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EXPERIMENTAL ARTICLES

Oligonucleotide Probes for the Detection of Representatives of the Genus *Thermoanaerobacter*

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Abstract—Based on the analysis of 16S rRNA nucleotide sequences, oligonucleotide probes were designed for the detection of representatives of the genus *Thermoanaerobacter.* To increase the specificity of detection, the genus *Thermoanaerobacter* was divided into three groups. The probe Tab 827 (5'-GCTTCCGCDYCCCA-CACCTA-3') detected all known representatives of the genus *Thermoanaerobacter*; the probe Tab_1 844 (5'-TTAACTACGGCACGRAATGCTTC-3') was specific for the first group of species of the genus (*T. wiegelii, T. siderophilus, T. sulfurophilus, T. brockii, T. kivui, T. ethanolicus, T. acetoethylicus,* and *T. thermohydrosulfuricus*); the probe Tab_2 424 (5'-CACTAMYGGGGTTTACAACC-3') targeted the second group (*T. thermocopriae, T. mathranii,* and *T. italicus*); and the probe Tab_3 184 (5'-TCCTCCATCAGGATGCCCTA-3') was specific for the third group (*T. tengcongensis, T. yonseiensis, T. subterraneus,* and *Carboxydibrachium pacificum*, an organism related to the genus *Thermoanaerobacter* according to its 16S rRNA sequence). The oligonucleotide probes were labeled with Dig-11-dUTP. Hybridization with the probes showed the affiliation with *Thermoanaerobacter* of several pure cultures that were morphologically similar to representatives of this genus but possessed metabolic features unusual for it (capacity for agarose hydrolysis, anaerobic oxidation of CO, growth at low pH values) or were isolated from habitats previously unknown for *Thermoanaerobacter* (deep-sea hydrothermal vents).

Key words: thermophiles, *Thermoanaerobacter, Carboxydibrachium pacificum*, oligonucleotide probes, detection, identification.

The genus *Thermoanaerobacter* comprises moderately thermophilic anaerobic gram-positive bacteria with a fermentative type of metabolism; some of them can reduce inorganic electron acceptors, such as thiosulfate, elemental sulfur, and Fe^{3+}.

Thermoanaerobacter representatives ferment various carbohydrates with the formation of acetate, H_2, CO_2, ethanol, and lactate; some species are homoacetogenic. Most members of the genus form endospores; however, for some species, sporulation has not been observed. At present, the genus *Thermoanaerobacter* comprises 14 valid species. These organisms were isolated from a wide variety of environments: hot springs [1], cyanobacterial mats [2], volcanic lakes [3], high-temperature oil reservoirs [4], etc. New properties are continually found in *Thermoanaerobacter* representatives. Thus, recently, extremely thermophilic representatives of this genus have been discovered [5, 6]. Hydrolytic enzymes produced by *Thermoanaerobacter* representatives have been characterized, such as keratinase [7] and a new protease [8].

The goal of this study was to develop an express method that would make it possible to identify *Thermoanaerobacter* representatives in pure cultures and enrichments without phenotypic characterization, as well as to detect them in natural samples.

MATERIALS AND METHODS

Pure and enrichment cultures. Type strains of the following species were used as reference strains: *Thermoanaerobacter siderophilus, T. sulfurophilus, T. wiegelii, T. mathranii, T. italicus, T. yonseiensis, Carboxydibrachium pacificum, Moorella glycerini, Thermoterrabacterium ferrireducens,* and *Thermotoga maritima.* The reference strains were cultivated according to the original methods used by the researchers who isolated them [3, 2, 9–11, 6, 12–15].

For the analysis, pure and enrichment cultures of anaerobic thermophilic organisms with rod-shaped cells were used (Table 3). Among the pure cultures, there were three strains capable of agarose hydrolysis (B5, K15, K67), a strain utilizing CO (2707), and strains isolated from deep-sea hydrotherms (518, 107). Among the enrichments, three (K44, 432, 416) were capable of ferric iron reduction and one (Kar) utilized CO. All pure and enrichment cultures were neutrophilic, except for the acidophilic organotrophic strains 711 and 761 and enrichment 816.

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Cultivation conditions. Pure unidentified cultures and enrichments were grown anaerobically according to an earlier described technique on medium 1 (Pfennig mineral base, trace elements, vitamins) [16] with various electron donors and acceptors. Thus, strains B5, K14, and K67 were cultivated on medium 1 supplemented with 3 g/l of maltose and 0.5 g/l yeast extract, pH 6.8–7.0. Strains 518 and 107, isolated from marine habitats, were grown on medium 2 of the following composition (g/l): KCl, 0.325; $MgCl_2$, 2.75; $MgSO_4$, 3.45; NH_4Cl, 0.25; $CaCl_2 \cdot 2H_2O$, 0.15; K_2HPO_4, 0.15; NaCl, 18; $NaHCO_3$, 1; Na_2S, 0.25; trace elements and vitamins [16], 1 ml/l each; the medium was supplemented with 2 g/l of maltose. For strain 518, 2 g/l of thiosulfate was added; for strain 107, the medium was supplemented with 10 g/l of sulfur, 3 g/l of peptone, and 0.5 g/l of yeast extract. The pH was 6.0 for strain 518 and 6.8–7.0 for strain 107. Enrichment cultures K44, 432, and 416 were associations of thermophilic anaerobic microorganisms that carried out dissimilatory reduction of Fe(III) in the presence of molecular hydrogen. The cultivation of iron reducers was performed on medium 1 supplemented with 10 g/l of peptone and 0.2 g/l of yeast extract; Fe(III) was added as Fe(III) oxide to a final concentration of 90 mM; the pH of the medium was 6.8. The acidophilic strains 711 and 761 were grown on medium 1 supplemented with 2 g/l of sucrose; the pH of the medium was 5.7 for strain 711 and 5.0 for strain 761. Enrichment 816 was grown on medium 1 supplemented with 2 g/l of yeast extract and 10 g/l of elemental sulfur; the pH of the medium was 3.5. Enrichment 746 was grown on medium 1 supplemented with 0.5 g/l of yeast extract, 2 g/l of sodium acetate, and 2 g/l of ferrous citrate; the pH of the medium was 4. Strain 2707 was grown on medium 1 supplemented with 0.5 g/l of yeast extract under an atmosphere of 100% CO; the pH of the medium was 6.8. Enrichment Kar was grown on medium 1, pH 7.0, under an atmosphere of CO. Enrichment GB2 was grown on an earlier described medium [12].

All pure and enrichment cultures were grown at 60°C, except for enrichments K44, 432, 416, and Kar (70°C), strain 2707 (80°C), and strain 711 (55°C).

Isolation and amplification of DNA. Cultures (10 ml) were centrifuged at 12000 *g* for 15 min, and DNA was isolated from the cell sediment by the Marmur method [18]. An alternative method of cell disintegration was boiling for 5 min in D buffer (4 M guanidine isothionate, 2.5 mM Na citrate, 0.1 M β-mercaptoethanol, 0.5% sarcosyl, pH 7). DNA was extracted from supernatant with a phenol–chloroform (1 : 1) mixture and then twice with chloroform and precipitated with ethanol. The precipitate was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4). The DNA isolated was used in a volume of 1 μl (1–10 ng) as the template for PCR.

Amplification of the template DNA was performed with the bacterial forward primer Bact 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal reverse primer Univ 1492R (5'-TACGGYTACCTTGTTACGACTT-3') [19]. PCR was run in a Model 80 Perkin Elmer Cetus DNA Thermal Cycler or in a multichannel Tertsik DNA amlifier (DNK-Tekhnologiya, Russia). The reaction mixture (50 μl) contained PCR buffer (Fermentas, Lithuania, 1×; 75 mM Tris–HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01 vol % Tween 20); 1.5 mM $MgCl_2$; dNTP, 200 μM each; primers, 0,5 μM each; 2.5 U of Taq polymerase (Fermentas, Lithuania, #FP0072), and 1–10 ng of template DNA. The reaction mixture was covered with a layer of mineral oil. The temperature program of the reaction was as follows: 5 min at 94°C; then 34 cycles of denaturation at 94°C; for 50 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min 50 s; and final extension at 72°C for 10 min.

The molecular weight of PCR products was determined by electrophoresis in 1% agarose gel (TAE buffer), stained with ethidium bromide (0.5 μg/ml), in the presence of a molecular weight marker. The electrophoresis was run at 4 V/cm. The gel was photographed in UV light with a wavelength of about 300 nm.

Outlining the target group for design of oligonucleotide probes. To outline the target group, we retrieved from GenBank nucleotide sequences of 16S rDNAs of all 14 valid *Thermoanaerobacter* species and of the type species of the genera placed in the RDP release 8.0 database ([20]; http://rdp.cme.msu.edu/html/) in the group 2.30.2 THERMOANAEROBACTER_AND_RELATIVES. In addition, we performed a search in GenBank with the use of the BLASTN software (http://www.ncbi. nlm.nih.gov/BLAST/) for nucleotide sequences of 16S rDNA having a high homology with the sequences of the type species of the genus *Thermoanaerobacter*, *T. ethanolicus*, or with sequences of recently described species that are relatively remote from it, *T. mathranii* and *T. tengcongensis*. The thus-revealed closely related sequences belonging to validly described organisms were also used for the construction of a phylogenetic tree.

Alignment of nucleotide sequences was performed using the MultAlin software (http://prodes.toulouse.inra.fr/multalin/). Further construction of the phylogenetic tree was carried out with the help of the TREECONW 1.3b software package [21] using the correction of Jukes & Cantor for the estimation of evolutionary distances, the neighbor-joining method for inferring tree topology, and bootstrap analysis of 100 samples for evaluation of the statistical significance of the branching order.

Design of oligonucleotide probes. The consensus sequence of the 16S rRNAs of the target group members was found using MultAlin software, and the search for probes was performed using an original software package, ProbeDesigner, compatible with a DOS or Windows environment (Lebedinsky, manuscript in preparation). In the present paper, we briefly describe the algorithms of the component programs of the package and the possibilities they offer. During the search for probes, consensus sequence fragments of a certain length taken with a step of one nucleotide are checked for their specificity with respect to the target group with the help of oligonucleotide vocabularies that are created in advance proceeding from the 18 331 sequences of 16S and 18S rRNAs from the files SSU_Prok.gb and SSU_Euk.gb of the RDP release 8.0 database. During the formation of these vocabularies, 25-meric oligonucleotides were taken with a step of one nucleotide from each sequence present in the database; each oligonucleotide was placed in two vocabularies. The position of an oligonucleotide in the first vocabulary is determined by the seven nucleotides in positions 3–9 (hereafter, seven 5'-subterminal nucleotides), and its position in the second vocabulary is determined by the seven 3'-subterminal nucleotides (for 25-mers, positions 17–23). The information on the organism to which the oligonucleotide belongs is put in the adjacent field of the record.

When the properties of a consensus sequence fragment are examined, a degenerated fragment is considered as a mixture for nondegenerated oligonucleotides, and each of the components of the mixture is checked in turn. The search for identical or similar oligonucleotides is conducted only among oligonucleotides having an identical sequence of seven 5'- or 3-'subterminal nucleotides. The fact that oligonucleotides with particular sequences of subterminal nucleotides occupy definite positions in the vocabularies allows the search for similar oligonucleotides to be accelerated $4^7/2$-fold (i.e., more than 8000-fold) as compared to a straightforward search among all oligonucleotides.

The comparison with vocabulary oligonucleotides is conducted with taking into account their origin (from target or nontarget sequences) and encompasses counting the number of mismatches between the probe complementary to the analyzed fragment and the vocabulary oligonucleotide and calculation, according to two models, of the melting temperatures of the duplexes formed. The tendency of the candidate probes to form hairpins and self-dimers is also assessed.

As a result, a list of probes is obtained, which are ranged according to certain parameters such as percent hit of target sequences; minimal number of mismatches with nontarget sequences; minimal difference between the melting temperature of the perfect duplex and the duplexes formed with nontarget sequences (the melting temperatures and their difference are calculated according to two models); the tendency to form hairpins and self-dimers.

The described method of comparison of consensus sequence fragments with vocabulary oligonucleotides has an intrinsic restriction: it does not allow comparison to be made with vocabulary oligonucleotides that differ from the fragment by one nucleotide in the 5'-subterminal seven plus by one nucleotide in the 3'-subterminal seven; hence, the method may underestimate the affinity of the probe to nontarget sequences. Therefore, the candidate probes stated at the first stage to possess the best characteristics are subjected to direct verification that involves determination of the reactions of the probe with each of the sites (taken with a one-nucleotide step) of each of the 18 331 SSU rRNA sequences from the files SSU_Prok.gb and SSU_Euk.gb of the RDP release 8.0 database. The minimal number of mismatches with nontarget sequences and the minimal difference between the melting temperatures of the perfect duplex and the duplexes formed with nontarget sequences are repeatedly calculated (when two or three probes are to undergo final verification, the duration of this straightforward procedure is acceptable).

Additionally, the probes designed were analyzed with respect to their specificity using the BLASTN NCBI facility and with respect to their tendency toward hairpin and self-dimer formation using the OligoAnalyzer 2.5 program (http://www. idtdna.com).

The oligonucleotides were synthesized by the company Syntol (Russia; http://www.syntol.ru), with purification in polyacrylamide gel. The probes were labeled with Dig-11-dUTP, which was introduced by the 3'-tailing method in an enzymatic reaction catalyzed by terminal deoxynucleotidyl transferase (Fermentas, Lithuania, #EP0162) and carried out according to the manufacturer's recommendations, by incubation for 15 min at 37°C of 20 μl of a reaction mixture containing 1× buffer for transferase (200 mM Ca cacodylate, pH 7.2, 1 mM $CoCl_2$, 0.1 mM DTT, and 0.01 vol % Triton X-100), 0.05 mM DIG-11-dUTP, 5 pmol/ml of the probe, 0.5 mM dATP, and 2.5 U/μl of deoxynucleotidyl transferase. The reaction was terminated by adding 2 μl of 0.5 M EDTA (pH 8.0), and the probes were stored at –20°C.

Hybridization of oligonucleotide probes and signal detection were performed according to protocols from a Boehringer Manheim laboratory manual ([22]; http://www.roche-applied-science.com/fst/products.htm?/prod_inf/manuals/dig_man/dig_toc.htm) with slight modifications; all reagents were from Boehringer Manheim. PCR products obtained on the studied and control DNA were transferred by the Southern method to a positively charged nylon membrane, fixed on the membrane by UV light for 3–4 min, and kept for 2 min in 6× SSC (1× SSC contains 150 mM NaCl and 15 mM Na citrate, pH 7.0). After that, membrane prehybridization was performed to prevent nonspecific sorption of

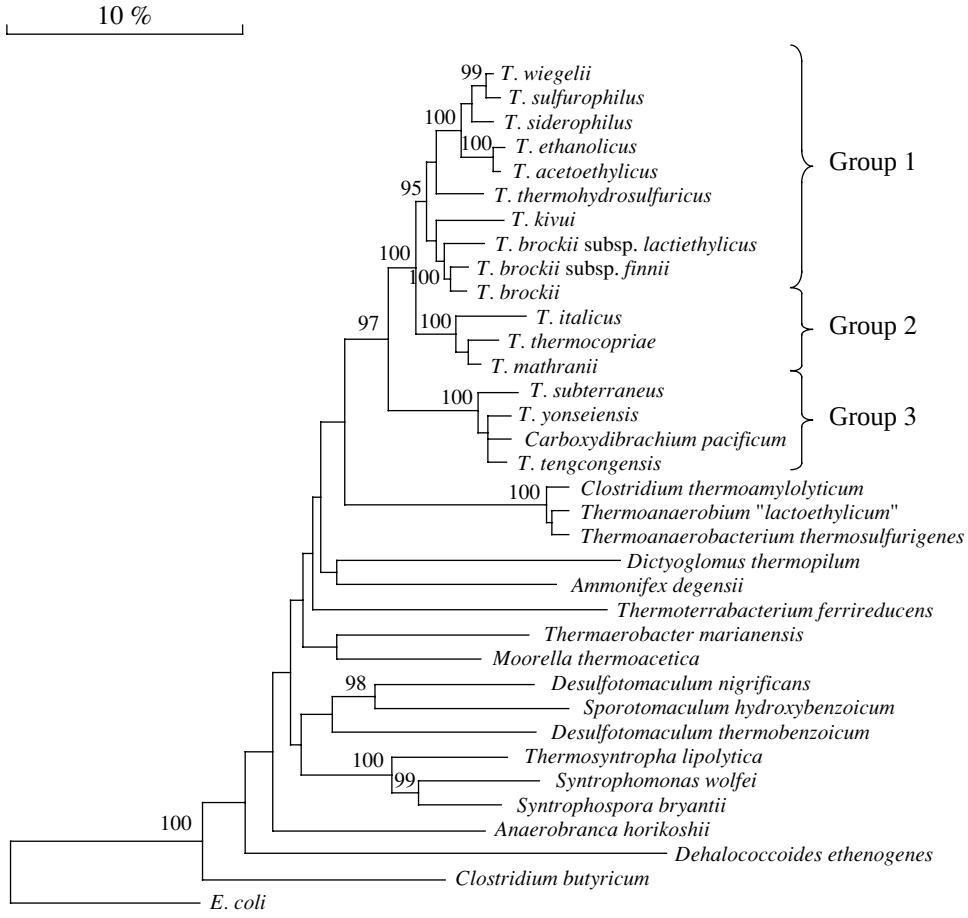

Fig. 1. Phylogenetic position and structure of the genus *Thermoanaerobacter*. The tree was constructed based on the analysis of nucleotide sequences of 16S rDNA. Bootstrap values lower than 95 are not shown.

the oligonucleotide probe: membranes were incubated in prehybridization buffer (5× SSC, 0.1% *N*-lauroyl sarcosine, 0.02% SDS, 1% Blocking reagent, and 100 μg/ml poly(A) or pancreatic DNA; 20 ml of prehybridization buffer per 100 cm^2 of the membrane). Prehybridization was carried out for 2 h at a temperature equal to the melting temperature of the probe, T_m, which was calculated according to the formula T_m = 81.5 + 16.6 logM + 0.41[%(G+C)] – 820/n.

Then, the prehybridization solution was replaced with an analogous solution that contained, instead of poly(A) or pancreatic DNA, 5 pmol/ml of labeled probe (the probe stock solution was preheated for 3 min at 70°C to eliminate possible self-dimers). The hybridization solution was applied in an amount of 3 ml per 100 cm^2 of the membrane. Hybridization was carried out for 12 h at a temperature calculated by the formula $T_{hyb} = T_m - 10°C$.

Then, the membrane was washed to remove unbound or nonspecifically bound probe. The optimal temperature of washing was found experimentally. To wash off the probe of negative controls, the membrane was first washed under low stringency conditions: at room temperature, first in 2× SSC + 0.5% SDS for 5 min, then in 1.5× SSC + 0.1% SDS for 15 min, and then in 0.5× SSC + 0.1% SDS for 15 min (40 ml of each solution per 100 cm^2).

After that, signal detection was carried out as follows. The membrane was washed two times in washing buffer (20 ml/100 cm^2; 100 mM disodium maleinate, 150 mM NaCl, pH 7.5, 0.3 vol % Tween 20). Then, the membrane was incubated in blocking solution (20 ml/100 cm^2; 100 mM disodium maleinate, 1% Blocking reagent), after which this solution was removed and the membrane was incubated for 30–60 min in a solution (20 ml/100 cm^2) prepared by adding the Fab fragments of antibodies against digoxygenin, bound with alkaline phosphatase, (Anti-Dig-AP, Fab fragments) to the washing buffer in a ratio of 1 : 20000. After that, the membrane was incubated twice for 15 min in washing buffer (20 ml/100 cm^2) and placed for 2 min in detection buffer (20 ml/100 cm^2, 100 mM Tris–HCl, 100 mM NaCl, pH 9.5). Then, the membrane was placed in a plastic bag. After that, 1% solution of chemiluminescent substrate (CDP-star) in detection buffer was applied to the membrane (0.5 ml/100 cm^2). To record the signal emitted by the labeled probe, the membrane was kept in the

Table 1. Oligonucleotide probes targeting the genus *Thermoanaerobacter* and the three groups of its species

Target group	Probe	
	designation	nucleotide sequence, 5' → 3'
The genus *Thermoanaerobacter*	Tab 827	GCTTCCGCDYCCCACACCTA
First group of species of the genus	Tab_1 844	TTAACTACGGCACGRAATGCTTC
Second group of species of the genus	Tab_2 424	CACTAMYGGGGTTTACAACC
Third group of species of the genus	Tab_3 184	TCCTCCATCAGGATGCCCTA

Table 2. Hybridization of oligonucleotide probes with PCR products obtained on DNA of reference strains with primers Bact 8F and Univ 1492R

Organism	PCR	Hybridization with probes			
		Tab 827	Tab_1 844	Tab_2 424	Tab_3 184
Thermoanaerobacter group 1					
Thermoanaerobacter siderophilus DSM 12299^T*	+	+	+	–	–
Thermoanaerobacter sulfurophilus DSM 11584^T *	+	+	+	–	–
Thermoanaerobacter wiegelii DSM 10319^T (DSMZ)	+	+	+	–	–
Thermoanaerobacter brockii DSM 1457^T *	+	+	+	–	–
Thermoanaerobacter, group 2					
Thermoanaerobacter mathranii DSM 11426^T (DSMZ)	+	+	–	+	–
Thermoanaerobacter italicus DSM 9252^T (DSMZ)	+	+	–	+	–
Thermoanaerobacter, group 3					
Carboxydibrachium pacificum DSM 12653^T*	+	+	–	–	+
Thermoanaerobacter yonseiensis DSM 13777^T (DSMZ)	+	+	–	–	+
Other bacteria					
Moorella glycerini DSM 11254^T*	+	–	–	–	–
Thermoterrabacterium ferrireducens DSM 11255^T*	+	–	–	–	–
Thermotoga maritima DSM 3109^T*	+	–	–	–	–

Note: "+" signifies positive reaction and "–" means lack of positive reaction.
* The collection of the Laboratory of Hyperthermophilic Microbial Communities, Institute of Microbiology, Russian Academy of Sciences.

dark in contact with an X-ray film (RT-1V, Svema, Russia). After exposure, the film was developed according to the manufacturer's recommendations.

After that, the same membranes were washed under high stringency conditions: at a temperature equal to T_m – 10°C, first in 0.2× SSC + 0.1% SDS for 5 min and then in 0.2× SSC for 1 min (40 ml of each solution per 100 cm^2). Then, the detection procedure was repeated.

Sequencing of 16S rDNA was performed using an ABI PRISM® BigDye™ Terminator v. 3.0 kit with analysis of the reaction products on an ABI PRISM 3100-Avant automatic sequencer.

RESULTS

Outlining the target group for design of oligonucleotide probes. Our search in GenBank with the use of the BLASTN software for nucleotide sequences of 16S rDNA having a high homology with the sequences of the type species of the genus *Thermoanaerobacter, T. ethanolicus*, or with sequences of the recently described species relatively remote from it, *T. mathranii* and *T. tengcongensis*, revealed (apart from valid species of *Thermoanaerobacter*) a number of invalid species, poorly characterized cultures, and clones. In addition, this search revealed a validly described species closely related by its 16S rDNA nucleotide sequence to the genus *Thermoanaerobacter*, namely *Carboxydibrachium pacificum*, which is a gram-positive thermophile capable of anaerobic oxidation of carbon monoxide with the production of hydrogen [12]. The phylogenetic position and structure of the genus *Thermoanaerobacter* and the position of *Carboxydibrachium pacificum*, investigated with the use of the TREECONW software package, are shown in Fig. 1. It can be seen that the genus *Thermoanaerobacter* is well separated from other genera and consists of three phylogenetic subgroups, one of which includes recently

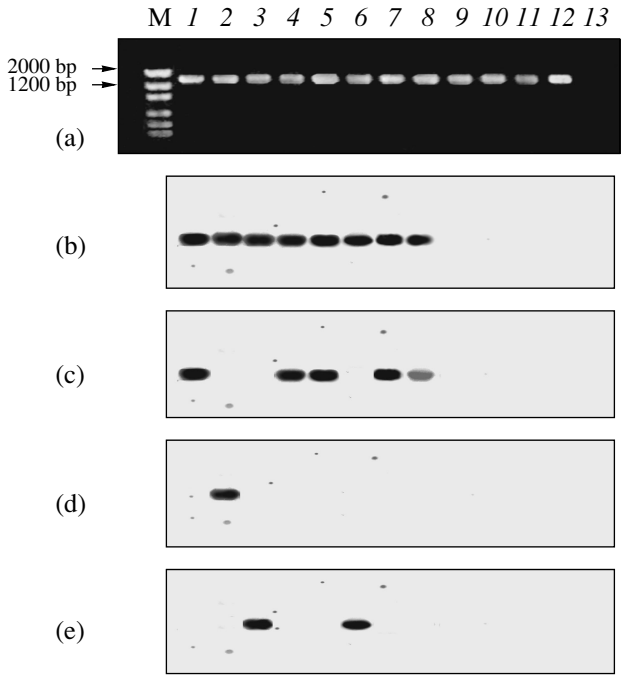

Fig. 2. Identification of members of the genus *Thermoanaerobacter* after (a) agarose gel electrophoresis of PCR products obtained with the primers Bact 8F and Univ 1492R with (b) the genus-level probe Tab 827 and group-specific probes (c) Tab_1 844, (d) Tab_2 424, and (e) Tab_3 184. Lanes: (M) molecular weight marker (2000, 1200, 800, 400, 200, and 100 bp); (*1*) *T. sulfurophilus*; (*2*) *T. italicus*; (*3*) *Carboxydibrachium pacificum*; (*4*) strain B5; (*5*) strain K14; (*6*) strain K67; (*7*) strain 518; (*8*) strain 711; (*9*) strain 761; (*10*) enrichment 816; (*11*) *Moorella glycerini*; (*12*) *Thermoterrabacterium ferrireducens*; (*13*) H_2O.

described species of *Thermoanaerobacter* and *Carboxydibrachium pacificum*. The main inference from the phylogenetic tree presented in Fig. 1 is that the probe targeting the genus Thermoanaerobacter should also target *Carboxydibrachium pacificum*.

The data on invalid species, poorly characterized cultures, and clones shown by BLASTN to be closely related to *Thermoanaerobacter* representatives were not used for the construction of the phylogenetic tree. However, this information was further used to distinguish between nonspecific reactions of the designed probes and reactions determined by phylogenetic relatedness.

Design of oligonucleotide probes. The probes that we designed (Table 1) targeted the genus *Thermoanaerobacter* and three groups of species within this genus. According to the results of the in silico analysis, each of the probes designed should form perfect duplexes with 16S rRNA of all validly described representatives of its target group and, at the same time, exhibit at least two nonterminal mismatches with nontarget organisms.

To verify the specificity of the probes, they were hybridized with *Thermoanaerobacter* spp. strains belonging to different groups and with strains of *Moorella* and *Thermoterrabacterium*, which are genera related to *Thermoanaerobacter*. The results of hybridizations confirmed the specificity of the probes (Fig. 2, Table 2).

Identification of thermophilic bacterial strains. Hybridization of probes targeting the genus *Thermoanaerobacter* and the three groups within it allowed several strains to be identified (Table 3). The three strains capable of agarose hydrolysis were identified as *Thermoanaerobacter* representatives; two of them belonged to the first group of *Thermoanaerobacter* species (strains B5 and K14), and one belonged to the third group (strain K67). A partial nucleotide sequence of the 16S rRNA gene of strain K67 was determined (between *E. coli* positions 1042 and 1434; GenBank accession number AY280871), and its analysis confirmed the affiliation of this strain with the third group of *Thermoanaerobacter* species (97% identities with 16S rDNA of *T. tengcongensis* according to BLASTN). Both of the strains isolated from deep-sea hydrotherms were identified as representatives of the first group of *Thermoanaerobacter* species. One of the two freshwater strains able to grow at low pH values belonged to the first group of *Thermoanaerobacter* species (strain 711), whereas the DNA of the other strain (761) failed to positively react with the genus-level probe. Strain 2707, a CO-utilizing anaerobe, turned out to be a representative of the third group of *Thermoanaerobacter* species.

Detection of *Thermoanaerobacter* representatives in enrichment cultures. Using the probe targeting *Thermoanaerobacter*, we detected representatives of this genus in one of the three iron-reducing enrichments (K44). However, in the two other iron-reducing enrichments tested *Thermoanaerobacter* representatives were not detected. *Thermoanaerobacter* representatives were also not detected in enrichment 816 grown at pH 3.5 or in the CO-utilizing enrichment Kar.

DISCUSSION

Molecular methods have been widely used for the analysis of natural microbial communities, including thermophilic ones [23]. In the present work, we used hybridization with oligonucleotide probes for express analysis of pure microbial cultures and enrichments. This method allowed us to assign several strains to the genus *Thermoanaerobacter*; moreover, these strains could be differentiated in terms of their affiliation to one of the three intrageneric groups of species. The results obtained extend our knowledge on the distribution of *Thermoanaerobacter* and on the physiology of this genus. Up to now, *Thermoanaerobacter* representatives have not been found in deep-sea hydrotherms [23]; the only exception is the closely related *Carboxydibrachium pacificum*. Both of the marine organotrophic East Pacific Rise isolates that we tested proved to belong to the genus *Thermoanaerobacter*.

Table 3. Hybridization of oligonucleotide probes with PCR products obtained on DNA of pure unidentified cultures and enrichments with primers Bact 8F and Univ 1492R

Culture	Source	Phenotypic peculiarities	PCR	Hybridization with probes			
				Tab 827	Tab_ 1 844	Tab_ 2 424	Tab_ 3 184
Strains							
B5	Baikal	Hydrolysis of agarose	+	+	+	–	–
K14	Kamchatka	Hydrolysis of agarose	+	+	+	–	–
K67	Kamchatka	Hydrolysis of agarose	+	+	–	–	+
107	Deep-sea hydrotherm	Growth on marine medium	+	+	+	–	–
518	Amistad Slope, West Pac	Growth on marine medium, pH optimum 6.0, pH minimum 3.8	+	+	+	–	–
711	Geyser valley, Kamchatka	Temperature optimum 55°C, pH optimum 5.7, pH minimum 3.8	+	+	+	–	–
761	Orange thermal field, Kamchatka	Temperature optimum 60°C, pH optimum 5.0, pH minimum 3.2	+	+	ND	ND	ND
2707	Kunashir Island	Anaerobic growth on CO with H_2 production	+	+	–	–	+
Enrichments							
GB 2	Guaymas Basin	Anaerobic growth on CO with H_2 production	+	–	ND	ND	ND
Kar	Kamchatka	Anaerobic growth on CO with H_2 production	+	–	ND	ND	ND
816	Mutnovski volcano, Kamchatka	Growth at pH 3.5	+	–	ND	ND	ND
746	Orange thermal field, Kamchatka	Temperature optimum 60°C, pH optimum 4, pH minimum ND	+	–	ND	ND	ND
K44	Geyser valley, Kamchatka	Fe(III) reduction in the presence of H_2	+	+	ND	ND	ND
416	Kurile Islands	Growth on acetate in the presence of Fe(III)	+	–	ND	ND	ND
432	Kurile Islands	Fe(III) reduction	+	–	ND	ND	ND

Note: "+" signifies positive reaction; "–" means lack of positive reaction; "ND" stands for "not determined."

We also found three *Thermoanaerobacter* representatives to exhibit a capacity for agarose hydrolysis, a property revealed in thermophilic anaerobes for the first time. This property was not specific fo a particular group of *Thermoanaerobacter* species but occurred both in the first and third groups.

All of the currently recognized species of *Thermoanaerobacter* are neutrophiles with a minimum growth pH of 4.7 [6]. We established an affiliation to *Thermoanaerobacter* of two anaerobic acidophilic strains capable of growth at pH 3.5 (strain 518 isolated from a deep-sea hydrotherm and strain 711 isolated from a Kamchatka hydrotherm).

The capacity for anaerobic utilization of CO with hydrogen formation is characteristic of many thermophilic prokaryotes [17]. Most of these organisms belong to gram-positive bacteria with a low G+C content. The results of our present work show that strain 2707, a rod-shaped anaerobic bacterium able to grow both organotrophically and at the expense of CO oxidation and isolated from a hydrotherm on Kunashir Island, belongs to the third group of species of the genus *Thermoanaerobacter.* This group consists of recently described species isolated from hydrotherms and characterized by extremely high growth temperatures (*T. yonseiensis*, *T. tengcongensis*). It also includes *Carboxydibrachium pacificum*, which was isolated from a deep-sea hydrotherm of the Okinawa trough and described as the sole representative of a new genus [12]. This marine non-spore-forming bacterium with branching cells is capable of anaerobic growth on CO with hydrogen formation. Thus, it does not seem accidental that one of the strains that we identified as a rep-

resentative of the third group of *Thermoanaerobacter* species is also capable of anaerobic growth on CO with the production of hydrogen. At the same time, we failed to obtain growth on CO of such representatives of the third group of *Thermoanaerobacter* species as *Thermoanaerobacter yonseiensis* and strain K67. Further investigations are needed to clarify what should be done to bring into order the taxonomy of the third group of *Thermoanaerobacter* species, which currently includes *Carboxydibrachium pacificum* and one additional carboxydotroph (strain 2707).

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