtgtttggcg aatcccttc tctccatcaa tcagggggcg atttaatcgc tttgctgaaa
541 acaagcatcg ccgaaaaaat caaactcccg cacctgtacc agtattgtgg aacggaggat 601 tttttgtatg atataaatac ctcttttcta
gaggagaccc ggcaagtagg cgcgtcttta 661 gtctattctg aagggccagg tggccataca tgggattatt gggatcgttg catcgacaac
721 tggcttttgt cgttgcgaaa agatgggtta ttgaaataa 759

1 tcgcggaccc tttgacccgc ctacgccctt tgattgggca tggcccaaat cgtttgagta 61 ctcgtttggc gggactaacg tcgcagagta
atcgtgggtg acgacatgcg acactgtttg 121 gctgttaatg tgccggcact tccgcatgta atcaggtcga tacgacattg accttccttc 181
ccgtcggacg cacattggac cccctgtgaa gattgatctc cgcagtgcta ttagctgtcg 241 tcggctgagc gaaaatgttc atccgacatg
actagtgatcc actacctcca cactaaccat 301 gtctgatcgg gcctcgattg acgattggat tagaccggct caacatccct taggcgtcaa
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actccgagac atgtactact tatcattgtc tcgagacaaa 481 actctgactt gcaaagtatg gacaatcttc ccacatttga acggtcaacc
acccgacaaa 541 actcagctgt ataagtatgt agcccgtgaa ctggacgaca atttcctcat gttcgacaccg 601attgtcctca
ggattatgac ccgtaagagc aagcgctgat gaatgctatt atactcctcc 661 tgtccaaagt gtattaactt cgctctgctc cgagttatta
tacctcccgc gagaccttgt 721 catgcccttа tataaaaagt agcaatctcg ctggtgtacc tgttgtatat gtgttcacac 781 cgagcccgaa
gaatcctctt gtgagcgaac agagaattgt tcacgttcat gtgaaaccca 841 ctacccgatt tcctccata tacttcagga actttcac 878

Fig. 4. Full sequence of the esterase-coding gene of *B. clausii* KSM-K16 (*upper*) that was extracted from the GenBank database (Accession No. AP006627).
PCR product was synthesized with primer I and genomic DNA of *B. clausii* SKAL-16 as a template (*lower*). The primer was designed based on the esterase-coding gene of *B. clausii* KSM-K16. The DNA fragments in boxes of upper sequence were sources for primers.

butyrate via esterase catalysis forms a colored dye *via* reaction with Fast Blue B. It is entirely possible that SKAL-16 may have esterase, because tributyrin is a lipid composed of short-chain fatty acids. In the qualitative analysis using activity staining with electrophoresis gel, however, a band indicative of esterase was not observed on the gel (data not shown). In order to confirm the presence of a gene encoding for esterase in SKAL-16, a specific DNA fragment was amplified. During the amplification of DNA with two primers, one product was acquired at 53.6°C of annealing temperature, and its homology was analyzed using the GenBank database system. Fig. 4 shows that 878 bases of the DNA fragment amplified with primer I was obtained; however, it was not identical with the esterase-coding gene of *B. clausii* KSM-K16. Furthermore, it was not similar to any of the other genes recorded in the GenBank database.

Metabolic Pathway

From all the results described above, it can be surmised that SKAL-16 may catabolize only glycerol as a carbon source *via* the TCA cycle, which is a common metabolic pathway in the majority of aerobic heterotrophs. PDH catalyzes the decarboxylation and dehydrogenation of pyruvate, by which pyruvate is converted to acetyl-CoA coupled to the reduction of NAD^+ to NADH. LDH catalyzes the oxidation of lactate to pyruvate, iCDH catalyzes the oxidation of isocitrate to α-ketoglutarate, and MDH catalyzes the oxidation of malate to oxalacetate in conjunction with the reduction of NAD^+ to NADH. As shown in Table 3, dehydrogenases other than

Table 3. Pyruvate dehydrogenase (PDH), lactate dehydrogenase (LDH), isocitrate dehydrogenase (iCDH), and malate dehydrogenase (MDH) activities estimated based on coupling reduction of NAD^+ with oxidation of pyruvate, isocitrate, or malate.

Intracellular crude enzyme	Specific activity (μM NADH/min/mg protein)			
	PDH	LDH	iCDH	MDH
Bacillus clausii SKAL16	2.33	0.0	2.07	2.47
Escherichia coli K12	13.8	22.6	9.6	10.8

E. coli K12 cell extract was used as a positive control.

LDH were detected, but at levels lower than those seen in *E. coli*, which was used as a positive control for the verification of enzyme activity. These results show that SKAL-16 harbors enzymes that operate in the TCA cycle.

DISCUSSION

In the present study, we observed an unusual phenomenon during the growth of *B. clausii* strain SKAL-16 on tributyrin. Tributyrin is a lipid composed of glycerol and butyric acid, a short-chain fatty acid. The distinctive smell of butyric acid increased in proportion with the duration of SKAL-16 culture. On the basis of the degree of butyric acid smell, the organic compounds contained in the culture were quantitatively and qualitatively analyzed, and the amount of butyric acid was found to increase in proportion to the incubation time; nevertheless, glycerol was not detected. Logically, this is in accordance with the result that SKAL-16 did not grow on butyric acid, but did grow on glycerol. SKAL-16 does not harbor esterase, although it can utilize tributyrin, which is composed of a short-chain fatty acid, butyric acid [36]. This indicates that the lipase of SKAL-16 may catalyze the hydrolysis of lipids unrelated to the molecular mass. SKAL-16 grew on organic nitrogen complexes, such as yeast extract and peptone, under conditions in which a carbon source was absent, and the strain utilized organic carbons under organic nitrogen-limiting conditions. This is a typical metabolic property observed in the most heterotrophic bacteria. However, the reason that SKAL-16 did not grow on the fatty acids remains difficult to explain.

One explanation could be that the SKAL-16 strain absorbs lipids outside, and then hydrolyzes lipids in the cytoplasm and selectively excretes fatty acids outside, if SKAL-16 did not harbor enzymes to catalyze the β-oxidation reactions of fatty acids. In general, acetate and butyrate are metabolites typically produced by fermentative bacteria, and they serve as substrates for a variety of heterotrophic bacteria [13, 20, 25, 27, 38]. The activation of acetate to acetyl-CoA and the β-oxidation reaction of fatty acids are difficult to estimate *in vitro*; however, the presence of these reactions was physiologically demonstrated by the results that SKAL-16 did not grow on acetate, but produced butyrate during growth on tributyrin.

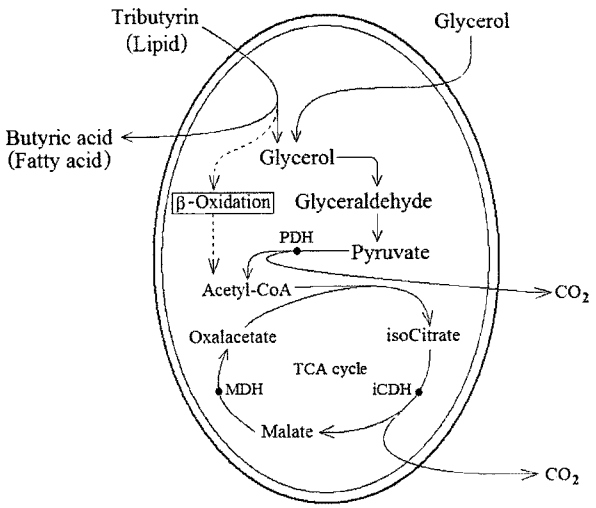

Fig. 5. Proposed metabolic pathway of *Bacillus clausii* SKAL-16, based on bacterial growth, metabolite, activity of specific enzymes, and substrate consumption.

PDH, MDH, and iCDH, which catalyze the oxidation of pyruvate, malate, and isocitrate, respectively, in conjunction with NAD^+ reduction to NADH, are key enzymes for the TCA cycle [23, 26]. LDH catalyzes the reduction of pyruvate to lactate, which is a key enzyme in lactic acid fermentation bacteria but not an essential enzyme for glycolysis or the TCA cycle. Accordingly, it appears reasonable that SKAL-16 does not harbor LDH. On the basis of the substrate utilization and enzyme activities of SKAL-16, we proposed a metabolic pathway from glycerol to carbon dioxide, and its schematics are provided in Fig. 5. Glycerol is oxidized to dihydroxyacetone *via* the catalysis of glycerol dehydrogenase in conjunction with the reduction of NAD^+, and dihydroxyacetone is converted to glyceraldehyde *via* the glycolysis pathway [6, 19, 31].

In conclusion, the metabolic properties of SKAL-16, which is capable of generating fatty acid *via* the catalysis of intracellular lipase, may be similar to fatty acid fermentation in appearance, but is actually a quite specific phenomenon, which has not been reported prior to this study. We are currently unable to speculate as to why SKAL-16 excretes fatty acids produced by the catalysis of the cytoplasmic enzyme, for which free energy must be wasted. The most plausible explanation for the reason of why SKAL-16 excretes fatty acid at a thermodynamic disadvantage could be the mutation of the gene encoding for the β-oxidation of fatty acids, thereby abrogating its catabolism function of fatty acids. This is the reason why the accumulation of fatty acids was induced in the cytoplasm. Furthermore, this may be an impetus for the change in pH of the cytoplasm to approximate a pK value of the fatty acid. The decline in pH in the cytoplasm can cause the scavenging of membrane potential in a fashion similar to the uncoupling reaction of metabolically generated organic acids [1]. Accordingly, fatty acid excretion may be the best response for survival of SKAL-16 during growth on tributyrin.

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