EXPERIMENTAL ARTICLES

Fervidicoccus fontis Strain 3639Fd, the First Crenarchaeon Capable of Growth on Lipids

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Abstract—Up to now, ability of prokaryotes to grow on lipids has been shown only for bacteria. *Thermococcus sibiricus*, member of the phylum *Euryarchaeota* isolated from a high-temperature oil well and capable of growth on olive oil, is the only exception. The present work reports isolation of a pure culture of a strictly anaerobic archaeon, strain 3639Fd (=VKM B-3509, =KCTC 25228) from a Kamchatka thermal spring, capable of growth on various lipids (tributyrin, triolein, and sesame, cottonseed, and sunflower oil) at 70°C and pH 5.5–6.0. Growth on tributyrin resulted in formation of butyrate, CO₂, and hydrogen. According to the results of the 16S rRNA gene sequence analysis and *in silico* DNA–DNA hybridization, the isolate was classified as a strain of *Fervidicoccus fontis*, an archaeon of the phylum *Crenarchaeota*. The closest characterized homologs of the α/β -hydrolases, encoded in the genomes of *F. fontis* 3639Fd and of the type strain of this species, Kam940^T, were various carboxyl esterases (EC 3.1.1), the enzymes responsible for lipid hydrolysis. Thus, *F. fontis* is the first crenarchaeon able to obtain energy by hydrolysis of lipid substrates.

Keywords: thermophilic archaea, extremophiles, lipases, esterases, lipid degradation, *Crenarchaeota*, triglycerides, esters, *Fervidicoccus fontis*

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Lipids are complex organic compounds, which, together with proteins, carbohydrates, and nucleic acids, are the main components of living cells. The enzymes responsible for lipid hydrolysis belong to the group of carboxylesterases (EC 3.1.1). Carboxylesterases cleaving the esters with short fatty acid chains (C₂–C₁₀) are often termed esterases, while those cleaving the esters with long fatty acid chains (over 10 carbon atoms) are known as lipases. Both groups of carboxylesterases have the same mechanism of action: their active centers contain the amino acid triad Ser–Asp(Glu)–His, which is involved in the cleavage of ester bonds (Bornscheuer, 2002). Moreover, carboxylesterases often possess both types of activity (Yang et al., 2019). Lipases and esterases have broad substrate specificity, do not require cofactors, and are highly active in organic solvents (López-Iglesias and Gotor-Fernández, 2015).

Lipases and esterases have a wide potential for application. They are used in wastewater treatment, synthesis of optically pure compounds (pharmaceuticals, esters, and amino acids), in production of biodiesel and biopolymers, cleaning agents, cosmetics, paper, leather, and foodstuffs (Bornscheuer, 2002; Anobom et al., 2014; Salihu and Alam, 2015; Cabrera

and Blamey, 2018). By 2020, lipase sales worldwide were as high as US\$ 590.5 million with an annual growth of 6.5% (Chandra et al., 2020).

Search for new lipases and/or microorganisms growing on lipids is among the urgent issues for modern biotechnology and microbiology. The sources of these enzymes for the current technologies are usually bacteria (members of the genera *Bacillus*, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Pseudomonas*, etc.) and fungi (e.g., members of the genera *Candida*, *Rhizomucor*, *Aspergillus*, and *Thermomucor*). Prokaryotic and fungal producers are preferable to plants and animals due to the ease of cultivation and genetic modification, as well as to the high yield and activity of the relevant enzymes (Chandra et al., 2020). Lipolytic archaea seem promising, since extremophiles living at the values of environmental parameters (temperature, pH, salinity, pressure, etc.), which are close to the known limits for life. Thus, these organisms and their enzymes are resistant to extreme conditions (Littlechild, 2015), which are required for a number of industrial processes. As far as lipolytic properties are concerned, halophiles are the best-studied group of archaea (Delgado-García et al., 2018). As for thermophilic archaea, carboxylesterase genes have been

detected in the genomes of some organisms: *Pyrococcus furiosus*, *Metallosphaera sedula*, *Pyrobaculum caldifontis*, *Aeropyrum pernix*, *Thermococcus kodakarensis*, *Archaeoglobus fulgidus*, *Picrophilus torridus* (Soni et al., 2019), as well as most *Sulfolobus* species—*S. solfataricus* (Mandrich et al., 2007), *S. acidophilus* (Soni et al., 2019), *S. shibatae* (Huddleston et al., 1995), *S. acidocaldarius* (Sobek and Gorisch, 1988), *S. tokodaii* (Suzuki et al., 2004), and *S. islandicus* (Stiefler-Jensen et al., 2017). Esterase and lipase activities of these archaea were shown for the extracts from their cells grown on complex peptide substrates (tryptone/peptone/yeast extract) or sucrose, or after expression of their carboxylesterase genes in *Escherichia coli* (Soni et al., 2019). The substrates most commonly used for determination of lipase and esterase activity are tributyrin (an ester of glycerol and three butyrate residues) and triolein (an ester of glycerol and three oleic acid residues) (Hotta et al., 2002). However, no results on cultivation of archaea on lipid substrates are presently available, except for a brief mention of the ability of a hyperthermophilic archaeon *T. sibiricus* to grow on olive oil (Mardanov et al., 2009), which did not include detailed microbiological and genetic confirmation of this property.

The present work describes an extremely thermophilic anaerobic strain 3639Fd, the first member of the phylum *Crenarchaeota* isolated on tributyrin and capable of growth on various lipids.

MATERIALS AND METHODS

Isolation and characterization of the thermophilic archaeon. Strain 3639Fd was isolated from an enrichment culture containing the sediments and water from the Vertoletnyi hot spring (N 54°30.005, E 160°00.732; 59°C, pH 6.5) in the Uzon caldera (Kamchatka, Russia) and incubated in situ for a week with 1% (vol/vol) tributyrin (Fluka, United States) and 0.1 g/L yeast extract (YE, Helicon, Russia). Subsequent work on the isolation and characterization of the pure culture was carried out using the strictly anaerobic modified Pfennig medium (Podosokorskaya et al., 2011). The medium was dispensed into test tubes and autoclaved at 121°C, 101 kPa for 60 min or, when the medium was supplemented with sulfur, at 50 kPa for 40 min. The following soluble substrates were used: glycerol (Himreactiv, Russia), butyrate (Sigma Aldrich, United States), YE, peptone (Helicon, Russia), tryptone (Dia-M, Russia), casein (Reachem, Russia), pyruvate (AppliChem, Germany), lactate (Sigma, United States), fumarate (Merck, Germany), tartrate (Fluka, United States), succinate (Reachem, Russia), malate (Sigma Aldrich, United States), glucose, xylose, rhamnose, maltose, sucrose (all from Reachem, Russia), cellobiose (Sigma Aldrich, United States), galactose (Serva, Germany), and arabinose (Fluka, United States). The substrates were added after autoclaving as sterile solutions to the final con-

centration of 1 g/L. Liposoluble substrates used were: triglycerides (tributyrin and triolein, Sigma, United States), edible oils (sesame, cotton, olive, and sunflower) and esters of long-chained fatty acids (palmate and stearate, Sigma, United States). These substrates were added to the sterile medium dropwise to 5–10 mL/L. Insoluble substrates: guar gum (CPKelco, United States), xylan (Megazyme, Ireland), and amorphous cellulose, were added prior to sterilization to the final concentration of 1 g/L. Strain 3639Fd was isolated in pure culture using the terminal dilutions method.

All experiments for description of the pure culture were carried out in 18-mL Hungate test tubes with 10 mL of the medium and N₂ as the gas phase, which were sealed with dense rubber stoppers and screw caps. The optimal growth temperature was determined by cultivating the strain for 3 to 6 days in the medium with tributyrin (pH 6.0) at 50, 55, 60, 65, 70, 78, and 83°C. The optimal pH for growth was determined by cultivating the strain at 70°C using the following buffers at the final concentration of 10 mM: acetate (pH range 3.0–5.5, Sigma Aldrich, United States), bicarbonate (5.5–6.5), MOPS (7.0–7.5, Dia-M, Russia), and HEPBS (8.0–8.5) (Sigma, United States). Ability to reduce various electron acceptors when grown on tributyrin was tested with SO₃²⁻, SO₄²⁻, and S₂O₃²⁻ (as sodium salts, final concentrations 10 mM), or S⁰ (1 g/L). Growth parameters and substrate preferences were determined under the optimal conditions (70°C, pH 5.5–5.6). To determine growth on specific substrates, the strain was sequentially transferred thrice under the same conditions. All experiments were carried out in triplicate.

Growth was assessed by direct cell count under an Olympus CX-41RF phase contrast microscope (Olympus, Japan). Sulfide production was determined colorimetrically with *N,N*-dimethyl-*p*-phenylenediamine as modified by Trüper and Schlegel (1964) using a Beckman model 35 spectrophotometer at $\lambda = 670$ nm. Volatile fatty acids were determined by gas–liquid chromatography on a Crystal 5000.2 chromatograph (Chromatec, Russia) equipped with a flame ionization detector. The separation was carried out on a Zebron ZB-WAXplus capillary column (Phenomenex, United States) in the temperature programming mode. Sugars and alcohols were determined by HPLC on a Stayer chromatograph (Akvilon, Russia). The separation was carried out on an Aminex HPX-87H column (BioRad, United States) in the isocratic mode with 25 mM H₂SO₄ as an eluent (0.6 mL/min). The signals were registered using two detectors: refractometric Smartline 2300 (Knauer, Germany) and ultraviolet UVV 104 at 210 nm (Akvilon, Russia) according to the sequential scheme. Gaseous products were determined on a 3700 modified gas chromatograph (ZIOC RAS, Russia) with the Phoenix v. 3.6.0 analytical software (BSoft, Russia).

Strain 3639Fd was deposited to the All-Russian Collection of Microorganisms (VKM) as VKM B-3509 and to the Korean Collection for Type Cultures (KCTC) as KCTC 25228.

Isolation of genomic DNA. To isolate genomic DNA of strain 3639Fd, early stationary-phase cells were collected by centrifugation for 15 min at 9000 rpm. To the pellet resuspended in the TNE buffer (10 mM Tris-HCl, 10 mM NaCl, 5 mM EDTA; pH 8.0), proteinase K (100 µg/mL), SDS (0.5% vol/vol), and EDTA (0.015 M) were added, and the mixture was incubated for 60 min at 50°C. After adding phenol-chloroform (1 : 1), the mixture was stirred and centrifuged for 15 min at 9000 rpm. The supernatant was extracted twice with an equal volume of chloroform, and 1/10 part of 3 M CH₃COONa (pH 5.2) and 2 parts of 96% ethanol were added. The obtained mucus was collected with a sterile spatula, washed twice with 70% ethanol, dried, and dissolved in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). DNA was then treated with RNase A (10 µg/mL) for 30 min at 37°C, extracted twice with an equal volume of chloroform, precipitated with 96% ethanol, washed with 70% ethanol, and dissolved in sterile water. DNA concentration was measured on a Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, United States).

Genome sequencing and assembly were carried out using the Illumina technology. Fragments (~500 bp) of genomic DNA were obtained by sonication on a BioRuptor® UCD-200 homogenizer (Diagenode, Belgium). The libraries were prepared using NEBNext® Ultra™ DNA Library Prep kit for Illumina® (New England Biolabs, United States) according to the manufacturer's recommendations. Clone libraries were sequenced on a MiSeq Sequencing System (Illumina, United States) using the 2 × 150 bp reagent kit. After quality filtration using CLC Genomic Workbench 10.0 (Qiagen, Germany) 1307626 reads were obtained.

The genome was assembled using SPAdes 3.13.0 (Antipov et al., 2016). At the final stage, ten contigs were obtained with the total size of 1248168 bp. The genome sequence was deposited to GenBank under accession no. JADEZV000000000.1.

The genome was annotated using the NCBI PGAP pipeline (Arefiev et al., 2020). Comparative analyses based on the complete genome sequence were carried out as follows: average nucleotide identity (ANI) was calculated using the pyani 0.2.8 module, choosing the ANIb method (Pritchard et al., 2016); average amino acid identity (AAI) was determined using the aai.rb script (Rodriguez-R and Konstantinidis, 2016); *in silico* DNA–DNA hybridization was performed using the GGDC 2.1 server (Meier-Kolthoff et al., 2013), with BLAST+ as an alignment method.

Pan-genomes of *F. fontis* strains Kam940^T and 3639Fd were determined using the Proteinortho 5.16 software package (Lechner et al., 2011) with the fol-

lowing parameters: 95% sequence identity (lowest threshold for a single species by AAI) and 50% mutual coverage. The proteins with sequence identity and coverage parameters below those indicated were considered unique (strain-specific). The analysis was visualized using the Venn software package (<https://cran.r-project.org/web/packages/venn/index.html>). Distribution along the COG (clusters of orthologous groups) functional categories was determined for unique proteins using webMGA (Wu et al., 2011). Gene sequences were aligned using the Mafft v. 7 (Katoh and Standley, 2002).

Ability of *Fervidicoccus* isolates to grow on lipids.

Ability to grow on lipid substrates was tested for the type strain *Fervidicoccus fontis* Kam940^T (=DSM 19380^T, =VKM B-2539^T), and for two strains of this genus (FoA-16 and 1507) from the collection of Laboratory of Metabolism of Extremophilic Prokaryotes (Research Center of Biotechnology, Russian Academy of Sciences). The same mineral medium was used (Podosokorskaya et al., 2011). Cultivation was carried out under optimal conditions for each strain: Kam940^T, at 70°C and pH 5.6, in the presence of 0.2 g/L YE; FoA-16, at 78°C and pH 4.8, in the presence of 0.1 g/L YE; strain 1507, at 70°C and pH 6.0, in the presence of 0.1 g/L YE. To confirm growth on a substrate, the strains were sequentially transferred thrice under the same conditions. All experiments were carried out in duplicate.

RESULTS AND DISCUSSION

Isolation and characterization of strain 3639Fd. The strain was isolated on tributyrin supplemented with YE (0.05 g/L) at 70°C and pH 5.5 by the terminal dilutions technique. The cells were small cocci of regular shape. The strain was an extremely thermophilic and moderately acidophilic microorganism, growing at the temperatures from 55 to 83°C and pH from 4.5 to 6.5 with the optimum at 70°C and pH 5.5–5.6. The organism required yeast extract in the medium at optimal concentration of 0.1 g/L.

Strain 3639Fd grew on tributyrin, producing butyrate (Fig. 1), with the maximum cell yield of $2.1 \pm 0.3 \times 10^7$ cells/mL after 67 h of cultivation under optimal conditions (70°C, pH 5.6, 0.1 g/L YE). Apart from butyrate, CO₂ and H₂ were produced (data not shown). Under these conditions, the minimal doubling time was 1.2 h. Cell yield in background medium with 0.1 g/L YE without tributyrin was $0.3 \pm 0.05 \times 10^7$ cells/mL (Fig. 2). Cultivation on tributyrin with sulfur resulted in a doubled cell yield ($5.4 \pm 0.5 \times 10^7$ cells/mL), and production of sulfide was observed. Thiosulfate had a weak stimulating effect on growth ($3.2 \pm 0.2 \times 10^7$ cells/mL), while sulfate had no effect, and sulfite inhibited the growth on tributyrin. The strain grew also on triolein ($1.4 \pm 0.03 \times 10^7$ cells/mL) and some oils: sesame ($1.1 \pm 0.1 \times 10^7$ cells/mL), cot-

Fig. 1. Growth of *Fervidicoccus fontis* 3639Fd (1) and butyrate formation (2) from tributyrin under optimal conditions (pH 5.6, 70°C, 0.1 g/L YE). Butyrate concentrations in the control samples: uninoculated medium with tributyrin (3) and in the medium with 1.0 g/L YE (4).

ton ($1.1 \pm 0.2 \times 10^7$ cells/mL), and sunflower ($0.9 \pm 0.15 \times 10^7$ cells/mL) (Fig. 2). Apart from lipids, strain 3639Fd fermented tryptone ($1.2 \pm 0.3 \times 10^7$ cells/mL), casein, YE ($2 \pm 0.5 \times 10^7$ cells/mL), organic acids, including butyrate ($1 \pm 0.05 \times 10^7$ cells/mL), and mono- and disaccharides. The

strain did not grow on glycerol, olive oil, peptone, and polysaccharides. Its inability to grow on olive oil, while it could grow on pure trioleate, was probably due to unknown impurities suppressing its growth.

Phylogenetic and taxonomic position of strain 3639Fd. The 16S rRNA gene sequence of strain 3639Fd exhibited 99.93% identity to that of *Fervidicoccus fontis* Kam940^T (Perevalova et al., 2010). The values of AAI, ANI, and *in silico* DNA–DNA hybridization for strains 3639Fd and *F. fontis* Kam940^T were 99.38, 99.63, and 97.1%, respectively. These values are above the threshold for individual species: 95% for AAI (Konstantinidis and Tiedje, 2005), 95% for ANI (Kim et al., 2014), and 70% for DDH (Wayne et al., 1987). Thus, based on the similarity of the 16S rRNA gene sequences and full genome comparison, strain 3639Fd was assigned to the species *F. fontis*.

Genome analysis of *Fervidicoccus fontis* 3639Fd and genetic confirmation of its ability to hydrolyze lipids. According to the PGAP annotation, the genome of *F. fontis* 3639Fd consisted of 1385 genes, including 1322 protein-encoding genes, 48 RNA (3 rRNA, 43 tRNA, 2 ncRNA), and 15 pseudogenes. Since 3639Fd differed phenotypically from the type strain Kam940^T, while the average similarity of their genomes and proteomes was relatively high (ANI and AAI of 99.63 and 99.38%, respectively), the common (core genome) and strain-specific parts of the *F. fontis* genome were determined. It was found that 1125 were common, while 159 occurred only in strain Kam940^T and 111, in strain 3639Fd (Fig. 3). The distribution of

Fig. 2. Growth of *Fervidicoccus fontis* Kam940^T (red/black columns) and 3639Fd (blue/gray columns) on lipid substrates.

unique proteins according to the COG functional categories (Fig. 4) showed approximately equal numbers of the proteins of most groups (F, I, K, L, M, and R). At the same time, the proteins of categories E (amino acid metabolism and transport) and H (cofactor metabolism and transport) were more numerous in strain 3639Fd. The proteins of category C (energy production and storage), P (metabolism and transport of inorganic ions), and S (unknown function) were more numerous in strain Kam940^T.

Since the present work focused on ability of strain 3639Fd to grow on triglycerides, we attempted to detect the genes encoding lipases/esterases. The genome of strain 3639Fd was found to contain the gene of family 6 α/β -hydrolase; its closest characterized homologs were various carboxylesterases (EC 3.1.1), including eukaryotic monoacylglycerol lipases (~30–33% similarity of amino acid sequences at ~60% coverage). Aligning of this carboxylesterase with members of 15 esterase families (Zarafeta et al., 2016) revealed its catalytic motif is being GASMGG, which corresponds to esterase families III and V (Zarafeta et al., 2016). The closest uncharacterized proteins are archaeal α/β -hydrolases, including one homolog from the type strain *F. fontis* Kam940^T. No genes encoding the enzymes of the known pathways for glycerol or fatty acid oxidation were detected in the genome of strain 3639Fd. While the strain did not grow on glycerol, we observed its growth on organic acids. Thus, other, presently unknown enzymes are probably involved in the oxidation of these substrates.

Testing ability of *Fervidicoccus* strains to grow on lipids. After 4 days of incubation at 70°C, cell yield of the type strain *F. fontis* Kam940^T was $1.6 \pm 0.1 \times 10^7$ cells/mL on tributyrin, $0.4 \pm 0.1 \times 10^7$ cells/mL on triolein, $1.4 \pm 0.4 \times 10^7$ cells/mL on olive oil, $2.0 \pm 0.1 \times 10^7$ cells/mL on cotton oil, $1.0 \pm 0.1 \times 10^7$ cells/mL on sunflower oil, and $0.8 \pm 0.3 \times 10^7$ cells/mL on the background control medium (without the substrate) (Fig. 2). No growth of strains *F. fontis* 1507 and FoA-16 was observed after 7 days of cultivation on tributyrin. Thus, apart from strain 3639Fd, only Kam940^T could grow on lipid substrates.

Fervidicoccus fontis is the only species within the order *Fervidicoccales* (phylum *Crenarchaeota*). The type strain of this species, Kam940^T, was isolated from a Kamchatka thermal spring and grew on proteins and peptides at 70°C (Perevalova et al., 2010). Molecular ecological studies revealed members of this genus in thermal springs of volcanic regions of the Yellowstone National Park, Kamchatka, Iceland, and New Zealand (Lebedinsky et al., 2013), as well as in China (Jiang et al., 2016); they are also predominant archaea in some mesophilic habitats, e.g., in swamp soils (Lv et al., 2014). This indicates a significant ecological role of members of this genus in anaerobic ecotopes, where they act as organic matter degraders and suc-

Fig. 3. Numbers of common (core genome) and unique proteins in *Fervidicoccus fontis* strains Kam940^T and 3639Fd.

cessfully competes with other chemoorganotrophic prokaryotes.

In the present work we showed a new feature of the genus *Fervidicoccus*, i.e., its ability to grow on various triglycerides. Moreover, the gene encoding a carboxylesterase, representing the family III or V, or a new esterase family was revealed in the genome of strain 3639Fd. A similar gene was found in the genome of *F. fontis* Kam940^T, which was subsequently shown to grow on lipid substrates.

Ability of two *F. fontis* strains to grow on lipids broadens our understanding of the ecological abilities of the genus *Fervidicoccus* and puts it apart among all known cultured *Crenarchaeota*, for which growth on lipids has not been shown so far. According to available data, only one archaeon is known to grow on lipids (olive oil), *Thermococcus sibiricus* (phylum *Euryarchaeota*). The genes encoding esterases and lipases were revealed in the genome of this archaeon (Mardanov et al., 2009). However, our analysis of four tentative esterases and lipases in this microorganism showed that none of them belonged to carboxylesterases, and none therefore could be involved in glycerolipid hydrolysis. Lack of information on growth of archaea on lipid substrates stems from the difficulty of isolating and maintaining archaeal pure cultures (Makarova et al., 2019).

Further in-depth analysis of the carboxylesterase revealed in the present work might reveal the mechanisms of lipolytic activity in archaea and will promote the isolation of new strains of lipolytic archaea with subsequent characterization of new lipases with a significant biotechnological potential.

Fig. 4. Distribution of strain-specific proteins in *Fervidicoccus fontis* strains Kam940^T (red columns) and 3639Fd (blue columns) according to the COG functional categories.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving ani-

mals or human participants performed by any of the authors.

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