

Trello Beffa · Michel Blanc · Michel Aragno

## Obligately and facultatively autotrophic, sulfur- and hydrogen-oxidizing thermophilic bacteria isolated from hot composts

Received: 20 July 1995 / Accepted: 25 September 1995

**Abstract** A variety of autotrophic, sulfur- and hydrogen-oxidizing thermophilic bacteria were isolated from thermogenic composts at temperatures of 60–80°C. All were penicillin G sensitive, which proves that they belong to the Bacteria domain. The obligately autotrophic, non-spore-forming strains were gram-negative rods growing at 60–80°C, with an optimum at 70–75°C, but only under microaerophilic conditions (5 kPa oxygen). These strains had similar DNA G+C content (34.7–37.6 mol%) and showed a high DNA:DNA homology (70–87%) with *Hydrogenobacter* strains isolated from geothermal areas. The facultatively autotrophic strains isolated from hot composts were gram-variable rods that formed spherical and terminal endospores, except for one strain. The strains grew at 55–75°C, with an optimum at 65–70°C. These bacteria were able to grow heterotrophically, or autotrophically with hydrogen; however, they oxidized thio-sulfate under mixotrophic growth conditions (e.g. pyruvate or hydrogen plus thiosulfate). These strains had similar DNA G+C content (60–64 mol%) to and high DNA:DNA homology (> 75%) with the reference strain of *Bacillus schlegelii*. This is the first report of thermogenic composts as habitats of thermophilic sulfur- and hydrogen-oxidizing bacteria, which to date have been known only from geothermal manifestations. This contrasts with the generally held belief that thermogenic composts at temperatures above 60°C support only a very low diversity of obligatory heterotrophic thermophiles related to *Bacillus stearothermophilus*.

**Key words** Compost · Thermophilic bacteria · *Hydrogenobacter* · *Bacillus schlegelii* · Sulfur- and hydrogen-oxidizing bacteria

### Introduction

Composting is a self-heating, aerobic, solid-phase biodegradative process of organic waste materials (Waksman et al. 1939; Finstein and Morris 1975; De Bertoldi et al. 1983). The composting process at the microbial level involves several interrelated factors, i.e., metabolic heat generation, temperature, ventilation (oxygen input), moisture content, and available nutrients.

Temperature reflects both the prior and the current rate of microbial activity. The temperature increase involves a rapid transition from a mesophilic to a thermophilic microflora. The compost ecosystem is limited by excessive heat accumulation. The terminal phase of composting is a cooling and maturation stage. The amount of readily available nutrients becomes a limiting factor that causes a decline in microbial activity and heat output. A high diversity of bacteria, fungi, and Actinomycetes has been reported during the short initial mesophilic phase and the cooling or maturation phase (Finstein and Morris 1975; De Bertoldi et al. 1983). However, the present knowledge of microbial diversity during the thermogenic (> 60°C) phase is surprisingly poor. The high temperatures reached (60–80°C) are often considered to reduce the microbial diversity dramatically (Strom 1985a, b; Nakasaki et al. 1985a, b; Fujio and Kume 1991). At the highest temperatures considered (65–69°C), previous studies focused only on obligately heterotrophic bacteria, and only strains related to *Bacillus stearothermophilus* were identified (Strom 1985a, b).

Microbial diversity could be expected during the thermogenic phase, where degradation and mineralization of complex organic matter also takes place (Schulze 1962; Nakasaki et al. 1985b). For example, autotrophic sulfur oxidizers oxidize (and, thus, detoxify) the hydrogen sulfide generated through the mineralization of organic sulfur compounds, whereas hydrogen-oxidizing bacteria use the molecular hydrogen produced by fermentative reactions during transitions from aerobic to anaerobic decomposition (Dugnani et al. 1986; Beffa et al. 1995). Such

T. Beffa (✉) · M. Blanc · M. Aragno  
Laboratoire de Microbiologie, Université de Neuchâtel,  
Rue Emile-Argand 11, CH-2007 Neuchâtel, Switzerland  
Tel. +41-38-232-261; Fax +41-38-232-231

transitions occur readily in the heterogeneous, biologically active, and oxygen-poor matrix in composts (Miller 1989).

The purpose of this study was to provide a better understanding of the taxonomic and functional diversity of highly thermophilic, facultatively or obligately autotrophic bacteria during the thermogenic stage of the composting process ( $> 60^{\circ}\text{C}$ ).

## Materials and methods

### Compost facilities and sampling

The industrial composting facilities studied (10 sites) represent the main types of composting systems used in Switzerland, such as classical open-air windrows, boxes in a semi-closed hall with automated turning and aeration, or closed bioreactors with automated aeration. Organic materials subjected to composting varied considerably and consisted mainly of green waste, wood chips, and kitchen waste, or sewage sludge. The compost facilities were located 30–250 km from the authors' laboratory. None of the compost facilities studied used seeding with a commercial compost-starter containing thermophilic bacteria.

### Enrichment and culture procedures

Enrichment, parallel serial dilutions, and cultures were performed in basal mineral medium (Aragno 1991). One gram fresh-weight organic material from hot ( $60\text{--}80^{\circ}\text{C}$ ) compost was placed in 10 ml of sterile basal mineral medium and shaken at 150 rpm for 30 min at room temperature. The parallel serial dilutions ( $10^{-1}\text{--}10^{-10}$ ) and cultures were incubated at  $70^{\circ}\text{C}$  for 1–14 days under micro-aerophilic conditions (5 kPa oxygen). Autotrophic hydrogen-oxidizing bacteria were grown under an atmosphere of  $\text{H}_2/\text{CO}_2/\text{O}_2$  (35 kPa: 10 kPa: 5 kPa, measured at room temperature) according to Aragno (1991). Autotrophic sulfur-oxidizing bacteria were grown with 20 mM thiosulfate or  $5\text{ g l}^{-1}$  crystalline elemental sulfur ( $\text{S}^0$ ) under an atmosphere of  $\text{N}_2/\text{CO}_2/\text{O}_2$  (35 kPa: 10 kPa: 5 kPa, measured at room temperature).  $\text{NaHCO}_3$  (60 mM) or a few grains of  $\text{CaCO}_3$  were added to prevent excessive acidification under sulfur-oxidizing conditions.

Pure colonies were isolated by successive plating on the same media solidified with  $13\text{ g l}^{-1}$  agar-agar (Oxoid, Fakola AG, Basel, Switzerland) with or without 20 mM thiosulfate. Gas mixtures were the same as for liquid cultures except that  $\text{O}_2$  was at 2.5 kPa. Distinct colonies appeared after 1–7 days. Obligately autotrophic strains that oxidize both hydrogen and sulfur were able to form colonies only when the solid medium was supplemented with 20 mM thiosulfate, as previously reported for hydrogen-oxidizing, thermophilic bacteria (Alfredsson et al. 1986). The purity of each culture was routinely checked microscopically and by plating on solid mineral and organic media. We report the minimal and maximal values of the parallel serial dilutions observed in hot compost samples from 10 different compost facilities.

Heterotrophic growth of pure strains of spore-forming bacteria was assessed in liquid basal mineral medium supplemented with 0.2% w/v organic compounds (acetate, pyruvate, D-glucose) or with 0.8% w/v nutrient broth (Merck, Darmstadt, Germany) under an atmosphere of  $\text{N}_2/\text{O}_2$  (45 kPa: 5 kPa, measured at room temperature) and under normal air conditions. Heterotrophic growth in liquid cultures of pure strains of non-spore-forming, obligately autotrophic bacteria was determined under an atmosphere of  $\text{N}_2/\text{O}_2$  (45 kPa: 5 kPa, measured at room temperature) in basal mineral medium at  $65^{\circ}\text{C}$  supplemented with 0.1% (w/v) organic compounds. The organic compounds tested were as follows: D-arabinose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannose, D-xylose, soluble starch, acetate, citrate, formate, fumarate,  $\beta$ -hydroxybutyrate, gluconate, DL-lactate, malate, pyruvate, succinate,

L-alanine, L-arginine, L-aspartate, L-glutamate, glycine, L-histidine, L-leucine, L-lysine, L-methionine, L-proline, L-serine, L-tryptophan, L-valine, ethanol, isopropanol, methanol, nutrient broth (Merck, Darmstadt, Germany), and yeast extract (Merck, Darmstadt, Germany).

The growth of obligately autotrophic, hydrogen-oxidizing thermophilic bacteria (e.g., *Hydrogenobacter thermophilus*) was severely inhibited by the presence of 20 mM pyruvate (Shiba et al. 1984). Accordingly, the effect of 2.5 and 25 mM pyruvate on the autotrophic growth of pure strains of non-spore-forming autotrophic bacteria was determined in the basal mineral medium under an atmosphere of  $\text{H}_2/\text{CO}_2/\text{O}_2$  (35 kPa: 10 kPa: 5 kPa, measured at room temperature). Cultures were incubated at  $75^{\circ}\text{C}$  during 10 days.

### Reference or type strains

Thermophilic bacteria related to the genus *Hydrogenobacter* were isolated from the following geothermal sources: *Hydrogenobacter thermophilus* strain TK-H (Kyshu, Japan; Kawasumi et al. 1984); *Hydrogenobacter* strain MF-3 (Etna, Italy; Aragno 1992), *Hydrogenobacter* strain T-3 (Tuscany, Italy; Bonjour and Aragno 1986), and *Hydrogenobacter* strain H-1 (Borgarfjörður, Iceland; Kristjansson et al. 1985); *Calderobacterium hydrogenophilum* strain Z-829, (Kamchatka, USSR; Kryukov et al. 1983). *Bacillus schlegelii* type strain (DSM 2000, Deutsche Sammlung von Mikroorganismen und Zellkulturen) was isolated from a cold environment (lake sediment, Le Loclat, Switzerland; Schenk and Aragno 1979).

### Characterization of isolates

Cell numbers and types were estimated by phase-contrast microscopic examination of enrichment cultures performed from serial dilutions and by the ability to form colonies on solid media, with either thiosulfate or hydrogen as electron source. Morphology and cell size were estimated on actively  $\text{H}_2$ -growing cultures at  $70^{\circ}\text{C}$  using phase-contrast microscopy. Sensitivity to penicillin was tested during 5 days of incubation at  $65^{\circ}\text{C}$  in liquid media containing  $10\text{ mg l}^{-1}$  benzyl-penicillin (Fluka Chemie, Buchs, Switzerland) according to the procedure described by Schenk and Aragno (1979). Temperature dependent growth was tested between  $50^{\circ}\text{C}$  and  $85^{\circ}\text{C}$  at  $5^{\circ}\text{C}$  intervals.

### DNA base composition and hybridization

DNA was isolated by the procedure (slightly modified) described by Jenni et al. (1987). Cells ( $1\text{--}2\text{ g}$  fresh weight) were collected by centrifugation at  $6,000 \times g$  for 20 min at  $4^{\circ}\text{C}$  and washed with 15 ml of 10 mM Tris-HCl buffer (pH 7.2) supplemented with NaCl (0.9% w/v). Washed cells were resuspended in 16 ml MUP buffer (7.5 M urea, 120 mM  $\text{NaH}_2\text{PO}_4$ , 120 mM  $\text{Na}_2\text{HPO}_4$ , final pH 7.5) and broken by ultrasonic treatment using a Branson model 450 sonicator for 10 min at 35–40 W on ice. The broken cells were mixed with 5 g hydroxylapatite (Fluka AG, Buchs, Switzerland) equilibrated with MUP buffer and left for 1 h with occasional agitation at room temperature. The hydroxylapatite was decanted, washed with additional MUP buffer, and decanted again. The hydroxylapatite-DNA complex was then poured onto a GF/A filter (Whatman, Springfield Mill, UK) in a vacuum-filtration system. The hydroxylapatite-DNA was washed twice with 10 ml of MUP buffer and then 4 times with 10 ml of 14 mM sodium phosphate buffer (pH 6.8). The DNA was eluted with 400 mM sodium phosphate buffer (pH 6.8). The DNA fractions were pooled and concentrated with 2-butanol to about 4 ml. The purified DNA was then dialyzed against saline citrate buffer (15 mM NaCl – 1.5 mM Na-citrate). The ratio of absorbance of the DNA preparations at 260 and 280 nm was around 1.9, which indicates that they were essentially free of proteins. The concentrated DNA solutions were stored at  $-20^{\circ}\text{C}$ . DNA

mol% G+C content was determined by the melting point ( $T_m$ ) method (Marmur and Doty 1962) and calculated according to the formula of Owen and Hill (1979). DNA-DNA homologies were measured spectrophotometrically by following the renaturation rates at  $T_m - 20^\circ\text{C}$  according to De Ley et al. (1970).

#### Protein gel electrophoresis

Cells (about 1 g fresh weight) growing actively under autotrophic conditions (hydrogen) were collected and washed as described above. Washed cells were resuspended in 1–2 ml of 100 mM Tris-HCl buffer (pH 8.0). Cells were broken by ultrasonic treatment using a Branson model 450 sonicator for 0.5–1.5 min at 10–20 W on ice. The broken-cell suspension was centrifuged at  $12,000 \times g$  for 10 min at  $4^\circ\text{C}$  and the supernatant (cell-free extract) was stored at  $-20^\circ\text{C}$ . Proteins (25–30  $\mu\text{g}$  per lane) were resolved by denaturing SDS-PAGE (5% stacking gel and 12.5% resolving gel) at  $22^\circ\text{C}$  according to Hames and Rickwood (1990). Gels were stained with Coomassie Brilliant blue R-250 after electrophoresis. Molecular mass standards (14.4–200 kDa) were purchased from Bio-Rad Laboratories (Hercules, Calif., USA).

#### Respiratory activity measurements

Cells for respiratory activity measurements were taken from actively growing cultures, washed, and resuspended gently in basal mineral medium according to Beffa et al. (1991a). The respiratory activities were measured polarographically with an oxygen electrode (Hansatech, model CBH<sub>2</sub>, adjustable volume) at  $60^\circ\text{C}$  as described previously (Beffa et al. 1991b, 1992). Oxygen consumption was calculated on the basis of 134 nmol O<sub>2</sub> ml<sup>-1</sup> in air-saturated medium at  $60^\circ\text{C}$  according to Beffa et al. (1991b). The final cell concentration in the respiratory cuvette was 0.02–0.2 mg protein ml<sup>-1</sup>. The respiratory substrates were supplied as follows (final concentration): 0.35  $\mu\text{g}$  H<sub>2</sub> ml<sup>-1</sup>, 10 mM thiosulfate; 10 mM hydrophilic S<sup>0</sup> (free of thiosulfate, obtained as described previously; Beffa et al. 1991a), 15 mM pyruvate. KCN (0.1 M) was freshly dissolved in 0.2 M sodium phosphate buffer at pH 7.5 and used at 1 mM final concentration. Cells were pre-incubated for 3 min at  $60^\circ\text{C}$  prior to addition of the substrate, and the respiratory activities were measured from constant respiratory slopes. Results are expressed as nmol O<sub>2</sub> (mg protein)<sup>-1</sup> min<sup>-1</sup>; corrected for the low endogenous oxygen uptake.

#### Analytical methods

Growth was followed turbidimetrically at 436 nm using 1-cm cuvettes in a Perkin-Elmer Lambda 6 spectrophotometer. Protein

concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

## Results and discussion

We report here the first evidence for the occurrence of sulfur- and hydrogen-oxidizing, facultatively or obligately chemoautotrophic bacteria growing between  $70^\circ\text{C}$  and  $75^\circ\text{C}$  in thermogenic ( $60$ – $80^\circ\text{C}$ ) composts. Thermophilic bacteria numbers in compost samples were estimated by enrichment of serial dilutions:  $10^4$ – $10^6$  cells (g compost dry weight)<sup>-1</sup> for non-spore-forming, obligately autotrophic sulfur- and hydrogen-oxidizing bacteria, and  $10^5$ – $10^8$  cells (g compost dry weight)<sup>-1</sup> for facultatively autotrophic hydrogen-oxidizing bacteria forming spherical spores.

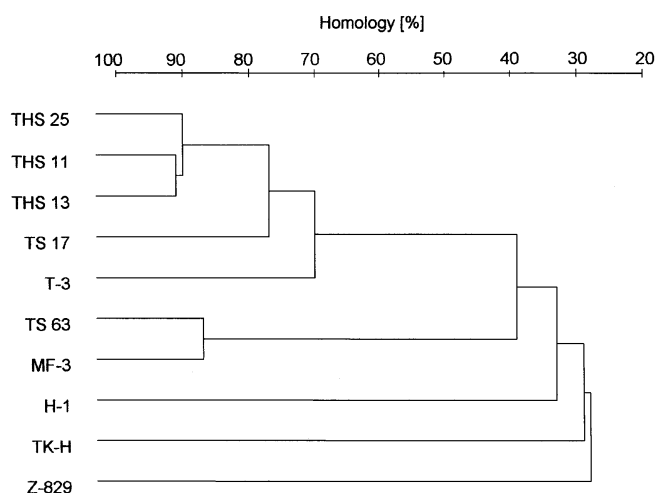
Obligately autotrophic bacteria could grow only under microaerophilic conditions (<10 kPa oxygen). They could not grow with the organic compounds and media tested as the sole energy and carbon sources. All isolates except strain THS-13 grew autotrophically on hydrogen in the presence of 25 mM pyruvate. In addition, organic compounds did not seem to significantly inhibit autotrophic growth with hydrogen and thiosulfate because the enrichments of these bacteria from compost samples that probably contained high concentrations of organic matter scored positive even at the first serial dilution. In contrast, the growth of obligately autotrophic, thermophilic hydrogen-oxidizing bacteria isolated from geothermal environments (e.g., *Hydrogenobacter thermophilus* TK-6) was strongly inhibited, particularly by 20 mM pyruvate (Shiba et al. 1984).

Facultatively autotrophic bacteria were able to grow on organic compounds or nutrient broth under microaerophilic and normal air pressure conditions. Bacteria related to the latter were unable to grow with inorganic reduced sulfur compounds or sugars as the sole electron donors.

All strains presented in this study were penicillin G sensitive, which proves that they belong to the Bacteria

**Table 1** Main characteristics of hydrogen- and sulfur-oxidizing autotrophic, thermophilic Bacteria strains isolated from hot compost piles (+ growth, – no growth)

Strains	Shape and size	Spores	Sensitivity to penicillin	Gram stain	Motility	Growth temperature ( $^\circ\text{C}$ )		mol % G+C	Growth substrates					
						(min)	(max)		H <sub>2</sub>	Thio-sulfate	S <sup>0</sup>	Ace-tate	Pyru-vate	Glu-cose
<i>Obligate autotrophic sulfur- and hydrogen-oxidizers</i>														
THS-11	Rod $0.5 \times 2$ – $3.5 \mu\text{m}$	–	+	–	+	60	80	35	+	+	+	–	–	–
THS-13	Rod $0.5 \times 2$ – $3.5 \mu\text{m}$	–	+	–	+	60	80	34.7	+	+	+	–	–	–
THS-25	Rod $0.5 \times 2$ – $3.5 \mu\text{m}$	–	+	–	+	60	80	35.6	+	+	+	–	–	–
TS-63	Rod $0.5 \times 3$ – $6 \mu\text{m}$	–	+	–	–	60	80	37.6	+	+	+	–	–	–
TS-17	Rod $0.5 \times 1.5$ – $3 \mu\text{m}$	–	+	–	+	60	80	36	+	+	+	–	–	–
<i>Facultative autotrophic hydrogen-oxidizers</i>														
THS-44	Rod $0.6 \times 3$ – $6 \mu\text{m}$	+	+	±	–	55	75	60	+	–	–	+	+	–
TH-102	Rod $0.6 \times 2.5$ – $7 \mu\text{m}$	–	+	±	+	55	75	60.4	+	–	–	+	+	–



**Fig. 1** Percent homology based on DNA:DNA hybridization between five strains isolated from compost (THS-25, THS-11, THS-13, TS-17, TS-63) and reference strains related to *Hydrogenobacter* isolated from geothermal areas in the world. Values are means of 3–5 runs. *Hydrogenobacter thermophilus* strain TK-H (Kyshu, Japan; Kawasumi et al. 1984); *Hydrogenobacter* strain T-3 (Tuscany, Italy; Bonjour et Aragno 1986, Aragno 1992); *Hydrogenobacter* strain MF-3 (Etna, Italy; Aragno 1992); *Hydrogenobacter* strain H-1 (Borgarfjörður, Iceland; Kristjánsson et al. 1985); *Calderobacterium hydrogenophilum* strain Z-829 (Kamchatka, Russia; Kryukov et al. 1983)

domain. The main features of these strains are presented in Table 1.

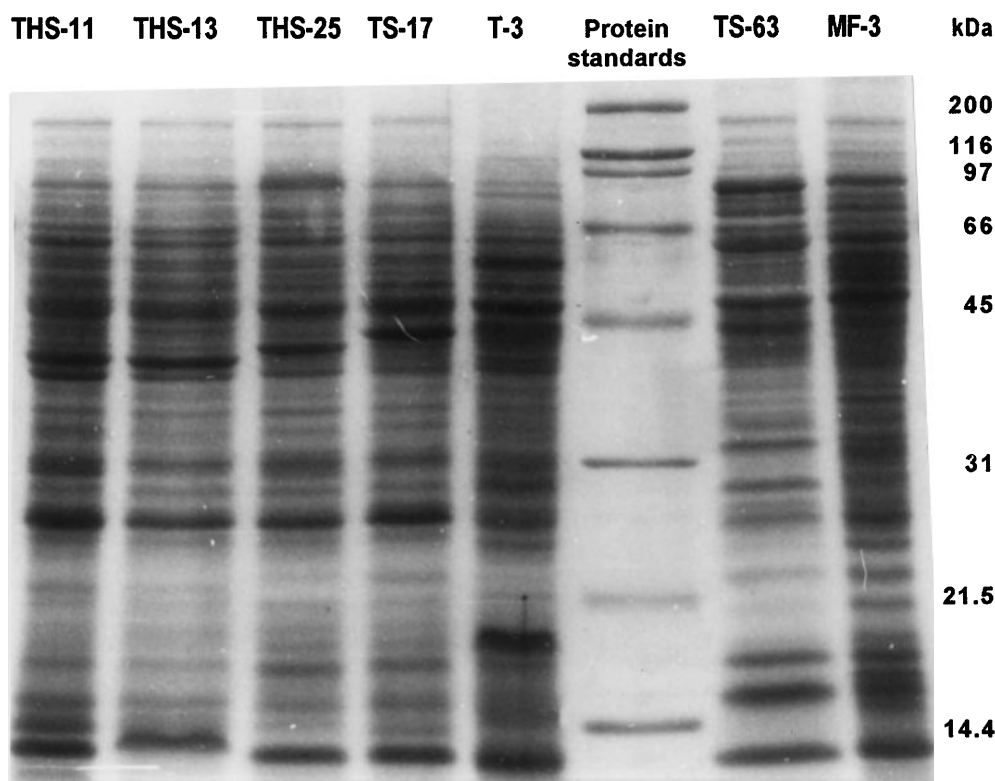
All sulfur- and hydrogen-oxidizing, obligately autotrophic strains had a 35–37.6 mol% G+C content (Table 1),

which is similar to those published for the strains related to *Hydrogenobacter* that have been isolated from geothermal areas (see Aragno 1991 and 1992 for a review). Strains THS-11, THS-13, THS-25, and TS-17 shared a high DNA:DNA homology (71–92%) with each other and with *Hydrogenobacter* reference strain T-3 (Fig. 1). This strain belongs to a DNA:DNA homology group found in geothermal springs in Italy and in the USA (Aragno 1991, 1992; M. Marchiani, Laboratory of Microbiology, University of Neuchâtel, Switzerland, personal communication). Strain TS-63 showed no significant homology with strain T-3, but a high homology (86%) with *Hydrogenobacter* reference strain MF-3, which belongs to another DNA:DNA homology group found in geothermal springs in Italy and in the Azores (Aragno 1991, 1992; M. Marchiani, personal communication).

Protein profiles showed that *Hydrogenobacter*-related strains divide into two groups (Fig. 2). Strains THS-11, THS-13, THS-25, and TS-17 showed patterns similar to that of strain T-3. However, slight differences among these strains appeared among proteins of 40–45 kDa. Strain TS-63 showed a profile similar to that of strain MF-3 if one takes into account that the differences observed probably depend on band intensities. Strains Z-829, TK-H, and H-1 showed significantly different profiles (data not shown). These results confirmed the DNA:DNA hybridization results.

Studies recently have been carried out on the phylogenetic position of the genus *Hydrogenobacter*, based on the 16S rRNA complete sequence similarities between three strains of *Hydrogenobacter* (including strain T-3) and *Aquifex pyrofilus* (strain Kol5a) (Pitulle et al. 1994). The

**Fig. 2** Coomassie brilliant blue-stained SDS-polyacrylamide gel showing proteins of total cell extracts of thermophilic bacteria related to the genus *Hydrogenobacter* isolated from geothermal areas (strains T-3 and MF-3) and from hot composts (strains THS-11, THS-13, THS-25, TS-17, and TS-63) Protein molecular size markers are indicated on the right



**Table 2** Respiratory activities of five strains isolated from compost [nmol O<sub>2</sub> consumed (mg protein)<sup>-1</sup> min<sup>-1</sup>]. Cells were grown for 2–3 days at 70°C in basal mineral medium supplied with thiosulfate, Na-pyruvate, or H<sub>2</sub> as the energy and electron source

Strains	Growth substrates	Respiratory substrates and activities [nmol O <sub>2</sub> (mg protein) <sup>-1</sup> min <sup>-1</sup> ]			
		Hydrogen	Thiosulfate	Elemental sulfur	Pyruvate
THS-25	Hydrogen	132	97	12	< 1
	Thiosulfate	< 1	488	358	< 1
TS-17	Hydrogen	118	< 1	< 1	< 1
	Thiosulfate	< 1	514	436	< 1
TS-63	Hydrogen	120	< 1	< 1	< 1
	Thiosulfate	< 1	694	135	< 1
THS-44	Hydrogen	154	266	< 1	< 1
	Pyruvate	67	330	< 1	82
TH-102	Hydrogen	113	22	< 1	< 1
	Pyruvate	148	75	< 1	91

present authors have confirmed that the genera *Aquifex* and *Hydrogenobacter* are closely related and support the proposal that the *Aquifex-Hydrogenobacter* complex be placed in the new order “*Aquificales*” and that the genera *Aquifex* and *Hydrogenobacter* belong to the same family, the “*Aquificaceae*” (Burggraf et al. 1992).

Our results present the first evidence that highly thermophilic, chemolithoautotrophic Bacteria related to the genus *Hydrogenobacter* occur in hot composts, which are short-term, high-temperature habitats and are not confined to the highly specialized, sparse geothermal habitats.

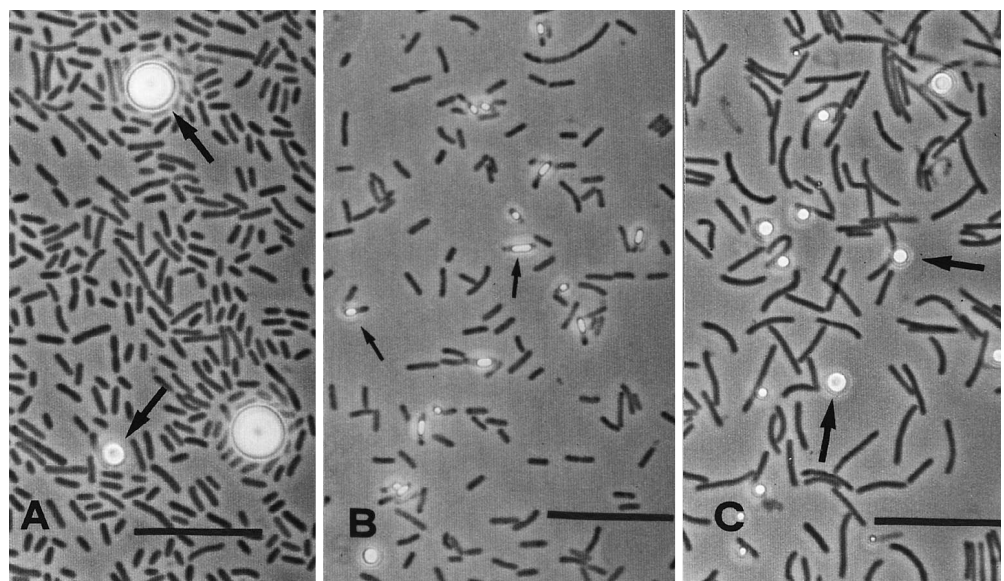
The eight facultatively autotrophic strains isolated from hot composts all showed similar G+C (60–63 mol%) and high DNA homology (75–90%) with each other and with the reference strain of *Bacillus schlegelii* DSM 2000. This confirms that all the strains isolated belong to the same species regardless of their geographical origin and environment (Aragno 1992). Table 1 shows the main taxonomic and metabolic features of one of the spore-form-

ing strains, THS-44, and those of the non-spore-forming strain, TH-102. *Bacillus-schlegelii*-related isolates also proved to grow well in nutrient broth under air.

When grown with thiosulfate as the sole energy source, strains isolated from compost and related to *Hydrogenobacter* possessed high thiosulfate- and elemental sulfur-oxidizing activities (Table 2). In contrast to strains TS-17 and TS-63, strain THS-25 possessed constitutive thiosulfate-oxidizing activity when grown on hydrogen. All respiratory activities were almost totally inhibited (> 85%) by 1 mM KCN, an inhibitor of the terminal cytochrome oxidase of the respiratory chain.

The number of types of thermophilic (optimum temperature > 65°C), aerobic, chemolithoautotrophic sulfur-oxidizing bacteria is very limited. *Hydrogenobacter* and related isolates, *Aquifex pyrofilus* (Huber et al. 1992) and *Thermothrix thiopara* (Caldwell et al. 1976; Mason et al. 1987) are the only well-characterized Bacteria to date. *T. thiopara* is however, no longer available from strain col-

**Fig. 3** Phase-contrast micrographs of three strains isolated from compost and related to *Hydrogenobacter* (A TS-17; B THS-25; C TS-63) Cultures were incubated at 65°C on solid basal mineral medium supplemented with thiosulfate and H<sub>2</sub> + CO<sub>2</sub> + O<sub>2</sub> as gas phase. When grown with H<sub>2</sub> and thiosulfate, cells released sulfur which appeared as white, refringent pellets (large arrows) and/or sheaths (small arrows) Bar: 10 µm



lections, and seems to have been lost (Kristjansson et al. 1994). Thermophilic, aerobic, autotrophic sulfur-oxidizing Bacteria are now most probably represented only by the strains related to *Hydrogenobacter* spp. and *Aquifex pyrofilus*.

The strains related to *Bacillus schlegelii* (THS-44 and TH-102) did not grow autotrophically with inorganic sulfur compounds as the sole energy and electron source. They possessed a constitutive thiosulfate-oxidizing activity but lacked elemental sulfur-oxidizing activity (Table 2). Some mesophilic heterotrophs have been reported to be able to oxidize thiosulfate (Mason and Kelly 1987). These bacteria may play a dominant role in the oxidation of sulfur compounds in soils and marine environments (Vishniac and Santer 1957; Tuttle and Jannasch 1972). To date, strains related to *B. schlegelii* constitute the first evidence of thermophilic, heterotrophic spore-forming bacteria able to oxidize sulfur compounds.

All autotrophic hydrogen oxidizers that grow on solid medium with thiosulfate alone or with thiosulfate plus hydrogen released sulfur, which appeared as white, refringent sheaths and/or pellets (Fig. 3). Reference strains of *Hydrogenobacter* and *B. schlegelii* grown under the same conditions also released S<sup>0</sup> in the growth medium (Beffa et al. 1993).

The results presented in this study contrast with the generally held belief that thermogenic composts at temperatures above 60°C support only a very low diversity of obligately heterotrophic thermophiles related to *Bacillus stearothermophilus* (Strom 1985a, b). The presence of high numbers of thermophilic, obligately and facultatively sulfur- and hydrogen-oxidizing bacteria in hot composts suggest that they may play a part in mineralization, and particularly in inorganic sulfur compound oxidation during the thermogenic phase (> 60°) of the composting process.

**Acknowledgements** We are indebted to Drs. T. Kodama, J. Kristjansson, K.A. Malik, G.A. Zavarzin, and F. Bonjour for sending us strains, and to J. Lott Fischer for correcting the manuscript. This research was supported by grants 31-28597-90 and 5002-038921 of the Swiss National Science Foundation.

## References

- Alfredsson GA, Ingason A, Kristjansson JK (1986) Growth of thermophilic, obligately autotrophic hydrogen-oxidizing bacteria on thiosulfate. *Lett Appl Microbiol* 2:21-23
- Aragno M (1991) Thermophilic, aerobic, hydrogen-oxidizing (Knallgas) bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) *The procaryotes*, 2nd edn. Springer, New York, pp 3917-3933
- Aragno M (1992) Aerobic, chemolithoautotrophic, thermophilic bacteria. In: Kristjansson JK (ed) *Thermophilic bacteria*. CRC Press, Boca Raton, pp 77-103
- Beffa T, Berczy M, Aragno M (1991a) Cytochromes and hydrogen-oxidizing activity in the thermophilic hydrogen-oxidizing bacteria related to the genus *Hydrogenobacter*. *Arch Microbiol* 156:497-500
- Beffa T, Berczy M, Aragno M (1991b) Chemolithotrophic growth on elemental sulfur (S<sup>0</sup>) and respiratory oxidation of S<sup>0</sup> by *Thiobacillus versutus* and another sulfur-oxidizing bacterium. *FEMS Microbiol Lett* 84:285-290
- Beffa T, Fischer C, Aragno M (1992) Respiratory oxidation of reduced sulfur compounds by intact cells of *Thiobacillus tepidarius* (type strain) *Arch Microbiol* 158:456-559
- Beffa T, Berczy M, Aragno M (1993) Elemental sulfur production during mixotrophic growth on hydrogen and thiosulfate of thermophilic hydrogen-oxidizing bacteria. *Current Microbiol* 27:349-353
- Beffa T, Blanc M, Lott Fischer J, Lyon P-F, Marilley L, Aragno M (1995) Composting: a microbiological process. In: Barrage A, Edelman X (eds) *Recovery, recycling and re-integration*. EMPA, Dübendorf, pp 139-144
- Bonjour F, Aragno M (1986) Growth of thermophilic obligatory chemolithoautotrophic hydrogen-oxidizing bacteria related to *Hydrogenobacter* with thiosulfate and elemental sulfur as electron and energy source. *FEMS Microbiol Lett* 35:11-15
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254
- Burggraf S, Olsen GJ, Stetter KO, Woese CR (1992) A phylogenetic analysis of *Aquifex pyrofilus*. *Syst Appl Microbiol* 15:352-356
- Caldwell DE, Caldwell SJ, Laycock JP (1976) *Thermothrix thioparus* gen. et sp. nov., a facultatively anaerobic facultative chemolithotroph living at neutral pH and high temperature. *Can J Microbiol* 22:1509-1517
- De Bertoldi M, Vallini G, Pera A (1983) The biology of composting: a review. *Waste Manage Res* 1:157-176
- De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* 12:133-142
- Dugnani L, Wyrsh I, Gandolla M, Aragno M (1986) Biological oxidation of hydrogen in soils flushed with a mixture of H<sub>2</sub>, CO<sub>2</sub>, O<sub>2</sub>, and N<sub>2</sub>. *FEMS Microbiol Lett* 38:347-351
- Finstein MS, Morris ML (1975) Microbiology of municipal solid waste composting. *Adv Appl Microbiol* 19:113-151
- Fujio Y, Kume S (1991) Isolation and identification of thermophilic bacteria from sewage sludge compost. *J Ferment Bioeng* 72:334-337
- Hames BD, Rickwood D (1990) *Gel electrophoresis of proteins: a practical approach*, 2nd edn. Oxford University Press, New York
- Huber R, Wilharm T, Huber D, Trincone A, Burggraf S, König H, Rachel R, Rockinger I, Fricke H, Stetter KO (1992) *Aquifex pyrofilus* gen. nov. sp. nov. represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. *System Appl Microbiol* 15:340-351
- Jenni B, Aragno M, Wiegel JKW (1987) Numerical analysis and DNA-DNA hybridization studies on *Xanthobacter* and emendation of *Xanthobacter flavus*. *Syst Appl Microbiol* 9:347-253
- Kawasumi T, Igarashi Y, Kodama T, Minoda Y (1984) *Hydrogenobacter thermophilus* n. gen. n. spec., an extremely thermophilic aerobic hydrogen-oxidizing bacterium. *Int J Syst Bacteriol* 34:5-10
- Kristjansson JK, Ingason A, Alfredsson GA (1985) Isolation of thermophilic autotrophic hydrogen-oxidizing bacteria similar to *Hydrogenobacter thermophilus* from Icelandic hot springs. *Arch Microbiol* 140:321-325
- Kristjansson JK, Hjörleifsdóttir S, Marteinson VT, Alfredsson GA (1994) *Thermus scotoductus* sp. nov., a pigmented-producing thermophilic bacterium from hot tap water in Iceland and including *Thermus* sp. X-1. *Syst Appl Microbiol* 17:44-50
- Kryukov VR, Saveleva ND, Pusheva MA (1983) *Calderobacterium hydrogenophilum* n. gen. n. spec., an extremely thermophilic hydrogen bacterium and its hydrogenase activity. *Microbiology* 52:611-618
- Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* 5:109-118
- Mason J, Kelly DP (1987) Thiosulfate oxidation by obligately heterotrophic bacteria. *Microb Ecol* 15:123-134

- Mason J, Kelly DP, Wood AP (1987) Chemolithotrophic and autotrophic growth of *Thermothrix thiopara* and some Thiobacilli on thiosulfate and polythionates, and a reassessment of the growth yields of *Thx. thiopara* in chemostat culture. *J Gen Microbiol* 133:1249–1256
- Miller FC (1989) Matrix water potential as an ecological determinant in compost, a substrate dense system. *Microb Ecol* 18:59–71
- Nakasaki K, Sasaki M, Shoda M, Kubota H (1985a) Change in microbial numbers during thermophilic composting sewage sludge with reference to CO<sub>2</sub> evolution rate. *Appl Environ Microbiol* 49:37–41
- Nakasaki K, Shoda M, Kubota H (1985b) Effect of temperature on composting of sewage sludge. *Appl Environ Microbiol* 50:1526–1530
- Owen RJ, Hill LR (1979) The estimation of base composition, base pairing and genome sizes of bacterial deoxyribonucleic acids. *Tech Ser Soc Appl Bacteriol* 14:277–296
- Pitulle C, Yang Y, Marchiani M, Moore ERB, Siefert JL, Aragno M, Jurtshuk P, Fox GE (1994) Phylogenetic position of the genus *Hydrogenobacter*. *Int J Syst Bacteriol* 44:620–626
- Schenk A, Aragno M (1979) *Bacillus schlegelii*, a new species of thermophilic, facultatively chemolithoautotrophic bacterium oxidizing molecular hydrogen. *J Gen Microbiol* 115:333–341
- Schulze KL (1962) Continuous thermophilic composting. *Appl Microbiol* 10:108–122
- Shiba H, Kawasumi T, Igarashi Y, Kodama T, Minoda Y (1984) Effect of organic compounds on the growth of an obligately autotrophic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus* TK-6. *Agric Biol Chem* 48:2809–2813
- Strom PF (1985a) Effect of temperature on bacterial species diversity in thermophilic solid waste composting. *Appl Environ Microbiol* 50:899–905
- Strom PF (1985b) Identification of thermophilic bacteria in solid waste composting. *Appl Environ Microbiol* 50:906–913
- Tuttle JM, Jannasch HW (1972) Occurrence and types of thiobacillus-like bacteria in the sea. *Limnol Oceanog* 17:532–543
- Vishniac W, Santer M (1957) The thiobacilli. *Bacteriol Rev* 21:195–213
- Waksmann SA, Cordon TC, Hulpoi N (1939) Influence of temperature upon the microbiological population and decomposition processes in composts of stable manure. *Soil Sci* 47:83–114