Rhodothermus marinus, gen. nov., sp. nov., a Thermophilic, Halophilic Bacterium from Submarine Hot Springs in Iceland

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Thermophilic, reddish-coloured heterotrophic bacteria different from *Thermus* were isolated from submarine alkaline hot springs in Iceland. The bacteria were obligately aerobic, moderately halophilic, Gram-negative rods, about 0·5 µm in diameter and 2–2·5 µm long. Neither spores, flagella nor lipid granules were observed, but a slime capsule was formed on carbohydrate-rich medium. Optimum growth was at 65 °C, pH 7·0, and at about 2% (w/v) NaCl. The bacteria were oxidase negative, catalase positive and contained a carotenoid pigment with the main absorbance peak at 476 nm and shoulders at 456 and 502 nm. The GC content of the DNA was about 64 mol %. Electron micrographs clearly showed an outer membrane, about 9 nm thick, and the cytoplasmic membrane together with the peptidoglycan layer was about 14 nm in thickness. The isolates were nutritionally different from *Thermus*. They utilized several common sugars but glutamate and aspartate were the only amino acids that most strains used. These bacteria are considered to represent a new genus which we name *Rhodothermus*, with the type species *Rhodothermus marinus*.

INTRODUCTION

In recent years there has been much interest in organisms which are associated with submarine hot springs (Corliss & Ballard, 1977; Jannasch & Nelson, 1984; Stetter, 1985). Most of the work has been done on the deep-sea hydrothermal vents in the Pacific Ocean, which support extensive populations of certain invertebrates and bacteria, including some thermophiles (Corliss & Ballard, 1977; Jannasch & Nelson, 1984). The best-known thermophilic submarine bacterium is *Pyrodictium*, isolated by Stetter (1982, 1985) from a shallow-water solfatara field in Italy.

In a recent study of submarine alkaline hot springs in Iceland we isolated salt-tolerant strains of *Thermus* (Kristjansson & Alfredsson, 1986; Kristjansson *et al.*, 1986). The halotolerant *Thermus* strains were very similar to their terrestrial counterparts, except in being adapted to the submarine thermal environment (Kristjansson *et al.*, 1986). Bacteria of the genus *Thermus* are the most common heterotrophs in terrestrial alkaline hot springs (Kristjansson & Alfredsson, 1983; Kristjansson *et al.*, 1985), and this also seems to be the case for submarine alkaline hot springs. There is a great diversity among strains of *Thermus* (Alfredsson *et al.*, 1985; Hudson *et al.*, 1986; Munster *et al.*, 1986) but only two species have so far been validly described (Brock, 1984; Loginova *et al.*, 1984). The only other aerobic thermophilic heterotrophs known to grow in alkaline hot springs at 70–80 °C are *Thermomicrobium roseum* (Jackson *et al.*, 1973) and several species of sporeformers (Kristjansson & Alfredsson, 1983).

In this paper we describe the isolation and characterization of a thermophilic and halophilic bacterium from shallow-water submarine hot springs in Iceland which differs substantially

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from strains of *Thermus* sp., *Thermus ruber* (Loginova *et al.*, 1984) and *Thermomicrobium* (Ramaley & Hixson, 1970). The new isolates are considered to represent a new genus and species of thermophilic bacteria, for which we propose the name *Rhodothermus marinus*.

METHODS

Study site. The hot springs are located off the coast of a small peninsula, Reykjanes, in the Isafjardardjup Bay, NW Iceland. A series of alkaline hot springs is located on the peninsula itself and down to sealevel. In a small inlet called Hveravik at the tip of the peninsula, several hot springs occur from a few to about 100 metres off the coast. In the inlet several hot springs emerge at low tide, but others are located further out, at a depth of 2–3 metres at low tide. The samples used in this study were taken from these outermost springs. The water in the hot springs is alkaline, and of low sulphide content, but slightly mixed with seawater (chloride 600 p.p.m.). The temperature in the hot-water duct ranges from 75 to 95 °C in different springs.

Sampling. The samples were collected by diving as previously described (Kristjansson *et al.*, 1986; Stetter, 1982). Samples were collected directly from the openings of five different submarine hot springs at a depth of 2–3 metres. It was not possible to exclude seawater completely when sampling. The samples, which consisted of fine gravel and water, were kept refrigerated until processed further.

Isolation of bacteria. Water samples were filtered directly but sand and gravel samples were washed with sterile seawater, which was then filtered through Millipore membrane filters with a pore size of 0·45 μm. The filters were put on plates containing nutrient agar medium 162 of Degryse *et al.* (1978) containing 3% (w/v) NaCl and incubated at 72 °C for 4 d (Kristjansson & Alfredsson, 1983). Red-pigmented colonies were picked and purified by streaking onto the same medium. Twenty-one strains, designated R-1 to R-21, were used for this study. All the strains were taken through the identification tests used for identification of *Thermus* spp. (Brock, 1984; Degryse *et al.*, 1978; Kristjansson & Alfredsson, 1983).

Media and culture conditions. The bacteria were routinely cultured on nutrient agar medium 162 containing 1% (w/v) NaCl but other concentrations of NaCl were used when appropriate. Strains were grown at a variety of temperatures, pH values and concentrations of NaCl in order to determine their basic physiological characteristics. Growth on single carbon sources was tested on minimal-agar plates inoculated with a multipoint inoculator as previously described (Alfredsson *et al.*, 1985). All growth tests were done at 65 °C. Growth on agar was scored positive if clearly visible and confluent growth had occurred after 7 d incubation and if the strain grew again upon subculturing onto the same medium. In liquid medium, growth was followed by measuring the increase in OD_{660} in a Hitachi spectrophotometer, model UV/VIS 100-20.

Electron microscopy. The cells were grown overnight on agar, scraped off and fixed with 3% (w/v) glutaraldehyde in 0·1 M-cacodylate buffer pH 7·4 (Ericsson *et al.*, 1978), then centrifuged for 10 min at 8000 *g*. The pellet was postfixed with 2% (w/v) OsO_4 in the same buffer, dehydrated with ethanol and embedded in Spurr resin. Ultrathin sections were cut with a Reichert ultramicrotome Om U3. The sections were stained in 2% (w/v) aqueous uranyl acetate for 20 min and in Reynolds' lead citrate for 5 min (Hayat, 1970). The electron micrographs were taken with a Philips EM 300 electron microscope (80 kV) by Johann Arnfinnsson, Department of Anatomy, University of Iceland.

DNA base composition. DNA was prepared according to Lauerer *et al.* (1986). The GC content of the DNA was calculated from the melting point in 0·1 × SSC (Marmur & Doty, 1962), using calf thymus DNA (42 mol % GC) as a reference. Direct analysis of the DNA base composition was done by HPLC chromatography after digestion of the DNA with nuclease P1 (Zillig *et al.*, 1980).

Determination of antibiotic sensitivity. Sensitivity to the following antibiotics was tested (the amount per disc is given in parenthesis): penicillin G (10 IU), ampicillin (10 μg), streptomycin (10 μg), kanamycin (30 μg), gentamicin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), polymyxin B (300 units), bacitracin (0·1 unit), nalidixic acid (30 μg), vancomycin (70 μg), lincomycin (19 μg), rifamycins (30 μg) and virginamycin (30 μg). The discs were standard 6 mm antibiotic discs except for vancomycin, lincomycin, rifamycins and virginamycin which were applied in 10 mm discs. These four antibiotics were from ROSCO Diagnostica, Denmark, bacitracin was from Mast Laboratories, and all the other antibiotics were from Oxoid. Bacteria were grown on nutrient agar medium 162 containing 1% (w/v) NaCl and the results scored after overnight growth at 65 °C. Antibiotic sensitivities of *Thermus* strains were determined on the same medium without added NaCl. No detectable inhibition was scored as 6 mm and 10 mm for the two different disc sizes. To check whether the absence or presence of NaCl affected the size of the inhibition zone, the antibiotic sensitivity of a salt-tolerant *Thermus* strain, IB-21 (Kristjansson *et al.*, 1986) was tested on standard nutrient agar medium 162 and on the same medium containing 1% (w/v) NaCl.

Spectrophotometry of pigments. For determination of the carotenoid pigments the bacteria were extracted with 90% (w/v) acetone as described by Brock & Brock (1967). After centrifugation the clear supernatant was taken and the absorption spectrum determined in a Perkin Elmer 550-S spectrophotometer.

RESULTS

Isolation. As a part of our studies on *Thermus* spp. from submarine hot springs, media containing varying concentrations of NaCl from 0 to 5% (w/v) were employed. With no added NaCl only yellow-pigmented colonies of *Thermus* were isolated. When 1% NaCl or more was added both yellow- and red-pigmented colonies appeared on the filters. The proportion of the red-pigmented colonies increased with increasing NaCl concentration up to 2% (w/v).

Morphology and pigments. On the nutrient medium 162 and at 65 °C, these bacteria formed reddish-coloured, low-convex colonies, 3–4 mm in diameter and with an entire edge. All strains were Gram-negative rods, normally about 0·5 μm in diameter and about 2–2·5 μm long. Filaments were never observed and neither spores, flagella nor lipid inclusions were seen by conventional staining methods or in the electron micrographs. When the bacteria were grown on carbohydrate-rich medium a distinct capsule was formed, as demonstrated by the India ink method. Electron micrographs of thin sections showed a normal Gram-negative cell envelope (Fig. 1). An outer membrane, about 9 nm thick, was clearly visible and the cytoplasmic membrane together with the peptidoglycan layer was about 14 nm in thickness.

Acetone extracts showed a spectrum typical of a carotenoid pigment with the maximum at 476 nm and shoulders at 456 and 502 nm. For *Thermus ruber* the maximum is at 483 nm, with shoulders at 455 and 513 nm (Loginova & Egorova, 1975; Loginova *et al.*, 1984); for *Thermus aquaticus* YT-1 the main peak is at 451 nm and the shoulders at 428 and 475 nm (Brock, 1978).

Physiological properties. The 21 reddish-coloured isolates were all very similar physiologically. Optimum growth temperature was 65 °C and optimum pH was 7·0. Minimum and maximum growth temperatures were about 54 °C and about 77 °C, respectively. None of the strains grew unless NaCl was present in the medium and adaptation to medium lacking NaCl was not possible (Kristjansson *et al.*, 1986). The optimum NaCl concentration for growth of strain R-10 in liquid culture medium 162 was about 2% (w/v) and the maximum above 6% (w/v) (Fig. 2). This was also shown to be true for all the other new isolates grown on solid medium. In contrast, the type strain *Thermus aquaticus* grows optimally without NaCl and shows a very low NaCl tolerance (Fig. 2). The same is true for *Thermus ruber* (Table 1). On plates the growth of the new isolates was much reduced at 5% (w/v) NaCl and only 6 of the 21 strains showed marginal growth at 6% (w/v) NaCl. The maximum specific growth rate (μ) in nutrient medium 162 with 1% (w/v) NaCl was 0·5 h^{-1}. All the strains were catalase positive but oxidase negative and did not hydrolyse casein. No nitrate reduction was detected. The bacteria did not grow anaerobically in nutrient salt medium 162 (1%, w/v, NaCl) and did not ferment sugars anaerobically.

The bacteria grew on minimal medium plates with several single compounds added as sole carbon and energy source. Their growth pattern on these single carbon sources was very different from that of strains of *Thermus*. Twenty strains were tested on 32 different compounds. The following compounds were used by most of the strains tested (the percentage of strains showing good or distinct growth is given in parenthesis): L-aspartate (100), L-glutamate (95), gelatin (100), acetate (60), pyruvate (100), glucose (70), galactose (75), starch (100), sucrose (95), lactose (95), raffinose (95) and maltose (85). The substrates not utilized by any of the 20 strains were: L-arginine, L-asparagine, L-glutamine, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine, L-valine, butyrate, gluconate, fumarate, malate, succinate, 2-oxoglutarate, citrate, sorbitol and glycerol. On DL-alanine and L-ornithine growth occurred only as a few well-separated colonies. This phenomenon has also been observed with *Thermus*, when the colonies were found to be mutants (Alfredsson *et al.*, 1985).

All organic acids tested were supplied as sodium salts, except butyric acid and malic acid (Alfredsson *et al.*, 1985). A high percentage of the strains grew well on several sugars, but of the amino acids tested only aspartate and glutamate were utilized.

DNA base composition. The GC content of DNA was determined for two strains, R-10 (64·4 mol %) and R-18 (64·7 mol %). Preliminary results from sequencing of 16S ribosomal RNA of strain R-10 showed that it was not related to *Thermus* X-1 (Ramaley & Hixson, 1970) or to
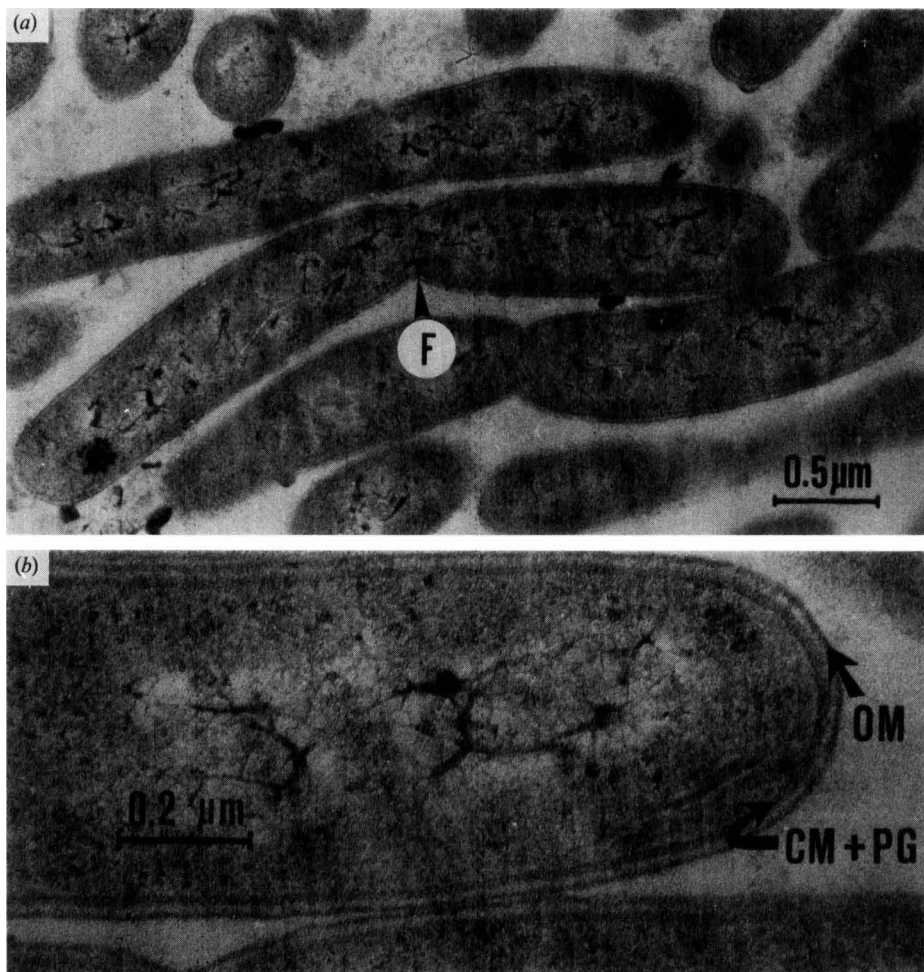
Fig. 1. Electron micrographs of *Rhodothermus marinus* strain R-18. (*a*) Thin longitudinal and cross-sections showing the width and length of typical cells as well as dividing cells with furrowing (F). (*b*) The cell envelope, consisting of a cytoplasmic membrane plus peptidoglycan layer (CM + PG), and a well-separated outer membrane (OM).
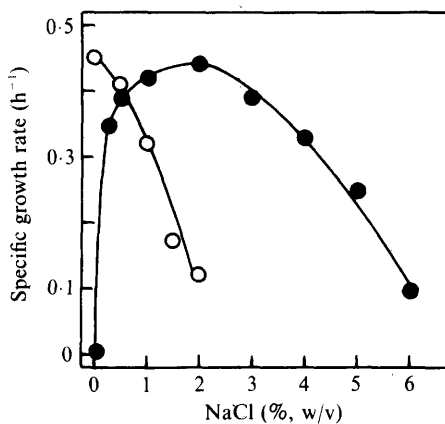

Fig. 2. Growth rate in nutrient medium 162 as a function of NaCl concentration in the medium. Growth temperature was 65 °C. ○, *Thermus aquaticus* strain YT-1; ●, *Rhodothermus marinus* strain R-10.

Table 1. *Main characters differentiating the new genus Rhodothermus from the genera Thermus and Thermomicrobium*

Character	Rhodothermus	Thermus	Thermus ruber*	Thermomicrobium†
Cell length, μm	2–2·5	5–10	3–6	3–6
Chains or filaments	−	+	+	−
Slime capsule	+	−	−‡	−
'Rotund bodies'	−	+	+§	−
Colony colour	Reddish	Yellow/orange	Red‡	Red
Main carotenoid peak	476 nm	451–456 nm	483 nm‡	494 nm
Nitrate reduction	−	+	−‡	
Oxidase reaction	−	+	+‡	
Exoprotease	−	Generally +	+‡	
Growth in NaCl:				
0%	−	+	+‡	+
3% (w/v)	+	−	−‡	−
6% (w/v)	+	−	−‡	−
Growth on:				
Glucose	+	Rare	+	−
Galactose	+	Variable	+	
2-Oxoglutarate	−	+	−‡	
Glutamine	−	+	−‡	+
Proline	−	+	−‡	
Aminoglycoside antibiotics	Resistant	Sensitive	Sensitive‡	Sensitive
Temp. optimum (°C)	65	70–75	60	70–75
Generation time	80 min	20–60 min	60 min	5·5 h

* Taken from Loginova *et al.* (1984). † Taken from Jackson *et al.* (1973).
‡ Result confirmed in this work. § Taken from Becker & Starzyk (1984).

other bacteria with available 16S rRNA sequences (Olafur S. Andresson, personal communication).

Antibiotic resistance patterns. The antibiotic inhibition zone diameters for *Thermus ruber, Thermus aquaticus* (YT-1) and eight Icelandic *Thermus* strains were all very similar to each other. The new isolates tested, R-10 and R-18, gave a pattern of results much like these *Thermus* strains, except in being resistant to the aminoglycosides streptomycin, kanamycin and gentamicin. For strain R-10, the diameters of the inhibition zones (mm) were as follows: penicillin G, 68; ampicillin, 65; streptomycin, 8; kanamycin, 6; gentamicin, 6; tetracycline, 30; chloramphenicol, 28; erythromycin, 53; polymyxin B, 11; bacitracin, 6; nalidixic acid, 6; vancomycin, 40; lincomycin, >70; rifamycins, 37; and virginamycin, 44. The *Thermus* strains showed inhibition zones of about 30, 23 and 32 mm for streptomycin, kanamycin and gentamicin, respectively. The control strain IB-21 gave identical resistance patterns on media with and without 1% (w/v) NaCl.

Comparison of the new isolates with other bacteria. The new isolates were compared with other known Gram-negative, aerobic, thermophilic heterotrophs, namely *Thermus aquaticus* (Brock, 1984), *Thermus ruber* (Loginova *et al.*, 1984) and *Thermomicrobium* (Jackson *et al.*, 1973). The main features of these bacteria are listed in Table 1 and compared with the new isolates (*Rhodothermus*). Many of the previously reported characterization tests were repeated for *T. ruber* and some new ones added (Table 1). We found *T. ruber* to produce exoprotease although it had previously been reported not to peptonize milk (Loginova *et al.*, 1984). In all other cases there was good agreement between our results and published data for *T. ruber*.

DISCUSSION

The 21 strains reported here were all isolated from the same submarine geothermal area in Iceland (Kristjansson *et al.*, 1986). Attempts to isolate them from other submarine hot springs in Iceland have not been successful (unpublished results). The isolates form a very homogeneous group, with only slight differences between strains in utilization of single carbon sources.

These new thermophilic bacteria have a temperature range for growth of about 54–77 °C and an optimum growth temperature of about 65 °C. This means that they can only grow very close to the openings of the submarine hot springs: only a few centimeters from the openings the water would be too cold for growth. Normally, geothermal water is anaerobic until it emerges from the ground or seabed and absorbs oxygen from its surroundings. These bacteria are obligate aerobes, and are thus confined to a very narrow zone near the openings of the hot springs where both the temperature and oxygen content of the water are suitable for growth. As these bacteria are heterotrophs they must also utilize the organic material present in the seawater. The optimum NaCl concentration for growth of strain R-10 in liquid culture was about 2% (w/v) and the maximum above 6% (w/v) (Fig. 2). These bacteria can therefore be considered as slightly halophilic or possibly moderately halophilic (Kushner, 1978; Russell & Kogut, 1985). Their requirement for oxygen, NaCl, organic material and temperature strictly defines a very narrow niche for these organisms in the hot spring ecosystem.

At present there are only two validly described species of the genus *Thermus*, i.e. *Thermus aquaticus* and *Thermus ruber* (Brock, 1984; Loginova *et al.*, 1984), but many strains have been isolated from different places and sources (Brock, 1978; Egorova & Loginova, 1975; Hudson *et al.*, 1986; Munster *et al.*, 1986; Oshima & Imahori, 1974; Pask-Hughes & Williams, 1977). Variations in optimum growth temperature, pigmentation, and nutrition have been reported among *Thermus* isolates (Alfredsson *et al.*, 1985; Degryse *et al.*, 1978; Loginova *et al.*, 1984; Pask-Hughes & Williams, 1977) and these differences are probably sufficient to warrant dividing the genus up into several species and possibly into more than one genus (Alfredsson *et al.*, 1985; Hudson *et al.*, 1986; Munster *et al.*, 1986).

Thermus ruber forms red colonies similar to our new isolates, so we made a careful comparison between this species and the new isolates. Many reported characterization tests were repeated for *Thermus ruber* and some new ones added (Table 1), and as expected, they showed it to belong to the *Thermus* group (Hensel *et al.*, 1986; Munster *et al.*, 1986). This is seen from important features like the cell size, the oxidase reaction, the sensitivity to aminoglycoside antibiotics and the effects of NaCl on growth. In contrast the new isolates are clearly different from *Thermus ruber*.

All the *Thermus* strains found in Iceland so far have shown the same general characteristics originally described for the genus *Thermus* (Brock, 1984). The morphology and other characteristics of *Thermomicrobium roseum* are very different from those of *Thermus* (Table 1 and Jackson *et al.*, 1973).

The new group of bacteria described in this paper is phenotypically very different from *Thermus* and other thermophilic and caldoactive bacteria (Table 1). Preliminary 16S rRNA sequence data furthermore indicate that it is phylogenetically distant from *Thermus* (Olafur S. Andresson, personal communication). Based on all this we propose a new genus *Rhodothermus* for these bacteria. All the isolates belong to a single species, *Rhodothermus marinus*. Formal descriptions are given below.

Rhodothermus Alfredsson, Kristjansson, Hjörleifsdottir and Stetter, gen. nov.

Rho.do.ther'mus, Gr. n. *rhodon* rose; Gr. fem. n. *therme* heat; M.L. masc. n. *Rhodothermus* the red thermophile. Cells are Gram-negative, non-motile straight rods with curved ends, about 2–2·5 μm long and 0·5 μm wide, occurring singly. A slime capsule is formed on carbohydrate-rich medium. Colonies are reddish-coloured, low convex, 3–4 mm in diameter with an entire edge. Aerobic thermophilic neutrophilic heterotrophs. Growth strictly salt-dependent. Oxidase negative, catalase positive. No dissimilatory nitrate reduction and no casein hydrolysis. Growth on most common sugars like glucose, sucrose, and lactose but not on most amino acids. Cells are

sensitive to penicillin, erythromycin, tetracycline and chloramphenicol, and resistant to streptomycin, kanamycin and gentamicin. The DNA base composition is about 65 mol % GC.

The type species is *Rhodothermus marinus*, DSM 4252 (ATCC 43812).

Rhodothermus marinus Alfredsson, Kristjansson, Hjörleifsdottir and Stetter, sp. nov.

ma.ri'nus, L. masc. adj. *marinus* thriving in the sea, describing its biotope. Description as for the genus. Cells never occur in chains or filaments. Optimal growth at 65 °C and pH 7 and about 2% (w/v) NaCl. Growth occurs at 6% (w/v) NaCl. The natural habitat is submarine alkaline hot springs.

The type strain is *Rhodothermus marinus*, DSM 4252 (ATCC 43812) (isolated from a submarine hot spring at Reykjanes, NW Iceland).

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