Extremophiles (2000) 4:305–313

ORIGINAL PAPER

Tony Atkinson · Steven Cairns · Don A. Cowan
Michael J. Danson · David W. Hough · D. Barrie Johnson
Paul R. Norris · Neil Raven · Clare Robinson
Rob Robson · Richard J. Sharp

A microbiological survey of Montserrat Island hydrothermal biotopes

Received: March 19, 2000 / Accepted: August 2, 2000

Abstract In March 1996, a survey of hydrothermal sites on the island of Montserrat was carried out. Six sites (Galway's Soufrière, Gages Upper and Lower Soufrières, Hot Water Pond, Hot River, and Tar River Soufrière) were mapped and sampled for chemical, ATP, and microbial analyses. The hydrothermal Soufrière sites on the slopes of the active Chances Peak volcano exhibited temperatures up to almost 100°C and were generally either mildly acidic at pH 5–7 or strongly acidic at pH 1.5–3, but with some hot streams and pools of low redox potential at pH 7–8. Hot Water Pond sites, comprising a series of heated pools near the western shoreline of the island, were neutral and saline, consistent with subsurface heating of entrained seawater. Biological activity shown by ATP analyses was greatest in near-neutral pH samples and generally decreased as acidity increased. A variety of heterotrophic and chemolithotrophic thermophilic organisms were isolated or observed in enrichment cultures. Most of the bacteria that were obtained in pure culture were familiar acidophiles and neutrophiles, but novel, iron-oxidizing species of *Sulfobacillus* were revealed. These species included the first mesophilic iron-oxidizing *Sulfobacillus* strains to be isolated and a strain with a higher maximum growth temperature (65°C) than the previously described moderately thermophilic *Sulfobacillus* species.

Key words Montserrat · Hydrothermal · Thermophiles · Acidophiles · Archaea

Communicated by W.D. Grant

T. Atkinson
Chimaeron Ltd., 8 Centre One, Lysander Way, Salisbury, UK

S. Cairns · R. Robson
School of Animal and Microbial Sciences, University of Reading, Reading, UK

D.A. Cowan (✉)
Department of Biochemistry and Molecular Biology, University College London, Gower St., London WC1E 6BT, UK
Tel. +44-20-7679-2246; Fax +44-20-7679-7193
e-mail: don.cowan@ucl.ac.uk

M.J. Danson · D.W. Hough
Centre for Extremophile Research, Department of Biology and Biochemistry, University of Bath, Bath, UK

D.B. Johnson
School of Biological Sciences, University of Wales, Gwynedd, UK

P.R. Norris
Department of Biological Sciences, University of Warwick, Coventry, UK

N. Raven · R.J. Sharp
Centre for Applied Microbiological Research, Salisbury, UK

C. Robinson
John Innes Centre, Norwich, UK

Introduction

The island of Montserrat (Fig. 1), one of the northeastern members of the Outer Antilles chain, is centered at approximately 62°10′ W, 16°44′ N, about 40 km southwest of Antigua in the West Indies. The island is approximately 14 by 6 km with topography dominated by the active Chances Peak volcano (914 m) and the eroded peaks of three extinct volcanoes, Centre Hills (676 m), Silver Hill (403 m), and South Soufrière Hills (756 m); soufrière is a local term, derived from the French word for sulfur, for regions of terrestrial hydrothermal and gas-venting activity. Montserrat has a history of volcano–seismic crises occurring at approximately 30-year intervals: 1933–1937, 1966–1967, and 1995–1998. The most severe eruption recorded previously, before the recent one, was in 1646 ± 46. The geology of Montserrat has been described in detail by MacGregor (1938) and Rea (1974). Previous interest in the hydrothermal sources (Tombs and Lee 1976) has been restricted to investigations of possible exploitation for geothermal power generation. Heat outputs of 1.3×10^6 and 3×10^5 cal s^{-1} have been recorded from Gages and Galway's Soufrières, respectively (Robson and Willmore 1955). Tombs and Lee (1976) concluded that heated groundwater was also likely to be accessible by borehole at a number of sites around the shore margins of southwestern Montserrat.

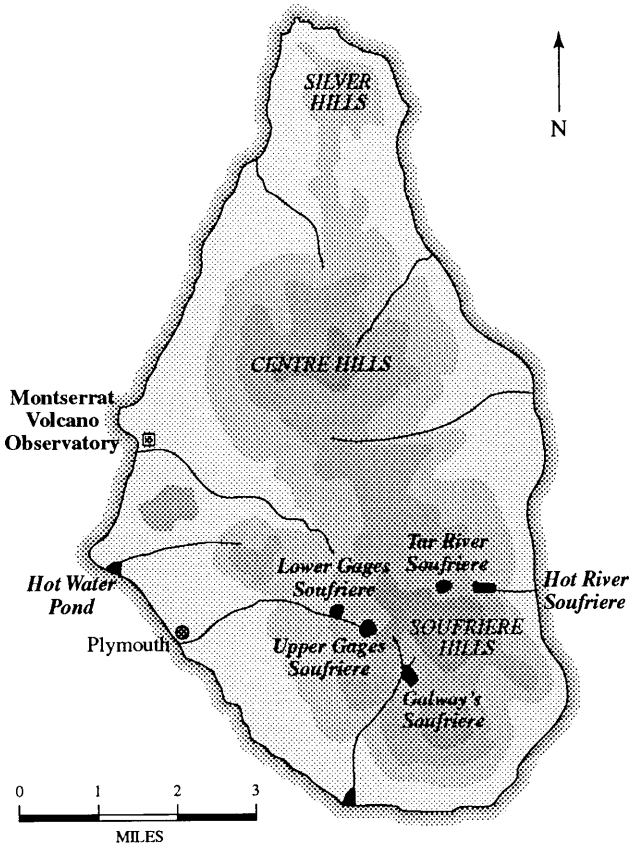

Fig. 1. The island of Montserrat. The thermal sites (Soufrières) are marked with *bold symbols*

Examples of other terrestrial and shallow marine hydrothermal sources have been extensively characterized, both chemically and microbiologically. For example, thermal sites of Yellowstone National Park (USA), Whakarewarewa (New Zealand), Krísuvík (Iceland), and Kamchatka (Russia) have been the subject of detailed ecological studies and have yielded numerous novel thermophilic taxa by classical microbiological methodology. However, direct isolations of DNA from thermal sources and sequencing of 16*S* rRNA genes have indicated that a large number of uncultured phylotypes exist in such biotopes (Yamamoto et al. 1998; Reysenbach et al. 1994; Barns et al. 1994). In general, the nature of the hydrothermal biotope (temperature/pH) is dictated by the subterranean geology and water flow (Keefer 1972; Brock 1986), particularly the degree of mixing of deep, volcanically heated water (buffered in the neutral–alkaline region by CO_2/HCO_3^-) with cold shallow groundwater (strongly acidified by microbial and chemical oxidation of sulfur compounds). Hydrothermal sources thus occur in a bimodal distribution: pH 1–3, sulfurous liquids, low flow rates; and pH 5–8, siliceous liquids, high flow rates.

Various studies (Moreira et al. 1995, 1997) have suggested that, at least within certain microbial genera such as the nonsporulating gram-negative bacteria and the highly oxygen-sensitive hyperthermophilic Archaea, geographically isolated hydrothermal sources harbor genetically distinct species. The apparent geographic isolation of an island source such as Montserrat might therefore suggest that novel thermophilic species should be evident.

Materials and methods

Surveying, sampling, and analyses

Base reference points (at the lowest point of each thermal area) were determined with a global positioning system (Magellan GPS 2000). Sites were mapped progressively from these reference points using a 30-m tape measure with both forward and reverse compass bearings. Observations of hydrothermal sample sites included visual estimations of liquid flow rates. Conductivity and redox values were measured with a Hanna Instruments 9033 conductivity meter. Temperature and pH were measured with a Solomat 520c digital thermometer equipped with a 10-cm steel probe and automatic temperature compensation. Temperature, pH, conductivity, and redox values were also measured using a Whatman Water Tester calibrated with standards as appropriate.

Water and sediment samples for various analyses were collected in sterile plastic bottles and used for immediate chemical analyses, maintained at ambient temperature before establishment of enrichment cultures, or transported into −20°C storage within 2–4 h of recovery. Chemical analyses were carried out using Merck Aquaquant (Mn^{2+}, Cl^-) and Merckaquant (NO_3^-, SO_4^{2-}, Fe^{2+}, K^+, Ca^{2+}) test kits. ATP concentrations were determined in triplicate with a luminometric assay using a SURE™ Y2K portable hygiene monitor (Celsis, Cambridge, UK), and the protocol, reagents, and consumables of the instrument's manufacturer (system SURE swabbing solution, luciferase buffer and lyophilized luciferase, sterile swabs, ATP-free Rohre tubes, ventilation caps, and ATP-free Rainin pipette tips).

Enrichment culture conditions

Samples collected from neutral pH environments in the temperature range 60°–75°C were used for isolation of heterotrophs. The medium used was based on growth media normally used for isolating terrestrial hyperthermophilic anaerobic Archaea and Bacteria. The medium contained, per liter: dextrin, 0.5 g; peptone, 0.5 g; yeast extract, 0.2 g; $(NH_4)_2SO_4$, 1.3 g; KH_2PO_4, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; $FeCl_3 \cdot 6H_2O$, 0.02 g; $MnCl_2 \cdot 4H_2O$, 1.8 mg; $Na_2B_4O_7 \cdot 10H_2O$, 4.5 mg; $ZnSO_4 \cdot 7H_2O$, 0.22 mg; $CuCl_2 \cdot 2H_2O$, 0.05 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.03 mg; $VOSO_4 \cdot 2H_2O$, 0.03 mg; $CoSO_4$, 0.01 mg; resazurin, 1 mg; l-cysteine, 0.5 g. The final pH was adjusted to 6.0 with NaOH. Eight aliquots from each environmental sample were cultivated in deep-well microtiter plates, one sample with each of the following electron acceptors: sulfur, cystine, sodium thiosulfate, sodium sulfite, sodium sulfate, sodium nitrate, sodium fumarate, or no specific acceptor. The microtiter plates were incubated at 65° and 75°C in

anaerobic jars, and samples were subcultured twice a week. Isolation of microorganisms from each well was performed on plates with the medium solidified with gellan gum (Phytagel, Sigma), $CaCl_2 \cdot 2H_2O$ (final concentration, 0.05% w/v) and $MgCl_2 \cdot 6H_2O$ (final concentration, 0.1% w/v).

Acidophiles were isolated by direct plating from samples onto selective solid media or after enrichment in various liquid media (Johnson 1995; Johnson and Roberto 1997). In addition, enrichment cultures were established at various temperatures and pH values (30°–80°C, pH 1.7–3) in a medium containing (in $g\,l^{-1}$) $MgSO_4 \cdot 7H_2O$ (0.4), $(NH_4)_2SO_4$ (0.2), K_2HPO_4 (0.1), and 10 mg $FeSO_4 \cdot 7H_2O$ l^{-1}. Substrates were ferrous iron (25 mM), sulfur flowers ($0.5\,g\,l^{-1}$), and yeast extract (0.01% w/v), both singly and in various combinations. Single-colony isolation of moderately thermophilic acidophiles used this mineral salts medium solidified with phytagel (0.4% w/v) and supplemented with ferrous iron and yeast extract. Preliminary identification of acidophilic isolates was made on the basis of colony characteristics (Johnson and Roberto 1997) and growth characteristics in liquid media. *Sulfobacillus* species were compared by visual inspection of whole-cell electrophoretic protein profiles following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously (Norris et al. 1996).

Results and discussion

Thermal site surveys

Hot Water Pond: base reference 16°43′4″ N, 62°13′83″ W

Hydrothermal sources were located in the upper portion of a 200-m-long, shallow valley, typically as siliceous pools 1–2 m in diameter and 10–30 cm deep. The conductivity and chloride and sulfate concentrations of samples (Table 1), together with the position of these sites at less than 1 m above sea level, were consistent with entrainment of seawater into the hydrothermal sources. In other respects, the hydrothermal sources appeared typical of the neutral pH, siliceous sites found in many thermal areas, but lacked sufficiently high steam volumes to produce very high temperatures and effective thermal mixing within the shallow pools.

Galway's Soufrière: base reference 16°40′06″ N, 62°10′95″ W

The Soufrière comprised a deep, narrow, 200-m-long valley on the southern slopes of Chances Peak with upper and lower elevations of 457 and 433 m, respectively (Fig. 2a). The major hydrothermal source exited as a spring with a flow rate of about 5–10 $l\,min^{-1}$ from a cliff face below Chances Peak. Numerous small thermal sources on the valley bottom and walls supplemented the central stream, giving an estimated flow rate of 20–50 $l\,min^{-1}$ at the lower end of the Soufrière. Different thermal sources showed visual evidence of widely differing mineralization, and analyses confirmed correspondingly wide ranges of temperature, pH, redox potential, and mineral content (Table 2). Lateral margins of the valley were composed of loosely cemented weathered pyroclastic deposits embedded with andesitic rocks. The friable nature of the tephra suggested that origins of individual hydrothermal sources, particularly on the valley floor, were transitory. Extensive gas evolution, sulfur deposition, and mineral salt extrusion were seen on the lower walls of the Soufrière.

Gages Upper and Lower Soufrières: base reference 16°44′73″ N, 62°13′48″ W

These Soufrières were situated in two branches of the upper reaches of Fort Ghaut, a steep narrow valley on the western slopes of Chances Peak (Fig. 2c,d), with midpoint

Table 1. Characteristics of Hot Water Pond hydrothermal sources and sample sites

Site	Description	Temp. (°C)	pH	Redox (mV)	Fe^{2+}	Ca^{2+}	SO_4^{2-}	Cl^-	Mn^{2+}	ATP (RLU)
					(all ions, $mg\,l^{-1}$)					
1	Small hydrothermal vent (3 cm in diameter) with black sediment	58	5.1	+79	3	50	200–400	7500	–	450
2	Hot pool (0.5 × 1 m, 10 cm depth)	73	6.4	+51	3–10	50	200–400	7500	10	210
3	Bubbling pool (1 m diameter, 5–8 cm depth), iron-rich sediment	61/92[a]	5.8–6.1	+29	3	10–25	200–400	7500	10	120
4	Hot pool (1 m × 0.5 m diameter, 10 cm depth)	88	5.9	−118	3	–	200–400	10000	15	129
5	Pool (2 m diameter, 10 cm depth); three central, heated regions	73	5.8	+42	3	25	200–400	7500	15	483[b]

RLU, relative lummosity unit
[a] Bulk water temperature/sediment temperature
[b] ATP measured in surface sample (60°C) with a crystalline sheen

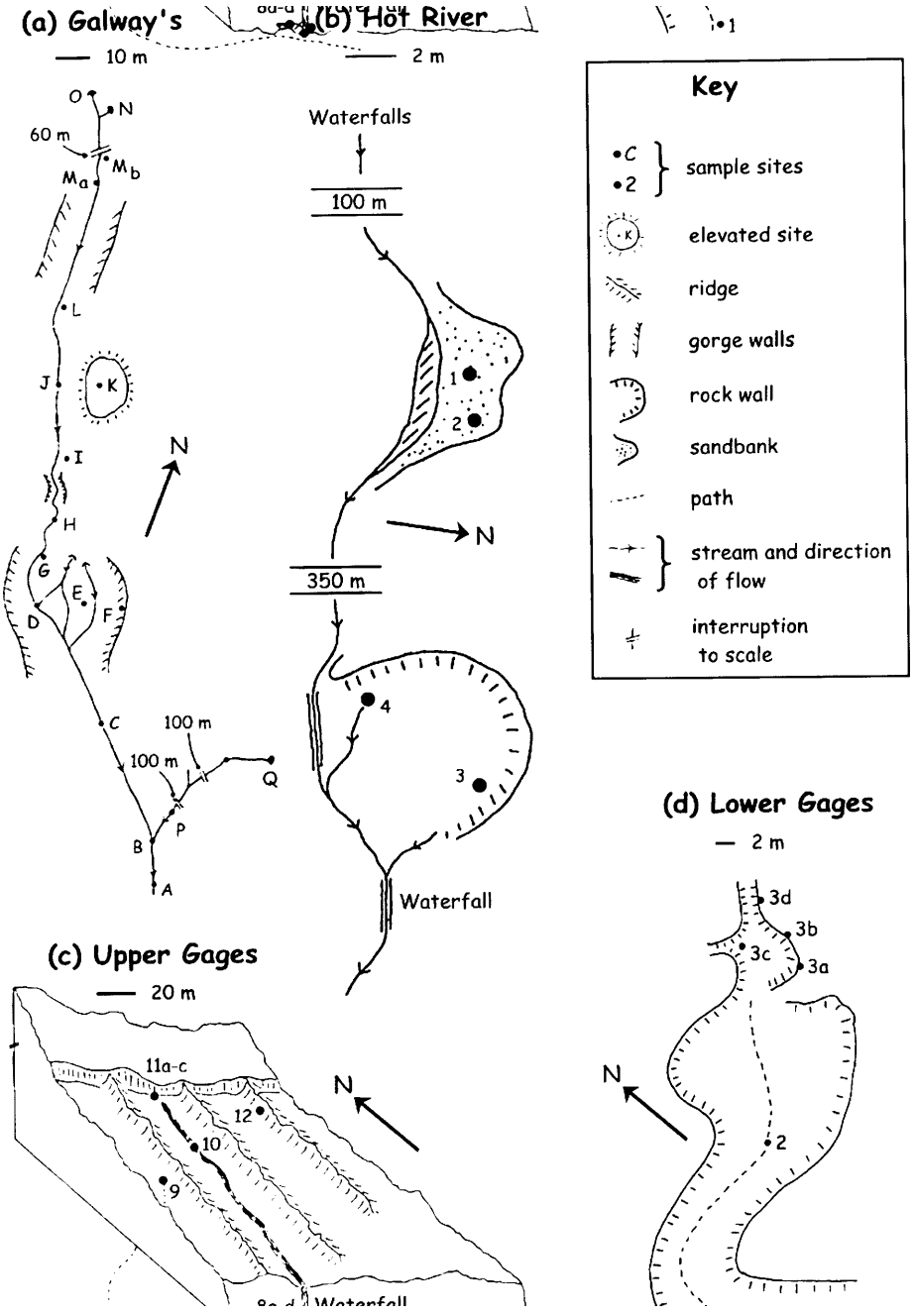

Fig. 2a–d. Site maps of (**a**) Galway's Soufrière, (**b**) Hot River, (**c**) Gages Upper Soufrière, and (**d**) Gages Lower Soufrière

elevations of 518 and 305 m, respectively. Both had numerous, small (typically 2–5 cm diameter), sulfur-encrusted steam vents with little or no hydrothermal flow. The only significant hydrothermal activities were in a 2-m-diameter pool under a 20-m cliff at the base of Gages Upper Soufrière (site 8) and in a small heated stream flowing at less than 5 l min^{-1} from a spring on the upper slopes of Gages Upper (site 11), less than 50 m from the upper rim of the Soufrière. Analyses (Table 3) indicated the sampled sites were typical of low-flow-rate, high-acidity thermal regions.

Hot River: base reference 16°44′69″ N, 62°13′55″ W

A small, fast-flowing stream (estimated flow rate, 500–1000 l min^{-1}) drained the eastern side of the Soufrière Hills via a narrow, deeply eroded valley (Fig. 2b). Thermal sites were reported to exist at several points at an elevation around 700 m (Rea 1974), but thermal activity appeared restricted to two hydrothermal sources with temperatures of less than 50°C and pH near neutrality (Table 4), probably indicative of only a low level of steam heating of deep groundwater.

Table 2. Characteristics of Galway's Soufrière hydrothermal sources and sample sites

Site	Description	Temp. (°C)	pH	Redox (mV)	Conductivity ($\mu S\,cm^{-1}$)	Fe^{2+}	Ca^{2+}	SO_4^{2-}	Cl^-	Mn^{2+}	ATP (RLU)
						(all ions, $mg\,l^{-1}$)					
A	Shoreline stream outfall, suspended light gray sediment	30	3.2	+500	1741	25	–	–	–	–	65
B	Stream bed at confluence of two streams	30	4.1	–	–	–	–	–	–	–	–
C	30 m upstream of B (stream bed)	40	4.2	−36	1812	–	–	–	–	–	–
D	60 m upstream of B (stream bed)	44	4.3	−126	1825	–	–	–	–	–	–
E	Bubbling pool, 0.8 m diameter; suspended gray sediment	93	5.9–6.3	−315	1810	0	100	400–800	20	1	24
F	Runoff channel from boiling source, high sulfur content	59	8.1	−345	1780	0	25–100	400–800	20	0.01	7
G	Bubbling pool, 0.5 m diameter; suspended gray sediment	98	3.0	−140	1902	3–10	50–100	>1600	150	8–10	151
I	Bubbling pool, 3 cm × 15 cm, by mineral salt-encrusted wall	94	1.7–2.6	+10	1865	–	–	–	–	–	–
K	Sulfur-rich mud platform, 7 m above stream, many steam and hydrothermal vents	86–96	1.7–2.3	−6	1980	100	50	>1600	20	3	63
L	Pool (0.7 m × 0.4 m), sulfur-rich sediment, no gassing	50–58	2.6–3.3	−120	1822	100–250	–	–	–	–	826
M_a	Small pool, ferric iron and gelatinous deposits	24	3.4–4.0	+235	1450	50–100	–	–	–	–	43
M_b	Pool with black sediment, 1 m diameter, 20 cm depth	68	5.7	–	–	–	–	–	–	–	31
N	Steam-heated pool, suspended black sediment	99	7.4	−403	1870	0	100	800–1200	40	10	22
O	Source spring of primary stream	94	6.3	−362	1851	0	100	1200–1600	20	3	7–13
P	Bed of side stream, massive sulfur deposition	43	3.9	+30	–	–	–	–	–	–	–
Q	Side stream source, clear water, rocks sulfur-encrusted	55	3.0	–	–	–	–	–	–	–	–

Table 3. Characteristics of Gages Soufrières hydrothermal sources and sample sites

Site	Description	Temp. (°C)	pH	Redox (mV)	Conductivity ($\mu S\,cm^{-1}$)	Fe^{2+}	Ca^{2+}	SO_4^{2-}	Cl^-	Mn^{2+}	ATP (RLU)
						(all ions, $mg\,l^{-1}$)					
3a	Steam vent with sulfur deposition	91	2.0	–	–	–	–	–	–	–	107
3d	Steam vent with sulfur deposition	80	1.0		–	–	–	–	–	–	68
6	Pool (50 cm diameter) with marginal steam vent	65	2.5		–	500	10–25	800–1200	20	20	40
7	Pool (0.7 m × 0.2 m), soft sediment	78	2.4	+423	1856	100–250	50–100	>1600	20	7.5	–
8a	Steam condensate on sulfur-encrusted roof of rock wall recess	97	2.2	–	–	–	–	–	–	–	20
8d	Heated region in 3 m × 2 m pool (20 cm depth)	78	3.4	+204	1791	–	–	–	–	–	2[a]
10	Bubbling pool (50 cm diameter)	82	1.5–2.0	–	–	–	–	–	–	–	55
11	Steam-heated pool (50 cm diameter)	95	1.6	–	–	–	–	–	–	–	11

[a] Apparent inhibition of luciferase reaction

Table 4. Characteristics of Hot River hydrothermal sources and sample sites

Site	Description	Temp. (°C)	pH	Redox (mV)	Conductivity ($\mu S\,cm^{-1}$)	Fe^{2+}	Ca^{2+}	SO_4^{2-}	Cl^-	Mn^{2+}	ATP (RLU)
						(all ions, $mg\,l^{-1}$)					
1	Spring from left bank of stream, with thick algal mat	44	6.5	+121	1423	0	–	–	–	–	27134
3	Cyanobacterial/algal mat in catchment pool (3 m above stream bed) in waterfall (13 m high)	48	6.7	+88	1665	0	100	200–400	75	0	55392
4	Stream bed above thermal source, massive ferric iron precipitates	35	6.4	−17	1130	5	–	–	–	–	5[a]

[a] Apparent inhibition of luciferase reaction

Tar River Soufrière: base reference 16°14′23″ N, 62°10′34″ W

The sample site, positioned on the northern face of the valley that drained the lower northeastern sector of the original Soufrière Hills crater was characterized by the apparent absence of hydrothermal sources but numerous small (1–10 cm diameter) steam vents, most of which were encrusted with recrystallized sulfur and provided samples of steam-heated soil (50°–75°C), sulfur crystals, and liquid condensate (95°–100°C). pH values of soil sample suspensions and condensates were between 1.0 and 3.0.

ATP analyses

The ATP content of the hot samples as determined by luciferase-dependent luminometric analysis was extremely low. Standardization of relative luminosity unit (RLU) values using ATP dilutions (D.A. Cowan, unpublished results) indicated that samples from Hot Water Pond, Galway's, and Gages Soufrières contained between 0.3 and 1 ng ATP ml^{-1}. Assuming a sample volume of approximately 100 ml and a cell ATP content of 0.16–2.25 fg (Fairbanks et al. 1984), an approximate cell density between 10^6 and $10^8\,ml^{-1}$ was indicated. These values compare well with estimates of thermophile biomass in other hydrothermal environments (Parkes et al. 1994; Baross and Deming 1995; Stetter 1998), but are substantially less than those for heated but moderate temperature sites such as the algal mats of Hot River sites 1 and 3 (2×10^8 to $3 \times 10^9\,ml^{-1}$ and 4×10^8 to $6 \times 10^9\,ml^{-1}$, respectively) or self-heating compost ($2 \times 10^{10}\,g^{-1}$; D.A. Cowan, unpublished results). Some caution must be exercised in interpreting these results because it might be argued that the ATP values reflect the degree of metabolic activity rather than the level of microbial biomass. However, it has been shown (D.A. Cowan and D. Sheppard, unpublished results; Karl and Bossard 1985) that ATP content does not necessarily vary widely with the growth phase of an organism. Surprisingly, variations in ATP content over three orders of magnitude have also been demonstrated in stationary-phase cells of different thermophilic bacteria and archaea (Raven et al. 1998), but this could have reflected efficiency of cell lysis rather than true intracellular ATP pools.

Isolation of neutrophilic thermophiles

Aerobic organisms were isolated at 60°–75°C from neutral pH sites at Hot Water Pond and Galway's Soufrière. Phenotypic characterization of 11 independent isolates indicated optimum growth conditions of approximately 65°C and pH 6–6.5. Microscopic examination showed all isolates to be gram-positive rods, some of which were spore forming. Partial 16*S* rRNA sequencing (data not shown) indicated that the spore-forming isolates were members of the genus *Bacillus*. Two non-spore-forming isolates were also isolated from various neutral pH samples and were tentatively identified as *Thermus aquaticus* and *Thermus ruber* on the basis of growth temperatures, cell and colony morphology, and pigmentation. Attempts to isolate thermophilic anaerobic neutrophiles in pure culture were unsuccessful.

Isolation of acidophiles

Acidic samples from Galway's Soufrière sites A, L, and M_a were examined for mesophiles and moderate thermophiles and sites G, I, K, and N for thermophiles. The sample from the site N area was taken from a small spring (pH 3.1, 93°C) with peripheral sulfur deposits rather than from a larger, adjacent pool of approximately neutral pH noted in Table 2. Samples from Gages Soufrière sites 6, 8, and 11 were used for establishing enrichment cultures over a wide temperature range. The site 8 sample was taken from the coolest region (44°C) of a heated pool (site 8d; Table 3). Samples were examined by microscopy several days after collection. Organisms appeared most numerous in samples from sites L (a variety of rods) and N (some rods and *Sulfolobus*-like organisms). The estimated ATP content of water at site L was far higher than at any of the other acidic sites (see Table 2). Relatively few rods were observed directly in samples from sites M_a and 8 and the relatively few organisms that were observed in samples from sites G and K resembled *Sulfolobus*-like organisms.

Mesophilic acidophiles

Colonies of heterotrophs, sulfur oxidizers, and iron oxidizers were obtained from all low-temperature site samples

Table 5. Characteristics of *Sulfobacillus*-type species and three novel species isolated from Montserrat samples

	Optimum temperature (°C)	Endospores	Growth on:		
			Ferrous iron plus CO_2	Ferrous iron plus yeast extract	Yeast extract
S. thermosulfidooxidans	50	+	+	+	+
S. acidophilus	50	+	+	+	+
Strain 6	58	+	−	+	+
Strain RIV-14	33	+	+	+	+
Strain L-15	37	+	+	+	+

that were incubated on solid media at 30°C. Of the iron-oxidizing mesophiles, *Leptospirillum ferrooxidans* was more numerous than *Acidithiobacillus* (formerly *Thiobacillus*) *ferrooxidans* on plates inoculated with sample L, while *A. ferrooxidans* outnumbered *L. ferrooxidans* by approximately 10:1 following direct plating of a sample from the cooler site, M_a. The relative distribution of these organisms cannot be assessed on the basis of a single sampling, but these observations corresponded to the higher temperature tolerance of *L. ferrooxidans*-like organisms compared to *A. ferrooxidans* (Norris 1990). A similar relative distribution of these bacteria in relation to temperature was reported for acidic mine waters (Hallman et al. 1992; Schrenk et al. 1998). Unexpectedly, previously undescribed mesophilic organisms that resembled moderately thermophilic, ferrous iron-oxidizing *Sulfobacillus* species were also isolated on plates inoculated with samples from sites M_a and L. These gram-positive strains were compared to the related moderate thermophiles from Montserrat (see later; Table 5). Two colony types of sulfur-oxidizing acidophilic rods were observed following plating of sample L, but whether these corresponded to *Acidithiobacillus* (formerly *Thiobacillus*) *thiooxidans* and the similar but thermotolerant *Acidithiobacillus* (formerly *Thiobacillus*) *caldus* was not determined. Analysis of rRNA genes amplified from DNA extracted from site L suggested that *A. caldus* was more likely to constitute a major portion of the in situ microflora (Burton and Norris 2000). Enrichment culture at 30°C for acid-tolerant sulfate-reducing bacteria in medium at pH 3–4 produced growth following inoculation with about a quarter of a wide range of samples from site A and various warmer sites.

Moderately thermophilic acidophiles

The previously described species of moderately thermophilic, acidophilic, iron- and/or sulfur-oxidizing acidophilic bacteria (*Acidithiobacillus caldus* and species of *Sulfobacillus* and *Acidimicrobium*; Norris and Johnson 1998) were all observed in Montserrat samples. Organisms resembling *A. caldus* were observed in 45°C sulfur-enrichment cultures of samples from several of the warm pools, including sites 6, 8, and L.

All samples from Galway's and Gages Soufrières that were used to inoculate enrichment cultures at pH 1.7 with ferrous iron as substrate, with or without yeast extract supplementation, produced growth and iron oxidation within 2 days at 48°C. Samples incubated at 60°C with ferrous iron and yeast extract also promoted rapid iron oxidation. Organisms resembling *Acidimicrobium ferrooxidans* and *Sulfobacillus* species were observed in 48°C cultures. Iron-oxidizing strains that were readily obtained in pure culture resembled *Sulfobacillus* species when standard (single-layer) phytagel-solidified medium was used to obtain single colonies. In contrast, both *Sulfobacillus* and *Acidimicrobium* types grew on overlay medium. Heterotrophic organisms resembling *Alicyclobacillus* species grew on both types of plates. Pure cultures of *Sulfobacillus* and *Alicyclobacillus* strains were selected and examined further.

Three different electrophoretic protein profiles (from SDS-PAGE) were found for the isolated *Sulfobacillus* strains. Two of the profiles, from strains isolated at 48°C, were essentially identical to those of the type strains of *Sulfobacillus thermosulfidooxidans* and *Sulfobacillus acidophilus*. The third profile was from a *Sulfobacillus*-like isolate from a 60°C ferrous iron/yeast extract enrichment culture. In contrast to *S. acidophilus* and *S. thermosulfidooxidans*, this isolate (strain 6) did not appear to grow autotrophically. The optimum and maximum growth temperatures for strain 6 were respectively 8°C and 5°C higher than those of the other moderately thermophilic *Sulfobacillus* species (see Table 5). Comparison of 16*S* rRNA gene sequences indicated that the closest relative to strain 6 was *S. acidophilus*, with 97% identity over 1467 nucleotides (N. Burton and P. Norris, unpublished data). Phylogenetic analysis of strains RIV-14 and L-15 has placed them with *Sulfobacillus* species (Yahya et al. 1999). Apart from their lower optimum temperatures for growth, they also differed from the other species in their tolerance of greater acidity, with good growth on ferrous iron at pH 1.0 and optimum growth at pH 1.5–1.6.

Heterotrophic acidophiles grew as white colonies on ferrous iron plates supplemented with yeast extract or tryptone soya. Three strains were isolated on the basis of different colony morphology. Their acidophily and endospore formation suggested an affiliation with *Alicyclobacillus* species, which was confirmed by 16*S* rRNA gene sequence analysis (data not shown). One strain was closely related to *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius* whereas the other two were related

separately to unnamed isolates from Yellowstone National Park, USA, and from acidic coal spoil in the UK (N. Burton and P. Norris, unpublished data). They were isolated from Gages and Galway's Soufrières, and all three were found in a single sample from Gages Soufrière site 8.

Thermoacidophiles

Growth of *Sulfolobus*-like organisms was observed within 48 h at 68°C and 78°C in sulfur (pH 3) and pyrite (pH 2) enrichment cultures that were inoculated with samples several days after their collection from Soufrière sample sites (including Galway's sites G, I, and K and Upper Gages sites 6, 8, and 11). Collected samples that showed the most organisms by microscopy generally produced the earliest enrichment culture growth. Sulfur-enrichment cultures at 88°C did not produce good growth, but a low number of *Sulfolobus*-like organisms were observed to survive for more than 3 weeks before growing rapidly when the temperature was reduced to 78°C. Growth was also established at 78°C, but more slowly (after 96 h), following inoculation with a sample of steam-heated solid and sulfur crystals taken from a moist gas vent at Tar River Soufrière.

Pure cultures of *Sulfolobus*-like cultures were not obtained, but after many serial subcultures on sulfur or pyrite, the electrophoretic profiles (from SDS-PAGE) of whole-cell proteins of several cultures were compared with those of known species and enrichment cultures from other countries (data not shown). Enrichment cultures at 68°C that were established with various samples showed protein profiles essentially identical to that of *Sulfolobus metallicus*. Enrichment cultures of samples at 78°C showed protein profiles that did not match those of known species, but long-term maintenance of a mixture of Montserrat cultures on pyrite at 78°C produced a protein profile essentially identical to that of a 78°C pyrite enrichment culture from Iceland (Norris et al. 2000).

16*S* rDNA analysis of acidic samples

With the exception of *Acidithiobacillus caldus*, the organisms isolated directly by plating and observed in enrichment cultures were different from those suggested to comprise major portions of the in situ populations after examination of 16*S* rRNA genes amplified from DNA extracted directly from the acidic samples (Burton and Norris 2000). For example, two novel 16*S* rRNA gene sequences from Galway's and Gages Soufrière samples that were placed phylogenetically within the thermoacidophilic Crenarchaeota (Norris et al. 2000) confirmed the protein profile indications (noted previously) of *Sulfolobus*-like organisms that remain to be cultured. The dominance of a few sequences in the limited clone banks presumably precluded revelation of genes from organisms that were present only as small fractions of in situ populations but which, like *S. metallicus*, may have been favored by the laboratory culture conditions.

Montserrat-specific location of novel acidophiles?

The specific conditions of acidic, geothermal sites can influence the nature of the indigenous microflora. For example, marine springs at Vulcano, Italy (Aeolian Islands), support chloride-tolerant, iron-oxidizing acidophiles such as *Thiobacillus prosperus* (Huber and Stetter 1989) rather than the chloride-sensitive *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* that were found in the low-salt, acidic springs on Montserrat. The physicochemical analyses of the Montserrat sites indicated conditions typical of geographically widespread freshwater hot springs, whether continental (e.g., those in Yellowstone National Park, USA; Brock 1978) or on isolated islands (e.g., the Azores). Consequently, the variety of organisms common to well-studied geothermal sites were found on Montserrat. Previously undescribed acidophiles were also isolated and indicated by environmental rRNA gene analysis, which may reflect geographic isolation and possibly the transient and variable nature of the hydrothermal sites.

The proximity and constant intermixing of hydrothermal outflows with widely differing pH and temperature characteristics may have provided an environment where the ability to adapt to rapidly changing environmental conditions was a key requirement for microbial survival. However, a wider distribution than Montserrat would probably be revealed for many of the novel organisms and phylotypes if similar surveys were conducted in other geothermal environments. Since this Montserrat survey, the novel thermotolerant *Sulfobacillus* species (strain 6) has also been found in samples from the Azores (P. Norris, unpublished results), a rRNA gene sequence first seen from Montserrat samples that represented a *Ferroplasma* strain and another that possibly represented a genus of undescribed sulfur-respiring anaerobes which have also been found at other locations (Burton and Norris 2000). Sulfate-reducing bacteria with at least as great tolerance of acid as the previously undescribed Montserrat strains were subsequently found at acidic, disused mine sites in the UK (Sen and Johnson 1999).

Within 6 months of this survey, the Soufrière Hills volcano entered a new phase of vigorous eruption. Rapid growth of the lava dome through the following 12 months culminated in a period of explosive and pyroclastic extrusive activity that devastated both the biological and social structure of southern parts of the island, the region where all but one of the hydrothermal sources were located. Repeated pyroclastic flows from the eastern, northwestern, and southwestern flanks of the dome completely engulfed Gages, Galway's, and Tar River Soufrières. The hydrothermal sources, with the exception of the Hot Water Pond saline thermal pools on the southwestern shoreline of the island, have ceased to exist in the forms that were mapped and sampled. The materials collected in March 1996 thus represent the last remnants of the preexisting thermophilic microbiology of these sites, on the working assumption that the impact of 600°–1000°C molten pyroclastics would effectively sterilize all contact surfaces and substrata to a significant (but undetermined) depth. The Montserrat

hydrothermal resource may therefore offer an opportunity for fundamental studies on microbial recolonisation. Reports from the Montserrat Volcano Observatory (see http://www.geo.mtu.edu/volcanoes/west.indies/soufriere/govt/) have indicated that within weeks of the first pyroclastic surges down Galway's Soufrière, the hydrothermal stream had reemerged from the cooling pyroclastic debris. Resampling of this source with reference to the preliminary survey results presented here and in the accompanying paper (Burton and Norris 2000) could illustrate pathways of recolonization of the geothermal biotopes.

Acknowledgments The authors thank the Biotechnology and Biological Sciences Research Council of the UK for financial support. We also wish to thank his Excellency the Governor of Montserrat, Frank Savage, The Montserrat National Trust, Dr. Simon Young of the Montserrat Volcano Observatory, the Commissioner of Police, Frank Hooper, John Harper of Montserrat Airways, and our guide, James Lee. We are grateful to Celsis Ltd., UK, for the loan of the ATP bioluminescence monitor and provision of the assay reagents.

References

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot-spring environment. Proc Natl Acad Sci USA 91:1609–1613

Baross JA, Deming JW (1995) In: Karl DM (ed) Microbiology of deep sea hydrothermal vent environments. CRC Press, Boca Raton, pp. 169–217

Brock TD (1978) Thermophilic microorganisms and life at high temperatures. Springer, New York

Brock TD (1986) Notes on the ecology of thermophilic archaebacteria. Syst Appl Microbiol 7:213–215

Burton NP, Norris PR (2000) Microbiology of acidic, geothermal springs of Montserrat: environmental rDNA analysis. Extremophiles 4:315–320

Fairbanks BC, Woods LE, Bryant RJ, Elliot ET, Cole CV, Coleman DC (1984) Limitations of ATP estimates of microbial biomass. Soil Biol Biochem 6:549–558

Hallman R, Friedrich A, Koops H-P, Pommerening-Röser A, Rohde K, Zenneck C, Sand W (1992) Physiological characteristics of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and physiochemical factors influence microbial metal leaching. Geomicrobiol J 10:193–206

Huber H, Stetter KO (1989) *Thiobacillus prosperus*, sp. nov., represents a new group of halotolerant metal-mobilizing bacteria isolated from a marine geothermal field. Arch Microbiol 151:479–485

Johnson DB (1995) Selective solid media for isolating and enumerating acidophilic bacteria. J Microbiol Methods 23:205–218

Johnson DB, Roberto FF (1997) Biodiversity of acidophilic bacteria in mineral leaching and related environments. In: IBS97/Biomine Conference Proceedings. Australian Mineral Foundation, Glenside, Australia, pp. P3.1–P3.10

Karl DM, Bossard P (1985) Measurement and significance of ATP and adenine nucleotide pool turnover in microbial cells and environmental samples. J Microbiol Methods 3:125–139

Keefer WR (1972) The geologic story of Yellowstone National Park, US Geol Surv Bull 1347

MacGregor AG (1938) The volcanic history and petrology of Montserrat, with observations on Mount Pelé in Martinique. Philos Trans R Soc Lond B 229:1–90

Moreira LM, Da Costa MS, Sacorreia I (1995) Plasmid RFLP profiling and DNA homology in *Thermus* isolated from hot-springs of different geographical areas. Arch Microbiol 164:7–15

Moreira LM, Da Costa MS, Sacorreia I (1997) Comparative genomic analysis of isolates belonging to the six species of the genus *Thermus* using pulsed-field gel electrophoresis and ribotyping. Arch Microbiol 168:92–101

Norris PR (1990) Acidophilic bacteria and their activity in mineral sulfide oxidation. In: Ehrlich HL, Brierley CL (eds) Microbial mineral recovery. McGraw-Hill, New York, pp. 3–27

Norris PR, Johnson DB (1998) Acidophilic microorganisms. In: Horikoshi K, Grant WD (eds) Extremophiles: microbial life in extreme environments. Wiley-Liss, New York, pp. 133–153

Norris PR, Clark DA, Owen JP, Waterhouse S (1996) Characteristics of *Sulfobacillus acidophilus* sp. nov. and other moderately thermophilic mineral sulphide-oxidizing bacteria. Microbiology 142:775–783

Norris PR, Burton NP, Foulis NM (2000) Acidophiles in bioreactor mineral-processing. Extremophiles 4:71–76

Parkes RJ, Cragg BA, Bale SJ, Getliff JM, Goodman K, Rochelle PA, Fry JA, Weightman AJ, Harvey SM (1994) Deep bacterial biosphere in Pacific Ocean sediments. Nature (Lond) 371:410–413

Raven N, Cowan D, Danson M, Hough D, Norris P, Johnson B, Cairns S, Sharp R, Atkinson A (1997) Measuring ATP in a volcano. In: Stanley PE, Smither R, Simpson WJ (eds) A practical guide to the industrial uses of ATP-luminescence in rapid microbiology. Cara Technology Publications, Lingfield, pp. 73–79

Rea J (1974) The volcanic geology and petrology of Montserrat, West Indies. J Geol Soc Lond 130:341–366

Reysenbach AL, Wickham GS, Pace NR (1994) Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl Environ Microbiol 60: 2113–2119

Robson GR, Willmore PL (1955) Some heat flow measurements in West Indian soufrières. Bull Volc 17:13–39

Schrenk MO, Edwards KJ, Goodman RM, Hamers RJ, Banfield JF (1998) Distribution of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*: implications for generation of acid mine drainage. Science 279:1519–1522

Sen AM, Johnson DB (1999) Acidophilic sulphate-reducing bacteria: candidates for bioremediation of acid mine drainage. In: Amils R, Ballester A (eds) Biohydrometallurgy and the environment toward the mining of the 21st century. Elsevier, Amsterdam, pp. 709–718

Stetter KO (1998) Hyperthermophiles: isolation, classification and properties. In: Horikoshi K, Grant WD (eds) Extremophiles: microbial life in extreme environments. Wiley-Liss, New York, pp. 1–24

Tombs JMC, Lee MK (1976) Geophysical surveys of Montserrat for geothermal resources. Report no. 27. Institute of Geological Science, London

Yahya A, Roberto FF, Johnson DB (1999) Novel mineral-oxidizing bacteria from Montserrat (W.I.): physiological and phylogenetic characteristics. In: Amils R, Ballester A (eds) Biohydrometallurgy and the environment toward the mining of the 21st century, part A. Elsevier, Amsterdam, pp. 729–739

Yamamoto H, Hiraishi A, Kato K, Chiura HX, Maki, Y, Shimizu A (1998) Phylogenetic evidence for the existence of novel thermophilic bacteria in hot spring sulfur-turf microbial mats in Japan. Appl Environ Microbiol 64:1680–1687