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Isolation of Halotolerant *Thermus* spp. from Submarine Hot Springs in Iceland

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Thermophilic, aerobic bacteria of the genus *Thermus* were isolated from submarine alkaline hot springs in Iceland. Five submarine hot springs were sampled, and all had viable counts of *Thermus* spp. of about 10^3 CFU/ml. All submarine strains grew in the presence of NaCl at 3% or higher, but no strains from terrestrial hot springs would grow at concentrations higher than 1% NaCl. The growth rate of submarine *Thermus* strains was not stimulated by NaCl and was reduced at NaCl concentrations higher than 1%. The pattern of growth of these isolates on single carbon sources was similar to that of terrestrial isolates.

Since the isolation by Stetter (17) of bacteria capable of growing at 110°C from a submarine solfatara field in Italy and the discovery of life associated with the deep-sea hydrothermal vents in the Pacific, there has been a greatly increased interest in the biology of submarine hot springs. The deep-sea vents are a continuous source of highly reduced compounds, like H_2S and H_2, which serve as substrates for chemosynthetic bacteria (9). These bacteria are therefore the main primary producers in this deep-sea ecosystem and in fact support extensive populations of certain invertebrates (5, 9).

The gas in submarine solfatara fields contains both H_2S and H_2, which can be utilized as an energy source by thermophilic chemoautotrophs (18). In submarine hot springs located in shallower waters, however, input of external organic material is probably substantial. Hot springs in terrestrial solfatara fields are usually very acidic owing to the oxidation of sulfides to sulfuric acid. In the sea, however, they contain seawater which seeps into the bottom and is heated by the rising steam.

Alkaline hot springs which emerge below sea level have been found in several places in Iceland. These hot springs are usually located on fissures going from the mainland and into the sea bottom. The water originates on the mainland and is essentially fresh water, although a minor mixing with seawater is sometimes detected. The water is anaerobic when it emerges but contains very little H_2S (J. Benjaminsson, Vestfirdir—*The Chemistry of the Geothermal Water*. Unpublished report of the Icelandic National Energy Authority, JHD06 1981, [in Icelandic]). Hydrogen in the geothermal water in this area has not been measured. Because of the low H_2S level, a large amount of chemosynthesis is unlikely. On land, alkaline hot springs are usually characterized by extensive growth of thermophilic cyanobacteria and other photosynthetic bacteria, at least up to 65 to 70°C. The most characteristic heterotrophs are usually bacteria of the genus *Thermus*, which are yellow obligate aerobes (3). They are found in great numbers in all terrestrial alkaline hot springs from 55 to 85°C (10).

In this paper, we report the first isolation from submarine hot springs of thermophilic, halotolerant bacteria of the genus *Thermus*. To our knowledge, this is the first study concerning the biology of submarine alkaline hot springs.

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MATERIALS AND METHODS

Study site. The hot springs are located off the coast of a small peninsula called Reykjanes in the Isafjardardjup bay in northwestern Iceland. There are a series of alkaline hot springs located on the peninsula itself and down to sea level. In a small creek called Hveravik, at the tip of the peninsula, several hot springs lie from a few to about 100 m off the coast. In the creek, the hot springs emerge at low tide, but other springs are located further out at a depth of 2 to 3 m. The samples used in this study were taken from these springs. The water in the hot springs is alkaline and low-sulfide but is slightly mixed with seawater (chloride, 600 μg/ml) (Benjaminsson, unpublished report). The temperature in the hot-water duct ranges from 75 to 95°C in different springs.

Bacterial strains. The bacteria were isolated from hot springs in several geothermal areas in Iceland. Seventy-five strains were from the submarine hot springs described above and were designated IB-1 to IB-75. A total of 50 *Thermus* strains from terrestrial hot springs were also used in this study. From the Borgarfjördur area (11) were isolated 13 strains, designated BO. Twenty-eight strains, designated HV and JK, were from the Hveragerdi-Hengill area (10). Four strains were from the geothermal municipal heating system of the city of Reykjavik (designated AR), and four strains designated IS were from the terrestrial hot springs near the submarine springs. The *Thermus aquaticus* reference strains YT-1 (ATCC 25104) and HB-8 (ATCC 27634) were kindly donated by E. Degryse.

Sampling and isolation of bacteria. Samples from the submarine hot springs were collected by a diver using a 50-ml syringe with a 1-cm-wide opening, as described by Stetter (17). Samples contained both fine gravel and water. The temperature at the collection site was measured by sticking the temperature probe into the openings of the hot springs when they could be located or into the sediment. Samples were processed within 1 week of the time of collection. *Thermus* strains were isolated from the water by the filter method on agar plates that had been incubated at 72°C as previously described (10). The isolation medium used was medium 162 of Degryse et al. (6), containing 0.25% yeast extract (Oxoid L21) and 0.25% tryptone (Oxoid L42). The normal medium which we use for routine isolation of *Thermus* strains does not contain extra salt. Therefore, no extra salt was added to the primary isolation medium. When growth on single carbon sources was tested, the minimal

TABLE 1. Characteristics of the submarine hot springs sampled in Isafjardardjup, Iceland

Sampling site	Temp (°C)	Chloride concn (μg/ml)	Seawater[a] in samples (%)	CFU/ml of sample water
Sand bottom	47	9.900	52	3,000
Sediment	70	7.640	40	1,500
Sediment	75–80	11.490	60	700
Crack in rock	78	7.450	39	2,500
Crack in rock	88	9.450	50	500

[a] Calculated from chloride concentration. Normal seawater contains about 19.000 μg of chloride per ml.

medium used was made from the same salt base as used for medium 162 with 0.05% NH_4Cl. Also added were 0.5 ml of the trace element solution of Badziong et al. (2) and 10 ml of vitamin solution A of Degryse et al. (6).

Identification of bacteria. In this study, *Thermus* spp. were identified by the criteria described by Brock (4) and Degryse et al. (6). All colony types on one plate were picked at random, purified by streaking, and tested for the following characteristics (reaction for *Thermus* spp. in parentheses): Gram reaction (−), carotenoid pigments (>90%+), colony morphology (on a filter 2 to 4 mm in diameter, low convex with a smooth surface), flagellation (−), pellicle in liquid culture (+), presence of spores (−), anaerobic growth (−), oxidase (+), and cell morphology (on plates at 72°C, thin rods a few micrometers long; in liquid at 75°C, long filaments).

Cloride measurements. The chloride concentration in the samples was determined by the Mohr titration method.

Determination of growth. The bacteria were routinely kept on nutrient agar medium 162, but different concentrations of NaCl were added when appropriate. Growth was scored positive on salt agar when clearly visible and confluent growth had occurred after 7 days of incubation and if the strain grew again upon being restreaked onto the same medium. All growth tests were done at 65°C. Growth on single carbon sources was tested with a multipoint inoculator on plates as previously described (1). When the bacteria were grown in liquid medium, growth was monitored by measuring the increase in turbidity at 660 nm in a Hitachi spectrophotometer, model UV/VIS 100-20.

Determination of salt tolerance. The salt tolerance of the bacteria was determined before and after salt adaptation. After the bacteria had been purified on nutrient agar 162, they were streaked directly onto the same agar containing various concentrations of NaCl to give the number of strains which grew at different salt concentrations without salt adaptation. The bacteria were adapted to salt in the following way. After growth had occurred at a specific salt concentration, the bacteria were restreaked onto plates containing sequentially increasing concentrations of NaCl. This was done until the specific salt concentration at which no more growth was observed had been reached. The number of strains which grew after salt adaptation was thus determined. When the effects of NaCl on growth rate in liquid nutrient medium 162 were tested, the strains were salt adapted in liquid medium before being used as inocula.

RESULTS

Sampling and isolation. Samples were collected directly from the openings of five different submarine hot springs. The samples consisted of fine gravel and water. It was not possible to totally exclude seawater when sampling. The percentage of seawater in the samples was calculated from the chloride content (see Table 1), but the normal geothermal water in this area contains very low concentrations of chloride. The viable count of thermophilic bacteria in the samples was estimated by counting colonies on the filters (Table 1). All colonies which grew in the primary isolations were yellow or orange. According to the identification scheme used, all of the 75 strains which were purified belonged to the genus *Thermus*. In later isolations, when NaCl was added to the isolation medium, the number of yellow colonies decreased with increasing salt concentration. At 2 and 3% NaCl, red colonies also appeared on the filters. When these were subjected to the identification scheme, they turned out to be significantly different from *Thermus* spp. They will be described elsewhere.

Salt tolerance of *Thermus* spp. The salt tolerance, without adaptation, of 75 submarine strains was compared with that of 50 strains from terrestrial hot springs. Twelve of the most salt-tolerant strains from each group were then carried through salt adaptation and tested again. Without adaptation, no terrestrial strains grew at a higher NaCl concentration than 1%. All submarine strains, however, grew at 3% NaCl (Table 2). Strains from both groups could be adapted to grow at a somewhat higher concentration of NaCl, but the difference between the two groups was still quite distinct.

To test the stability of the salt tolerance of the submarine isolates, strain IB-21 was passaged 10 times by restreaking of single colonies onto fresh agar medium 162. After 10 passages on medium without NaCl, the strain was tested again and showed exactly the same degree of salt tolerance as before (data not shown).

Effect of salt on growth rate. To find whether the submarine strains were halophilic or only halotolerant, the effect of NaCl on growth rate was determined. Strain IB-21 was compared with *T. aquaticus* YT-1, which is a typical terrestrial strain. The growth rate of strain YT-1 was lowered at all NaCl concentrations tested (0.5 to 2%). The growth rate of strain IB-21 was not increased by NaCl and was in fact lowered at NaCl concentrations higher than 1% (Fig. 1).

Growth on single carbon sources. The growth pattern of the submarine strains on single carbon sources was very similar to that of the terrestrial strains (1). In both groups, the majority (more than 2/3) of the strains grew on acetate (68 and 77% for submarine and terrestrial strains, respectively),

TABLE 2. Salt tolerance of *Thermus* strains from submarine and terrestrial hot springs

NaCl concn in growth medium (%)	% of strains showing growth[a]			
	Terrestrial strains		Submarine strains	
	Without adaptation (n = 50)	With adaptation (n = 12)	Without adaptation (n = 75)	With adaptation (n = 12)
0.0	100	100	100	100
0.5	64	100	100	100
1.0	20	50	100	100
1.5	0	42	100	100
2.0		33	100	100
2.5		17	100	100
3.0		0	100	100
4.0			89	92
5.0			0	67
6.0				0

[a] n, Number of strains tested.

L-asparagine (72 and 81%), butyrate (76 and 50%), L-glutamate (87 and 92%), α-ketoglutarate (65 and 92%), L-proline (79 and 79%), and pyruvate (71 and 85%). Less than 1/3 of the strains in both groups grew on L-arginine (16 and 31%), citrate (29 and 27%), fumarate (3 and 23%), glycerol (0 and 23%), glucose (17 and 8%), malate (1 and 21%), L-ornithine (36 and 8%), L-phenylalanine (19 and 12%), succinate (19 and 31%), and L-valine (4 and 12%). The percentage of strains utilizing L-aspartate (9 and 46%), galactose (1 and 35%), L-glutamine (16 and 78%), lactose (15 and 58%), L-leucine (20 and 54%), and L-serine (21 and 42%) was low for the submarine strains but high or intermediate for the terrestrial strains. Of the terrestrial strains, 32% produced extracellular proteolytic enzymes, whereas 12% of the submarine strains did so (unpublished results).

DISCUSSION

Although at present there are only two approved species of the genus *Thermus*, i.e., *T. aquaticus* and *T. ruber* (4, 13), a large number of strains have been isolated from different places and sources (3, 7, 10, 14, 15). *Thermus* spp. are heterotrophic aerobes, which are easy to grow in the laboratory, and many strains have been studied quite extensively. Variations in optimum growth temperature, pigmentation, and other traits among *Thermus* isolates have been reported (6, 15, 16). *Thermus* spp. are ubiquitous and can be isolated from both natural and man-made thermal sources. It must be assumed, however, that the natural habitats of *Thermus* spp. are the geothermal areas of the world. These bacteria have been found in both neutral and alkaline hot springs around the world (3, 6–8, 10, 14, 15). In Iceland, *Thermus* spp. were present in considerable numbers in all terrestrial hot springs with temperatures of 55 to 85°C and pH values higher than 6.5 (10). *Thermus* spp. can utilize a variety of organic substrates for growth and may well be the most important heterotrophs in the hot-spring ecosystem (1, 11).

In the present work, we used the medium developed by Degryse et al. (6) to isolate a large number of *Thermus* strains from submarine alkaline hot springs. In terms of nutrition and other characteristics, the isolates were very similar to *Thermus* spp. which have been isolated from terrestrial hot springs. The only distinct difference was that they could grow in the presence of a much higher concentration of NaCl than could the terrestrial strains. The growth of the submarine strains was, however, not stimulated by NaCl, and they can therefore be considered halotolerant but not halophilic (Fig. 1) (12).

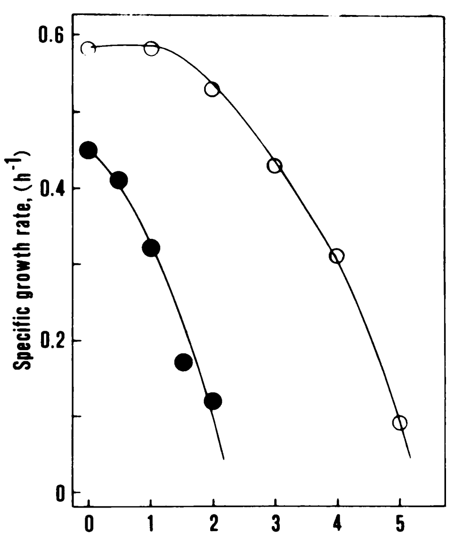

FIG. 1. Growth rate of *Thermus* spp. as a function of NaCl concentration in the medium. Symbols: ○, submarine strain IB-21, ●, terrestrial strain YT-1.

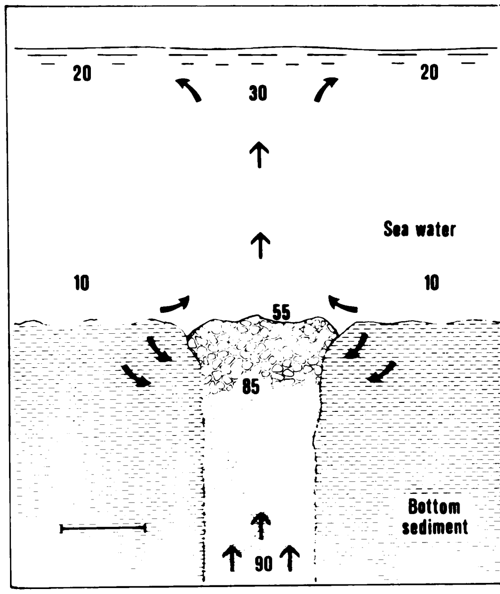

FIG. 2. Schematic drawing of a submarine alkaline hot spring. Numbers indicate temperature in degrees centigrade. The shaded top area in the hot-water duct indicates the possible growth zone of thermophilic, obligately aerobic bacteria. Thin arrows show the flow of hot water, and thick arrows show the flow of cold seawater. The bar represents approximately 5 cm and applies only to the bottom sediment.

Extremely thermophilic bacteria have been isolated from submarine solfatara fields, but we know of no study reporting isolations of thermophilic bacteria from submarine freshwater hot springs. The fact that these bacteria were isolated from such locations is very interesting and immediately poses many questions. *Thermus* spp. have an optimum growth temperature of 70 to 75°C, and the minimum growth temperature is about 55°C (3, 10). This means that they can only grow inside the submarine hot springs. Only a few centimeters from the openings, the temperature would be too low for growth. *Thermus* spp. are obligately aerobic, but the geothermal water, however, is anaerobic until it reaches the surface and absorbs oxygen from the surroundings (Benjaminsson, unpublished report). In the submarine hot springs, the only source of oxygen is the seawater that mixes in. The geothermal water is both hot and essentially fresh. It therefore rises right through the colder and denser seawater and forms a thin, warm layer on the surface. In calm weather, these layers can be quite extensive, and we have measured temperatures up to 50°C in such a layer; the temperature of the seawater underneath was only 10°C (unpublished results). An oxygenated zone in the submarine hot springs which is sufficiently hot for bacterial growth would therefore be very thin. Such a zone could be a few centimeters thick at most. This would also explain why the bacteria must be halotolerant to survive. The structure of a hypothetical thermophilic ecosystem of a submarine alkaline hot spring is shown schematically in Fig. 2.

The submarine *Thermus* spp. seem to be nutritionally similar to their terrestrial counterparts. The nutritional diversity is large, as has been observed for terrestrial strains, and the same organic compounds seem to be the preferred substrates (1). This is somewhat surprising, since one might expect a very different range of substrates to be available in the sea than in terrestrial hot springs.

Any thermophilic organisms growing in a submarine hot spring would be subjected to great fluctuations in the environment and stresses from the cold and salty seawater. Further studies on how organisms can adapt to such an extreme ecosystem would be interesting. Some other interesting organisms have been found in or associated with these hot springs. These include a red, moderately halophilic thermophile and a *Lyngbya*-type cyanobacterium normally not found in terrestrial hot springs.

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