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Occurrence and Detection of *Thermoanaerobacterium* and *Thermoanaerobacter* in Canned Food

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Summary

In order to determine the reason for loss of vacuum in canned food, obligately anaerobic, spore forming thermophilic organisms were isolated from shelf-stable canned food containing vegetables, noodles and potatoes as main ingredients. Thermophilic bacteria from 44 canned food samples that had been stored under anaerobic conditions at 37 °C for at least 7 days were isolated. In addition, organic fertilizer used for the cultivation of some of the foods' ingredients was examined and anaerobic, thermophilic bacteria could also be isolated from this source. Identification of bacterial strains was carried out by partial and complete 16S-rRNA-gene sequencing. Some of the obtained gene sequences showed a high level of similarity to existing 16S-rRNA gene sequences towards strains of the genera *Thermoanaerobacter*, *Thermoanaerobium* and *Thermoanaerobacterium* respectively, which have not yet been reported to be of importance as food spoilers. In the course of identification of these thermophilic bacteria we developed genera specific PCR-based approaches for detecting isolates belonging to the genera *Thermoanaeroacterium* and *Thermoanaerobacter*. Direct capturing of free DNA from contaminated samples using oligonucleotides coupled with paramagentic beads allowed the reduction of the detection time to six hours with a lower limit of 10^4 cells/mL.

Key words: thermophilic bacteria, canned food, anaerobic thermophiles, PCR, *Thermoanaerobacter*, *Thermoanaerobacterium*

Introduction

In the last two decades much research has been carried out on thermophilic bacteria. In particular enzymes isolated from thermophilic organisms have gained much attention, since these proteins are heat stable and do not easily denature at higher temperatures (*1,2*). Therefore those enzymes are of potential scientific and commercial interest. In the course of screening for possible biotechnological applications of thermophilic bacteria, a closer look was also taken on the taxonomy of relevant genera. Extensive isolation studies on thermophiles from a variety of environmental sources have been carried out (*3, 4*). As a result a number of species had to be reclassified and some new genera have emerged. In particular the genus *Clostridium* has undergone substantial rearrangements (*5,6*). A vast amount of new thermophiles has been isolated from a number of biotopes, among them hot springs as well as compost and soil (*7–10*). While some reclassified thermophiles like *Thermoanaerobacterium thermosaccharolyticum*, formerly known as *Clostridium thermosaccharolyticum* (*11*), have been recognized as food spoilers, most of the recently described thermophilic organisms were not known to be of any relevance in the food industry as spoilage organisms. In this paper we demonstrate the occurrence of strictly anaerobic thermophilic bacteria belonging to the genera *Thermoanaerobacter*, *Thermoanaerobacterium* and *Thermoanaerobium* in

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canned food. In addition a fast detection method for the genus specific identification of the first mentioned genera is presented.

Material and Methods

Bacterial strains and culture conditions

Bacterial strains were isolated from spoiled canned food samples, containing potatoes, noodles, vegetables and tomato puree as main ingredients. Main characteristic of spoilage was loss of vacuum and decrease in pH of approximately 1 unit. Thermophilic strains were also isolated from organic fertilizer samples used in cultivation of some of the foods' ingredients. Reference strains were acquired from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). As reference strains, *Thermoanaerobacterium aotearoense* DSM 8692, *Thermoanaerobacterium saccharolyticum* DSM 7060, *Thermoanaerobacterium xylanolyticum* DSM 7097, *Thermoanaerobacterium thermosulfurigenes* DSM 2229, *Thermoanaerobacterium thermosaccharolyticum* DSM 571, *Thermoanaerobium lactoethylicum* DSM 9003, *Thermoanaerobacter ethanolicus* DSM 2246, *Thermoanaerobacter thermohydrosulfuricus* DSM 567 and *Thermoanaerobacter brockii* DSM 1457 were used. The medium used for enrichment was Oxoid Schaedler media (Oxoid, Nasingstoke, Hampshire, England). pH was adjusted to 7.5 and the broth was autoclaved at 121 °C for 20 min. Unless otherwise stated, all cultures were incubated at 37 °C for at least 7 days and at 65 °C for 24 to 72 hours in 10 mL culture tubes under anaerobic conditions (30 % N_2, and 70 % CO_2).

Isolation of bacteria

One gram of food sample was transferred under anaerobic conditions to a culture tube containing 10 mL of liquid Schaedler broth. After incubation, the enrichment cultures were individualized by dilution on solidified media (10 g purified Oxoid agar Agar per 1 L Schaedler broth). Single colonies were picked and transferred at least twice to 10 mL culture tubes with liquid media and incubated for 24 to 72 hours. Pure cultures were stored at –80 °C in a glycerol-medium mixture (50:50 w/v).

DNA isolation

Cells for DNA isolation were harvested by centrifugation at 5000 g for 10 min and subsequently washed twice with saline-EDTA-buffer (0.15 M NaCl, 0.01 M EDTA, pH=8.0). Genomic DNA was isolated from washed cells according to Mamur (*12*). DNA purity was determined by the ratio of absorbance at 260 nm and absorbance at 280 nm.

Sequencing and analysis of 16S-rRNA genes sequence data

16S-rRNA genes were amplified using the primers 616VII and 630R (see Table 1). Amplified DNA products were purified with the Quiagen QIA-quick PCR purification Kit (Quiagen, Hilden, Germany) and subsequently sequenced using a Thermo Sequenase fluorescent labeled primer sequencing kit with 7-deaza-dGTP (Amersham Life Science, Buckinghamshire, England). For partial sequencing the oligonucleotide 610RII was used. Complete 16 S-rRNA gene sequencing was carried out with the primers 606RII, 607V, 609RII, 610RII and 612RII. The properties of all primers are summarized in Table 1.

Table 1. Oligonucleotides used for 16S-rRNA gene amplification and sequencing.
Primers 616VII and 630R were used as capture probes with biotinylated 5´-termini (see Material and Methods)

Primer	5' – sequence – 3'	Application	Binding position[a]
606RII	TRACGGSCTGTGTGTACA	sequencing	1390
616VII	AGAGTTTGATYMTGGCTCAG	PCR/sequencing	8
630R	CAKAAAGGAGGTGATCCAGC	PCR/sequencing	1526
607V	GGGCTACACACGTGC	sequencing	1220
609RII	ACTACYVGGGTATCTAAKCC	sequencing	784
610RII	ACCGCKRCTGCTGGCAC	sequencing	514
612RII	GTAAGGTTYTNCGCGT	sequencing	968

[a] The positions shown in the table correspond to their locations on the rRNA of *E. coli* (*13*)

Sequencing electrophoreses was performed on an ALFexpress Sequencer (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The alignment of 16S-rRNA gene sequences was performed using the ARB software (*15*), which contains a set of representative 16S-rRNA gene sequences of the genera *Clostridia*, *Themoanaerobacterium*, *Thermoanaerobacter* and *Thermoanaerobium*. In addition online analysis was carried out on the website of the Ribosomal Database Project II (*16*).

PCR assay

Development of genus specific PCR

Genus specific PCR assays were developed for the identification of the two genera *Thermoanaerobacter* and *Thermoanaerobium*. For the *Thermoanaerobacter* specific PCR primers (Table 1 and Table 2), previously described by Erbeznik *et al.* (*14*), were modified by shortening Table 1 for eight and Table 2 for five nucleotides, respectively.

Primers ThmV1 and ThmR1 used for the detection of the genus *Thermoanaerobacterium* were newly designed by comparative sequence analysis of hitherto avail-

Table 2. Primer for genus specific PCR for the detection of *Thermoanaerobacterium* and *Thermoanaerobacter* targeted against the 16S-rDNA

Primer	5' – sequence – 3'	target organism	Nucleotide position[a]
ThmV1	GAAGGGAGTACTACGGTAC	*Thermoanaerobacterium*	68
ThmR1	TATGGTACCGTCATTTCTTT		451
Table 1[b]	TGGATAAGCTCCTTGTAGGGC	*Thermoanaerobacter*	176
Table 2[b]	TCCTTTCGGCTCGCTACTACCTGC		999

[a]The positions shown in the table correspond to their locations on the rRNA of *E. coli* (*13*);
[b]primers originally developed by Erbeznik *et al.* (*14*), but have been modified to meet the requirements

able 16S-rRNA sequences of known species of this genus (for primer sequences see Table 2). PCRs were carried out using an Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany). The reaction mixture (final volume 100 μL) consisted of 0.5 μL ThmV1 Primer (10 pmol/μL), 0.5 μL ThmR1 primer (10 pmol/μL), 10 μL 10x reaction buffer (containing 1.5 mM $MgCl_2$), 1 μL deoxynucleoside triphosphate (Pharmacia-Biotech, 10 mM each), 1,0 μL DNA solution and 1 U Taq polymerase (Boehringer, Mannheim, Germany). A mastermix of all components (except DNA samples and Taq polymerase) was prepared. The following temperature profile was used for both specific reactions: initial denaturation 94 °C for 4 min, addition of 1 U Taq polymerase, followed by 94 °C for 45 s; 61 °C for 1 min and 72 °C for 45 s for 24 cycles. A final cycle at 94 °C, 45 s and 72 °C, 4 min was performed. The amount of 25 μL of the amplicons were electrophoresed on a 1.5 % agarose gel in 0.5 % TBE buffer and stained in an 0.1 % ethidium bromide solution.

Isolation of free bacterial DNA with paramagnetic particles and capture probes

The Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs) were obtained from Promega, Mannheim, Germany. All food samples needed to be diluted to extract a reasonable amount of bacterial DNA. Five hundred microliter of food sample were diluted in 500 μL sterile water. For the annealing of the biotinylated eubacterial-specific 16S-rRNA oligonucleotides (616VII and 630R, see Table 1) to free 16S-rDNA and capturing of these hybrids to streptavidin-coated paramagnetic particles the tube containing the diluted food sample was placed in a thermoblock at 65 °C for 15 minutes. Three microliter of the 5'-biotinylated oligonucleotides (100 pM) as well as 13 μL of 20 × SSC buffer (87.7 g NaCl and 44.1 g trisodium citrate dihydrate were dissolved in 400 mL nuclease free water, pH=7.2) was subsequently added to the food sample. After cooling for 10 min at room temperature the entire content of the tube was added to the pre-washed SA-PMPs (washing of the paramagnetic particles was performed according to the manufacturer's manual). To enable the formation of a SA-PMP-oligonucleotide-DNA complex, the mixture was incubated at room temperature for at least 30 minutes. Incubation time depended on the kind of food sample and had to be optimized separately for each kind of sample. For best results, incubation time was 50 minutes. SA-PMPs were then captured in a magnetic stand for 45 seconds. The supernatant was carefully removed, without disturbing the SA-PMPs. Subsequently the particles were washed and captured four times with 0.1 × SSC (300 μL per wash). To elute the captured bacterial DNA, 100 μL of sterile preheated water (50 °C) were added the SA-PMPs and particles were resuspended by gently flicking the tube. Following this, the SA-PMPs were magnetically captured and the eluted DNA aqueous phase was transferred to a sterile tube. This elution step was repeated and the eluates were pooled. The purified DNA was subjected to PCRs as described above.

Results

Forty-four samples of spoiled canned food or food related sources were screened for microbial growth under strictly anaerobic conditions. The examined food cans were characterized by loss of vacuum and decrease in pH value. We were able to identify 33 anaerobic thermophiles from the examined samples. In the first step, isolation was executed by means of enrichment cultures. Optimal growth temperature for all isolates was between 60 and 65 °C. Identification of 14 isolates was carried out by comparative sequence analyses of partial (500 bp on average) and complete 16S-rRNA gene sequences towards sequences of reference strains. A list of all isolates identified by sequence comparison as well as the isolates' origin is presented in Table 3.

Eight of these isolates could be assigned to the species *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium thermosaccharolyticum* and *Thermoanaerobacterium thermosulfurigenes* by means of database comparison. A single isolate was identified as *Thermoanaerobium lactoethylicum* but could not be found elsewhere. Therefore, further studies were concentrated on the occurence of organisms belonging to the genera *Thermoanaerobacter* and *Thermoanaerobacterium*. Furthermore, the similarity values of five additional isolates did not match with any reference sequence. Thus, a doubtless allocation to any existing species was not possible. These bacteria originate from food samples with different ingredients like spinach (TMW 2.457), tomato puree (TMW 2.437), or potatoes (TMW 2.430, TMW 2.433 and TMW 2.345). Strains TMW 2.437 and TMW 2.457 showed their highest level of sequence similarity towards the extreme thermophile *Thermoanaerobacterium aotearoense,* which was originally isolated from a hot spring in New Zealand (*17*), but it is not known to be of any importance in food preservation. TMW 2.345, TMW 2.430 and TMW 2.433 present their highest similarity values towards *Thermoanaerobacterium saccharolyticum* which originated from geothermal

Table 3. Isolates from food samples identified by comparative 16S-rRNA gene sequence analyses

Strain	Identified as	Main food ingredient or source of organism
TMW 2.343	*Thermoanaerobacterium saccharolyticum*	potatoes
TMW 2.345	*Thermoanaerobacterium* spp.*	potatoes
TMW 2.350	*Thermoanaerobium lactoethylicum*	potatoes
TMW 2.430	*Thermoanaerobacterium* spp.*	potatoes
TMW 2.433	*Thermoanaerobacterium* spp.*	potatoes
TMW 2.437	*Thermoanaerobacterium* spp.*	tomato puree
TMW 2.450	*Thermoanaerobacterium thermosaccharolyticum*	noodles/vegetables
TMW 2.451	*Thermoanaerobacterium thermosaccharolyticum*	noodles/vegetables
TMW 2.457	*Thermoanaerobacterium* spp.*	spinach
TMW 2.463	*Thermoanaerobacterium thermosulfurigenes*	potatoes
TMW 2.464	*Thermoanaerobacterium thermosulfurigenes*	potatoes
TMW 2.465	*Thermoanaerobacterium thermosulfurigenes*	spinach
TMW 2.466	*Thermoanaerobacterium thermosaccharolyticum*	organic fertilizer
TMW 2.468	*Thermoanaerobacterium thermosaccharolyticum*	organic fertilizer

* similarity values of 16S sequences do not match more than 98.6 % towards reference sequences of valid type species

sites in Wyoming, USA (*5*) and is also not known as food relevant organism up to now.

Based on these results and in combination with 16S-rRNA gene sequences of reference strains a genus specific PCR for the identification of *Thermoanarobacter* and *Thermonaerobacterium* was developed. Primer Table 1 and Table 2 were used for detection of *Thermoanaerobacter* strains, with a length of 850 bp for the amplification product (Fig.1). For the proof of *Thermoanaerobacterium* species, the primers ThmV1 and ThmR1 with an amplification product of 400 bp were applied (data not shown). All isolates that could be identified by the means of the genus specific PCRs are presented in Table 4.

The combination of the genus specific PCR with a paramagnetic particle based DNA isolation method, allowed us to scan heterogenic food samples for anaerobic, thermophilic bacteria of the genus *Thermoanaerobacterium* and *Thermoanaerobacter* respectively in a short period of time. The detection limit for this method is currently 10^4 cells/mL.

Discussion

Thermophilic, anaerobic bacteria have been known to be involved in food spoilage for quite a long time (*18*). Since phenotypic identification of those strains has always been problematic, only in recent years has the employment of modern techniques like PCR and 16S-rRNA gene sequencing led to new insights in the structure of the taxonomy of thermophiles (*5,11,19*). By using database comparison in order to determine the 16S-sequence similarities of isolates from food and food rela-

Table 4. Isolates from spoiled food samples and fertilizers identified by genus specific PCR

TMW	Identified as	main ingredient or source
TMW 2.384	*Thermoanaerobacter* spp.	meat/vegetables
TMW 2.385	*Thermoanaerobacter* sp.	diet food with meat and rice
TMW 2.386	*Thermoanaerobacter* spp.	diet food with meat and rice
TMW 2.438	*Thermoanaerobacterium* sp.	tomato puree
TMW 2.439	*Thermoanaerobacterium* spp.	tomato puree
TMW 2.452	*Thermoanaerobacterium* sp.	noodles/vegetables
TMW 2.453	*Thermoanaerobacterium* spp.	noodles/vegetables
TMW 2.454	*Thermoanaerobacterium* sp.	noodles/vegetables
TMW 2.459	*Thermoanaerobacterium* spp.	spinach
TMW 2.460	*Thermoanaerobacterium* sp.	spinach
TMW 2.461	*Thermoanaerobacter* spp.	potatoes
TMW 2.462	*Thermoanaerobacter* sp.	potatoes
TMW 2.467	*Thermoanaerobacterium* spp.	organic fertilizer
TMW 2.469	*Thermoanaerobacterium* sp.	organic fertilizer
TMW 2.470	*Thermoanaerobacterium* spp.	organic fertilizer
TMW 2.471	*Thermoanaerobacterium* sp.	organic fertilizer
TMW 2.472	*Thermoanaerobacterium* spp.	organic fertilizer
TMW 2.473	*Thermoanaerobacterium* sp.	organic fertilizer
TMW 2.474	*Thermoanaerobacterium* spp.	organic fertilizer

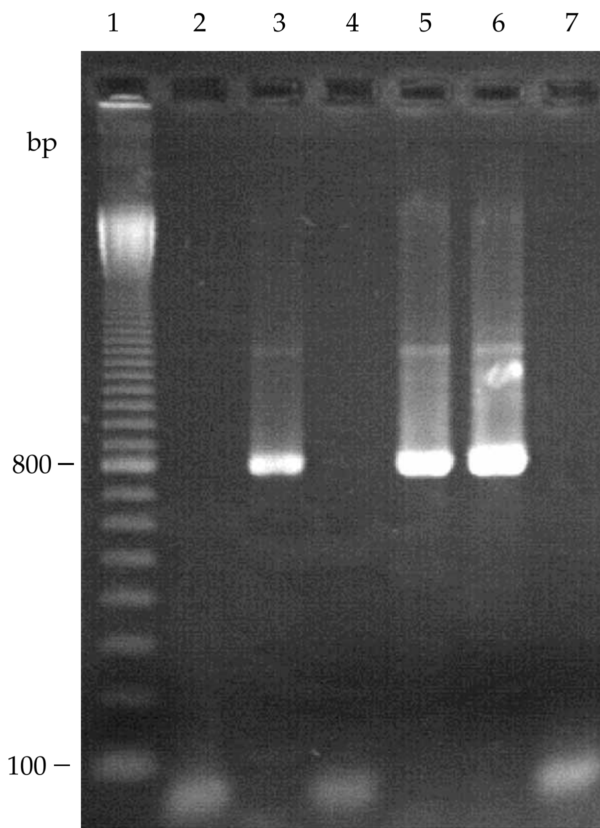

Fig. 1. Specific PCR for *Thermoanaerobacter* spp. with primer Table 1 and Table 2. Lane 1, 100 bp DNA-ladder; lane 2, *Thermoanaerobacterium saccharolyticum* DSM 7060; lane 3, *Thermoanaerobacter ethanolicus* DSM 2246; lane 4, *Thermoanaerobacterium aotearoense* DMS 8692: lane 5, TMW 2.461 (food isolate) Lane 6, *Thermoanaerobacter thermohydrosulfuricus* DSM 567; Lane 7, negative sample (no DNA added)

ted products, we were able to show that some thermophilic bacteria which have not been detected in food so far, like *Thermoanaerobacterium saccharolyticum* or *Thermoanaerobacterium thermosulfurigenes*, can be present in food. In addition, the thermophilic isolates TMW 2.345, TMW 2.430, TMW 2.433, TMW 2.437 and TMW 2.457 could not be allocated towards the 16S-rRNA sequences of any known species without ambiguity. Thus, we suppose that some of these isolates may represent hitherto undescribed species belonging to the genera *Thermoanaerobacterium* and *Thermoanaerobacter*.

The occurence of such organisms might be triggered by the use of modified agricultural techniques (*e.g.* special fertilizers) in fruit and vegetable cultivation. One hint might be the simultaneous identification of *Thermoanaerobacter thermosaccharolyticum* in organic fertilizer used for cultivation of tomatoes and in products containing them. This may indicate a possible route of contamination.

Since 16S-rRNA gene sequencing is rather time consuming we designed genus specific PCRs for both genera, *Thermoanaerobacter* and *Thermoanaerobacterium*. This technique allowed us to screen many food isolates in a short period of time. Moreover, a direct detection method was developed based on the enrichment of free bacterial DNA from food samples. Using the universal 16S-rRNA primers (616VII and 630R) as DNA capture probes, the need for cultural preenrichment from food samples was overcome. Coupling these primers to streptavidin coated magnetic beads, made it possible to bind free DNA of spontaneously lysed bacterial cells in samples without previous enrichment. As a precondition cell count within the sample had to be at least 10^4 cells/mL. After magnetic separation of the beads-probe-DNA complex and elution of the captured DNA a genus specific PCR could be performed. This whole detection process takes about 6 hours, which is quite fast compared to the usual succession of preenrichment and a subsequent identification with cultural techniques.

Since five of the organisms we detected did not match very well towards any known species, we also assume the involvement of yet undescribed species in anaerobic food spoilage.

Conclusions

The conclusions drawn from our results are that we have either detected anaerobic, thermophilic organisms in foods that were already present there but simply could not have been detected up to now, or we have demonstrated the occurrence of a new kind of food spoilage caused by bacteria whose occurrence is provoked by agricultural conditions and/or production parameters. Further research has to be done to clarify contamination routes, growth conditions, taxonomic status and health aspects of the isolated organisms.

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Pojava i određivanje *Thermoanaerobacterium* i *Thermoanaerobacter* u konzerviranoj hrani u limenkama

Sažetak

Da bi se odredio uzrok nestanka vakuuma u limenkama konzervirane hrane, obligatni anaerobi, termofilni organizmi koji stvaraju spore, izolirani su iz hrane u limenkama s glavnim sastojcima: povrće, rezanci i krumpir. Izolirane su termofilne bakterije iz 44 uzorka limenki uskladištenih pod anaerobnim uvjetima pri 37 °C barem 7 dana. Osim toga, ispitana su organska gnojiva upotrijebljena za uzgoj navedenog povrća pa su i iz tog izvora izolirane anaerobne termofilne bakterije. Identifikacija bakterijskih sojeva provedena je djelomičnim i potpunim sekvencioniranjem 16S-rRNA gena. Neke od dobivenih genskih sekvencija pokazale su visoki stupanj sličnosti s postojećim sekvencijama 16S-rRNA gena sojeva rodova *Thermoanaerobacter*, *Thermoanaerobium* i *Thermoanaerobacterium*. Do sada još nije bila ustanovljena važnost tih sojeva kao onečišćavača hrane. Tijekom identifikacije navedenih termofilnih bakterija autori su razvili genetički specifičan pristup utemeljen na PCR za određivanje izolata koji pripadaju rodovima *Thermoanaerobacterium* i *Thermoanaerobacter.* Izravno vezanje slobodne DNA iz onečišćenih uzoraka, koristeći oligonukleotide povezane s paramagnetskim zrncima omogućilo je smanjenje vremena detekcije na 6 sati s donjom granicom od 10^4 stanica/mL.