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D. L. Balkwill · T. L. Kieft · T. Tsukuda
H. M. Kostandarithes · T. C. Onstott · S. Macnaughton
J. Bownas · J. K. Fredrickson

Identification of iron-reducing *Thermus* strains as *Thermus scotoductus*

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Abstract *Thermus* strain SA-01, previously isolated from a deep (3.2 km) South African gold mine, is closely related to *Thermus* strains NMX2 A.1 and VI-7 (previously isolated from thermal springs in New Mexico, USA, and Portugal, respectively). *Thermus* strains SA-01 and NMX2 A.1 have also been shown previously to grow using nitrate, Fe(III), Mn(IV) or S^O as terminal electron acceptors and to be capable of reducing Cr(VI), U(VI), Co(III), and the quinone-containing compound anthraquinone-2,6-disulfonate. The objectives of this study were to determine the phylogenetic positions of the three known metal-reducing *Thermus* strains and to determine the phylogenetic significance of metal reduction within the genus *Thermus*. Phylogenetic analyses of 16S rDNA sequences, BOX PCR genomic fingerprinting, and DNA–DNA reassociation analyses indicated that these strains belong to the previously described genospecies *T. scotoductus*. The morphologies and lipid fatty acid profiles of these metal-reducing strains are consistent with their identification as *T. scotoductus*; however, the *T. scotoductus* strains tested in this study evinced a wide intraspecies variability in some other phenotypic traits, e.g., carbon substrate utilization and pigmentation. Iron reduction occurred in all strains of *T. scotoductus* tested except the mixotrophic, sulfur-oxidizing strain IT-7254. *Thermus* strains belonging to other species did not reduce Fe(III) to Fe(II) or reduced it only poorly.

Keywords Bacteria · Deep subsurface · Iron reduction · *Thermus scotoductus*

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D. L. Balkwill (✉)
Department of Biomedical Sciences,
Florida State University,
Tallahassee, FL 32306-4300, USA
E-mail: david.balkwill@med.fsu.edu
Tel.: +1-850-6449219
Fax: +1-850-6445781

T. L. Kieft
Department of Biology,
New Mexico Institute of Mining and Technology,
Socorro, NM 87801, USA

T. Tsukuda · H. M. Kostandarithes · J. K. Fredrickson
Pacific Northwest National Laboratory,
P.O. Box 999, Richland, WA 99352, USA

T. C. Onstott
Department of Geosciences,
Princeton University,
Princeton, NJ 08544, USA

S. Macnaughton
AEA Technology Environment,
National Environmental Technology Centre,
Culham, OX14 3DB, Abingdon, Oxon, UK

J. Bownas
Center for Environmental Biotechnology,
University of Tennessee,
Knoxville, TN 37932, USA

Introduction

Increasing interest in the microbiology of extreme environments has resulted in the isolation and characterization of a great diversity of thermophilic bacteria, including several novel species within the genus *Thermus*, such as *Thermus scotoductus* (Kristjansson et al. 1994), *Thermus brockianus* (Williams et al. 1995), *Thermus oshimai* (Williams et al. 1996), *Thermus igniterrae,* and *Thermus antranikianii* (Chung et al. 2000). Members of the genus *Thermus* were formerly considered to be strictly aerobic, based on the characteristics of the species *Thermus aquaticus* (Brock 1984). However, many of the more recently described *Thermus* isolates have been shown to grow anaerobically using nitrate as the terminal electron acceptor (Ramirez-Arcos et al. 1998a, 1998b; Williams and Sharp 1995; da Costa et al. 2001).

Kieft et al. (1999) isolated *Thermus* strain SA-01 from groundwater in a deep South African gold mine and characterized it as a facultative anaerobe capable

of coupling the oxidation of organic substrates to reduction of a wide range of electron acceptors, including nitrate, Fe(III), Mn(IV), and S^O. Suspensions of *Thermus* strain SA-01 could also reduce Co(III)-EDTA, Cr(VI), U(VI), Tc(VII), and the quinone-containing compound anthraquinone-2,6-disulfonate (AQDS) (Kieft et al. 1999). Similar metabolic versatility has been demonstrated in *Thermus* strain NMX2 A.1, which was isolated from a thermal spring in New Mexico (Hudson et al. 1989). Analysis of 16S rDNA sequences showed that strains SA-01 and NMX2 A.1 are very closely related (Kieft et al. 1999). *Thermus* strain VI-7, which was isolated from a thermal spring in Portugal (Santos et al. 1989), is also a close phylogenetic relative of strains SA-01 (Kieft et al. 1999) and NMX2 A.1 (Williams and Sharp 1995) and can also reduce Fe(III) (Kieft et al. 1999). Another close relative is *Thermus scotoductus* strain IT-7254, which was isolated from a thermal spring in Iceland and was shown to be capable of mixotrophic growth using S^O or thiosulfate as energy sources (Skirnisdottir et al. 2001). Oxidation of arsenite and reduction of arsenate have also been shown recently in the genus *Thermus* (Gihring and Banfield 2001; Gihring et al. 2001). Clearly, some species of *Thermus* are more metabolically versatile than was known heretofore.

The objectives of this study were to determine the phylogenetic positions of three closely related metal-reducing *Thermus* strains—SA-01 (Kieft et al. 1999), NMX2 A.1 (Hudson 1986), and VI-7 (Santos et al. 1989)—and to determine the phylogenetic significance of metal reduction in the genus *Thermus*. We compared the morphological, biochemical, and phylogenetic characteristics of SA-01, NMX2 A.1, and VI-7 with those of related *Thermus* strains, including *T. scotoductus*, with particular focus on the occurrence of iron-reducing capabilities.

Materials and methods

Sources of bacterial strains and culture maintenance

The *Thermus* strains included in this study are listed in Table 1. *Thermus* strain SA-01 [ATCC 700910; Subsurface Microbial Culture Collection (SMCC; Balkwill 1993) LX-001] was isolated as described previously (Kieft et al. 1999) from groundwater collected at a depth of 3.2 km below land surface in a South African gold mine, where the ambient rock temperature is approximately 60°C. *Thermus* strain NMX2 A.1 was provided by Hugh Morgan, University of Waikato, Hamilton, New Zealand. Strains CG-2, D1, NH, and VI-7 were provided by Milton S. da Costa, Universidade de Coimbra, Portugal. *T. scotoductus* strain IT-7254 was provided by Sigurlaug Skirnisdottir, Prokaria Ltd., Reykjavik, Iceland. All other *Thermus* strains were obtained from the American Type Culture Collection (http://www.atcc.org/).

Thermus strains were routinely cultured under aerobic conditions in TYG medium (5 g tryptone, 3 g yeast extract, and 1 g glucose per liter) (strains SA-01, VI-7, T-351, *Thermus filiformis* Wai33 A.1^T, *T. thermophilus* HB8^T, and *T. aquaticus* YT-1^T), ATCC growth medium 461 (*T. scotoductus* strain SE-1^T), ATCC growth medium 697 (strains NMX2 A.1 and X-1), ATCC growth medium 1598 (IB-57), and medium 166 (Skirnisdottir et al. 2001) (strains NH, D1, and IT-7254). Stock cultures were stored at –80°C in 16% glycerol.

Physiological characteristics

Single carbon growth tests were performed using medium 162 of Degryse et al. (1978), with the various carbon substrates present at a concentration of 20 mM and with yeast extract and tryptone omitted. These cultures were incubated aerobically at 65°C. Anaerobic growth with nitrate as the electron acceptor was evaluated in TYG broth cultures containing 1 g KNO_3 l^{-1} and purged with O_2-free N_2. A defined basal medium formulated for cultivating *Geobacter chapellii* and described by Kieft et al. (1999) was used for anaerobic Fe(III) reduction analyses. For these analyses, 10 mM Fe(III) was added as Fe(III)-nitrilotriacetate (NTA) to maintain Fe solubility. Carbon sources (sodium lactate or sodium formate) were added at 10 mM. The headspace gas for these experiments was N_2:CO_2 (80:20). Cells were first cultivated aerobically in appropriate

Table 1 List of strains included in this study

Taxon/other designations	Culture collection accession no.	Original source of strains	Reference
Thermus sp.			
SA-01	ATCC 700910	Witwatersrand, South Africa	Kieft et al. 1999
NMX2 A.1		Jemez Springs, New Mexico, USA	Hudson 1986
VI-7		Caldas de Vizela, Portugal	Santos et al. 1989
CG-2		S. Miguel, Portugal	Santos et al. 1989
IB-57	ATCC 43814	Submarine hot spring, Iceland	Kristjansson et al. 1986
T. filiformis			
T351	ATCC 31674	Rotorua, New Zealand	Hudson et al. 1989
Wai33 A1^T	ATCC 3814	Waimangu, New Zealand	Hudson et al. 1987
T. scotoductus			
X-1	ATCC 27978	Bloomington, Indiana, USA	Ramaley and Hixson 1970
SE-1^T	ATCC 51532	Selfoss, Iceland	Kristjansson et al. 1994
IT-7254	ATCC BAA-292	Hveragerdi, Iceland	Skirnisdottir et al. 2001
D1	NCIMB 11246	London, England	Pask-Hughes and Williams 1975
NH	NCIMB 11245	London, England	Pask-Hughes and Williams 1975
T. thermophilus			
HB8 ^{T}	ATCC 27634	Japan	Oshima and Imahori 1974
T. aquaticus			
YT-1 ^{T}	ATCC 25104	Yellowstone NP, USA	Brock and Freeze 1969

media and then inoculated, after purging with O_2-free N_2, into the anaerobic basal medium to achieve a cell density of 5×10^7 cells ml^{-1}. Cultures were incubated at 65°C with shaking. Reduction of Fe(III)-NTA was determined by measuring Fe(II) in 0.5 N HCl extracts (Kieft et al. 1999) using the ferrozine assay (Stookey 1970).

Lipid analysis

Thermus strains were grown in TYG (strains SA-01 and VI-7), ATCC medium 697 (NMX2 A.1 and *T. scotoductus* SE-1^T), or ATCC medium 461 (*T. scotoductus* X-1). Each culture was freeze-dried prior to lipid analysis. All solvents were GC grade (Fisher, Pittsburgh, Pa.). All glassware was washed in a 10% (v/v) Micro cleaning solution (VWR Scientific, Pittsburgh, Pa.), rinsed ten times in tap water, then ten times in deionized water. The glassware was then heated at 450°C for 4 h prior to use. Lipids were extracted from duplicate samples (50 mg) using the modified Bligh and Dyer technique (White et al. 1979). Total lipids were fractionated into glyco-, neutral and polar lipids; the polar lipid fraction was then transesterified with mild alkali to recover the phospholipid fatty acids (PLFA) as methyl esters in hexane (Guckert et al. 1985). Trimethyl silyl (TMS) derivatives of glycolipids were generated as described in Wait et al. (1997). The PLFA and TMS-glycolipids were separated, quantified, and identified by GC-MS (Ringelberg et al. 1994). Fatty acids and glycolipids were identified by relative retention times, by comparison with authentic standards (Matreya Inc., Pleasant Gap, Pa.), and by the mass spectra (collected at an electron energy of 70 mV). Fatty acid nomenclature is as described by White and Ringelberg (1998). PLFA and glycolipid mole percent data were arcsine transformed (White and Ringelberg 1998), and then hierarchical cluster analyses were performed with a single linkage method using Minitab version 11 (Minitab, State College, Pa.).

Molecular genetic analyses

Genomic DNA was isolated from cultures incubated overnight in TYG at 65°C. Cells were harvested by centrifugation and washed once with 5 M NaCl. The DNA isolation and purification DNA was carried out with the QIAGEN Genomic Tip 100G kit (Qiagen Inc., Valencia, Calif.). The isolated genomic DNA was mixed with 0.1-mm glass beads and sheared by bead beating (Mini BeadBeader, BioSpec Products, Bartlesville, Okla.) to a size range of 3 to 6 kb. DNA base compositions were determined (by Marin Biologic Contract/Research Laboratories, Tiburon, Calif.) by measuring the thermal melt temperatures (Marmur and Doty 1962).

Repetitive DNA fingerprinting was performed following the method of Louws et al. (1994). The PCR primer derived from the repetitive sequence BOX A1R (5′-CTACGGCAAGGCGACGCTGACG-3′) was used in PCR amplification of *Thermus* DNA (purified with the QIAGEN Genomic Tip column as described above). The PCR mixtures contained 2.5 units of *Taq* polymerase, 1.5 mM $MgCl_2$, 50 mM KCl, and 10% dimethyl sulfoxide in a 50-µl reaction volume. The PCR protocol was 80°C for 5 min (hot start); 35 cycles of 94°C for 1 min, 44°C for 30 s, and 65°C for 6 min; followed by a final extension step at 65°C for 20 min. PCR products (25 µl of each) were electrophoresed in 1.5% agarose to resolve the amplified DNA fragments.

Thermus cultures were sent to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), GmbH, Braunschweig, Germany (http://www.dsmz.de/) for DNA-DNA reassociation analyses. DSMZ uses the hydroxyapatite chromatography method for DNA isolation (Cashion et al. 1977) and the method of De Ley et al. (1970) for DNA-DNA reassociation as modified by Huss et al. (1983) and Escara and Hutton (1980).

16S rRNA gene sequences for *Thermus* strains SA-01, NMX2 A.1, and VI-7 were obtained from the GenBank/EMBL database. These sequences were initially aligned to all sequences for *Thermus* strains available from both the Ribosomal Database Project (RDP; Maidak et al. 2001) and GenBank/EMBL databases. The sequences were aligned by hand, based on the secondary structure of the 16S rRNA molecule (Gutell 1994; Gutell et al. 1994). The resulting alignment was analyzed using the distance matrix technique to generate a preliminary phylogenetic tree (not shown). Duplicate sequences and those not needed for comparison to strains SA-01, NMX2 A.1, and VI-7 (based on evaluation of the tree) were then removed, leaving 26 sequences in the alignment for a more detailed analysis. The final alignment of 1,368 bases included sequences for *Thermus* strains SA-01, NMX2 A.1, and VI-7; representative sequences for all recognized species of *Thermus*; and sequences for several other eubacteria that were used for comparison or as an outgroup during analyses. The phylogenetic positions of *Thermus* strains SA-1, NMX2 A.1, and VI-7 were analyzed using the distance matrix, maximum-likelihood, and parsimony methods. Distance matrix analysis was performed with the PHYLIP group of computer programs (Felsenstein 1993). Maximum-likelihood analysis was performed with the fastDNAml program (Olsen et al. 1994). The PAUP* software program (PAUP* 4.0, Beta Version 4.0b4a; Swofford 2000) was used for parsimony analysis.

Results and discussion

Morphological and biochemical characteristics

Thermus strains SA-01, NMX2, and VI-7 and *T. scotoductus* strains X-1 and SE-1^T varied in their pigment production; they also showed considerable variability in their ability to utilize various simple sugars, amino acids, and organic acids as sole C sources (Table 2). None of the substrates tested could be utilized by all five of these strains.

Metal reduction

A previous study demonstrated that *Thermus* strains SA-01 and NMX2-A.1 were able to couple oxidation of lactate and acetate to Fe(III) reduction for energy and

Table 2 Physiological characteristics[a] of *Thermus* strains

Physiological characteristic	*Thermus* strain				
				T. scotoductus	
	SA-01	NMX2 A.1	VI-7	X-1	SE-1^T
Pigmentation	Cream	Yellow	Light tan	Cream	Tan
Utilization of:					
Glucose	+/–	–	+	+	+
Fructose	+/–	+	–	–	–
Galactose	–	–	–	–	–
Mannose	+	–	+	+	+
Melibiose	–	–	–	–	–
Maltose	–	–	–	+	–
Lactose	–	–	–	–	–
Trehalose	+	–	+	+	–
Glutamate	+	+	+	–	–
Proline	+	+	–	+/–	+/–
Succinate	–	+/–	–	–	–
Pyruvate	+	+/–	–	–	–
Acetate	+	+	+	+	–
Reduction of nitrate	+	+	+	–	+

[a]Tests scored as positive (+), negative (–), or weak (+/–)

growth (Kieft et al. 1999). These strains could also couple oxidation of formate to reduction of Fe(III) to Fe(II) (Fig. 1). As noted previously, both strains reduced some Fe(III), even in the absence of an exogenous electron donor (Figs. 1, 2a). This was most likely due to oxidation of storage carbohydrates, oxidation of the NTA used to complex and maintain solubility of Fe(III), or both (Kieft et al. 1999). Neither H_2 nor acetate stimulated Fe(III) reduction beyond that observed for the cultures that did not receive an electron donor, whereas pyruvate was equally or more effective than lactate as an electron donor (data not shown).

Various strains of *Thermus* showed differences in their abilities to reduce Fe(III) (Fig. 2). Of the *T. scotoductus* strains tested, only IT-7254 was unable to reduce Fe(III). Strains of *T. aquaticus* (YT-1^T), *T. thermophilus* ($HB8^T$), and *T. filiformis* (strains T351 and Wai33 $A1^T$) reduced Fe(III) poorly. Nearly all of these *T. scotoductus* strains are capable of dissimilatory Fe(III) reduction, the only exception in this study being strain IT-7254. In addition to the ability to grow with Fe(III)-NTA as the sole terminal electron acceptor, SA-01 and NMX2 A.1 can reduce Fe(III)-citrate, hydrous ferric oxide (HFO), Mn(IV), Cr(VI), U(VI), Co(III)-EDTA, and the quinone-containing compound anthraquinone-2,6-disulfonate (AQDS) (Kieft et al. 1999). It remains to be seen whether the other *T. scotoductus* strains shown here to be iron reducers share this metabolic diversity with SA-01 and NMX2 A.1. Curiously, IT-7254 shows metabolic versatility of a different sort in that it can grow mixotrophically while oxidizing S^O or thiosulfate.

Fatty acid composition

The membrane PLFAs of the five strains tested in this study (Table 3) were dominated by terminally branched saturated fatty acids, as has been shown previously for other members of this genus (Hensel et al. 1986; Tenreiro et al. 1995; Wait et al. 1997; Chung et al. 2000; da Costa et al. 2001). Terminally branched saturated fatty acids comprised over 95 mol% of the PLFAs in the five strains tested, with iso-branched 15:0 and 17:0 PLFAs being the dominant forms. Cluster analysis of PLFAs placed *T. scotoductus* strain SE-1^T somewhat distant from the other four strains (data not shown). The glycolipid fatty acid profiles also showed slight differences among *Thermus* strains SA-01, NMX2 A.1, and VI-7 and the two *T. scotoductus* strains (Table 4). The finding of long-chain 1,2-diols in *T. scotoductus* strain X-1 confirms the work of Wait et al. (1997) and extends it to four closely related strains. The function of these diol-linked glycolipids remains unknown. Cluster analysis of the glycolipid fatty acid profiles separated *Thermus* strains SA-01, NMX2 A.1, and VI-7 from the *T. scotoductus* strains (data not shown).

Molecular genetic analysis

The mole percent G+C contents, as determined by thermal denaturation, were 65.0 (±0.4) for SA-01, 65.8

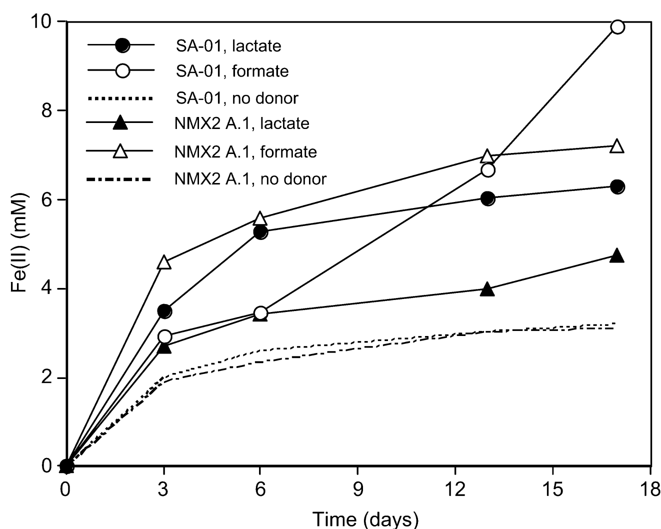

Fig. 1 Fe(III)-NTA (10 mM) reduction by *Thermus* sp. strains SA-01 and NMX2 A.1 with 10 mM lactate or formate or in the absence of electron donor

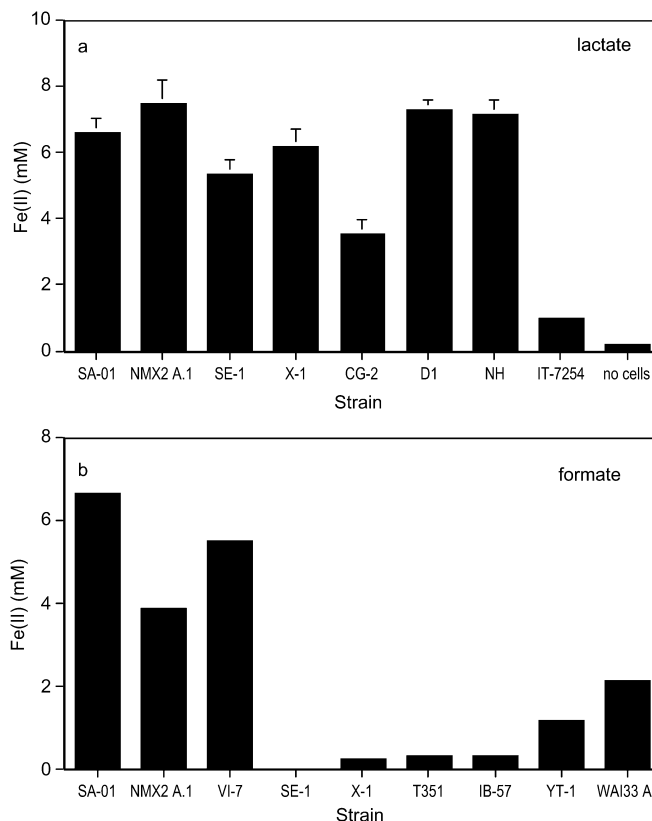

Fig. 2a, b Reduction of 10 mM Fe(III) to Fe(II) during 7 days' incubation at 65°C. **a** Reduction by various *Thermus* strains with 10 mM lactate as the electron donor. *Error bars* represent one standard deviation (n = 3). **b** Reduction with 10 mM formate as the electron donor (n = 1). Values have been corrected for the amount of Fe(II) produced in the absence of lactate or formate as electron donor

Table 3 Phospholipid fatty acids of *Thermus* strains grown at 65°C (mole percents)

Fatty acid	*Thermus* strain				
	SA-01	NMX2 A.1	VI-7	*T. scotoductus* SE-1[T]	*T. scotoductus* X-1
Normal saturated atty acids					
15:0	< 0.5	< 0.5	< 0.5	< 0.5	0.5
16:0	2.7	0.6	0.9	< 0.5	1.2
17:0	< 0.5	< 0.5	< 0.5	0.6	0.6
Terminally branched fatty acids					
i13:0	< 0.5	< 0.5	0.7	< 0.5	< 0.5
i14:0	< 0.5	0.6	< 0.5	< 0.5	0.5
i15:0	26.5	35.3	42.1	15.3	20.2
a15:0	9.5	12.0	11.3	18.5	9.1
i16:0	2.1	3.2	1.6	2.1	8.0
i17:0	44.4	37.7	34.8	29.8	42.9
a17:0	12.4	8.4	6.3	31.3	13.8
i18:0	< 0.5	< 0.5	< 0.5	< 0.5	0.8
i19:0	< 0.5	< 0.5	< 0.5	< 0.5	0.5
a19:0	< 0.5	< 0.5	< 0.5	0.8	< 0.5
Others [a]	2.4	1.9	2.3	1.6	0.8

[a]The sum of other fatty acids, each with < 0.5 mol%

Table 4 Glycolipid fatty acids from *Thermus* strains cultured at 65°C (mol%)

Fatty acid	*Thermus* strain				
	SA–01	NMX2 A.1	VI-7	*T. scoto-ductus* SE-1[T]	*T. scoto-ductus* X-1
Normal saturated fatty acids					
16:0 and coeluted 15:0 TMS	8.3	9.0	4.0	1.8	3.3
17:0	< 0.5	< 0.5	< 0.5	0.6	0.6
18:0	0.9	< 0.5	< 0.5	0.8	< 0.5
Terminally branched fatty acids					
i15:0	14.4	20.9	32.6	7.1	9.0
a15:0	5.1	7.1	10.6	9.6	3.7
i16:0	1.6	3.4	1.7	1.1	4.7
i17:0	33.3	36.7	33.5	17.5	29.8
a17:0	8.9	7.6	6.4	22.9	8.9
i18:0	< 0.5	< 0.5	< 0.5	< 0.5	0.7
i19:0	< 0.5	< 0.5	< 0.5	0.6	0.4
a19:0	< 0.5	< 0.5	< 0.5	1.0	< 0.5
Terminally methylated and mid-chain methylated fatty acids					
br15:0 TMS a and coeluted phthalate	2.7	3.6	2.0	< 0.5	1.2
16:0 TMS	< 0.5	< 0.5	< 0.5	< 0.5	0.7
17:0 TMS	7.2	5.0	0.5	1.1	4.1
br17:0 TMS a	1.6	0.9	< 0.5	1.7	1.2
Hydroxy fatty acids					
i3OH 15:0	0.8	0.6	3.7	0.5	< 0.5
a3OH 15:0	< 0.5	< 0.5	0.5	< 0.5	< 0.5
i3OH 17:0	0.8	< 0.5	1.1	< 0.5	< 0.5
Diol fatty acids					
i16:0 1,2-diol	< 0.5	< 0.5	< 0.5	0.6	< 0.5
i17:0 1,2-diol or 16methyl hepta deca-1,2-diol	8.4	< 0.5	< 0.5	15.8	0.6
a17:0 1,2-diol or 15methyl hepta deca-1,2-diol	0.9	< 0.5	< 0.5	10.3	< 0.5
17:0 1,2-diol	< 0.5	< 0.5	< 0.5	0.6	< 0.5
ND or (p) i19:1 1,2-diol[a]	< 0.5	< 0.5	< 0.5	1.2	< 0.5
ND or (p) a19:1 1,2-diol	< 0.5	1.2	< 0.5	< 0.5	< 0.5
Unknown (p) iso branched 1,2-diol	< 0.5	< 0.5	1.2	< 0.5	< 0.5
(p) i20:0 1,2-diol	< 0.5	< 0.5	< 0.5	< 0.5	0.5
(p) i21:0 1,2-diol	< 0.5	< 0.5	< 0.5	< 0.5	21.7
(p) a21:0 1,2-diol	< 0.5	< 0.5	< 0.5	< 0.5	4.8
Others[b]	5.1	4.0	2.2	5.2	4.8

[a](p) = possible or tentative identification; ND = identity not determined
[b]The sum of other fatty acids, each with < 0.5 mol%

(± 0.6) for NMX2 A.1, and 65.9 (± 0.6) for VI-7. These values are very close to the 64–65% reported for *T. scotoductus* strains X-1 and SE-1 (Kristjansson et al. 1994). Phylogenetic trees of 16S rRNA gene sequences generated by parsimony, maximum-likelihood, and distance methods showed that strains SA-01, NMX2 A.1,

and VI-7 clustered with previously described strains of *T. scotoductus*. *Thermus* SA-01, NMX2 A.1, and VI-7 sequences were 98.4–99.5% similar to *T. scotoductus* sequences and <96.8% similar to sequences from strains other than *T. scotoductus* (1,368 bases compared). The 16S rRNA gene sequence-based phylogenetic analysis thus supports the identification of the metal-reducing strains SA-01, NMX2 A.1, and VI-7 as *T. scotoductus*.

Because bacterial phylogeny cannot be resolved solely by analysis of 16S rRNA gene sequences if those sequences are more than 97% similar, DNA-DNA reassociation studies and/or other analyses are needed to make species-level comparisons (Fox et al. 1992; Stackebrandt and Goebel 1994). Whole genome DNA-DNA hybridizations revealed significant similarities (76.7–89.9% reassociation) between strains X-1, SE-1, NH, D1, IT-7254, and CG-2 and genomic DNA from *Thermus* strains SA-01 and NMX2 A.1. Thus, all eight strains appear to belong to the species *T. scotoductus*, according to the 70% DNA-DNA reassociation criterion (Wayne et al. 1987). Genomic fingerprinting of the BOX A repeat sequence showed close similarities between the three metal-reducing strains (SA-01, NMX2 A.1, and VI-7) and *Thermus scotoductus* strains X-1 and SE-1^{T} (Fig. 3). In general, the BOX fingerprints data were consistent with the 16S rDNA-based phylogenetic analysis.

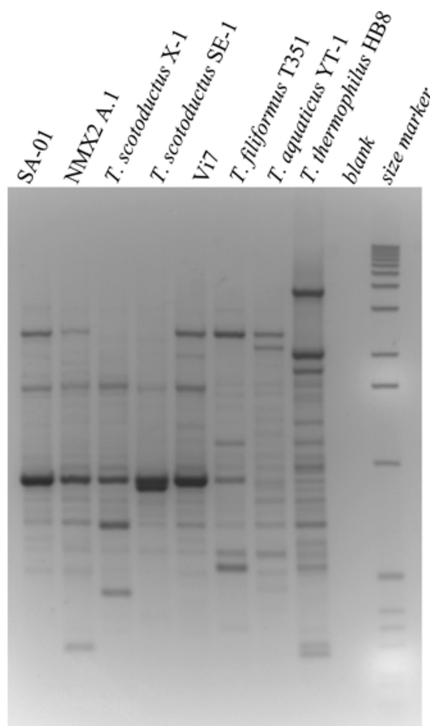

Fig. 3 BOX-PCR fingerprints of *Thermus* sp. SA-01, *Thermus* sp. NMX2 A1, *T. scotoductus* X-1, *T. scotoductus* SE-1, *Thermus* sp. VI-7, *T. filiformis* T351, *T. aquaticus* YT-1, and *T. thermophilus* HB8

Phylogenetic significance and geographic distribution

The morphological, biochemical, and physiological characteristics of metal-reducing strains SA-01, NMX2 A.1, and VI-7 confirm their placement in the genus *Thermus*. Lipid profiles of SA-01, NMX2 A.1, and VI-7 confirm their tight phylogenetic relationship to each other and further demonstrate a similarity to *T. scotoductus* strains X-1 and SE-1^{T}. Phylogenetic analyses based on 16S rDNA sequences and genomic DNA-DNA hybridization clearly show that the closely related metal-reducing strains SA-01, NMX2 A.1, and VI-7, plus strain CG-2, fall within the previously described species *T. scotoductus*. *Thermus* strains NMX2 A.1 and VI-7 were suggested (though never formally proposed) by Williams and Sharp (1995) to comprise a separate species, "*T. imahorii*," based on 16S rRNA gene sequences.

While *T. scotoductus* comprises a phylogenetically distinct group at the species level, it is phenotypically variable, probably more so than most *Thermus* species described to date. For example, the organic substrates that can be used for growth vary greatly among *T. scotoductus* strains (Table 2; Kristjansson et al. 1994). Pigmentation is also variable and thus appears not to be a useful diagnostic character for discriminating *T. scotoductus* strains, or for all metal-reducing strains within the species. The yellow pigmentation observed in NMX2 A.1 is common in most other *Thermus* spp., including *T. aquaticus*, *T. thermophilus*, *T. filiformis*, *T. brockianus*, *T. oshimai*, *T. igniterrae*, and *T. antranikianii* (Chung et al. 2000), but is not a characteristic of previously described *T. scotoductus* strains (Tenreiro et al. 1995). The soluble melanin-like pigment reported for *T. scotoductus* strains X-1 and SE-1^{T} (Kristjansson et al. 1994) was not observed in the metal-reducing strains SA-01, NMX2 A.1, and VI-7. Nearly all *T. scotoductus* strains can reduce iron, while other species appear to reduce iron poorly; thus, dissimilatory metal reduction may be a useful phylogenetic trait for identifying this species and may also suggest a useful strategy for obtaining strains of this species in enrichment culture. However, unequivocal identification of *T. scotoductus* must rely on phylogenetic analysis.

It is interesting to note the broad global distribution of the metal-reducing *T. scotoductus* strains: SA-01 was isolated from ground water collected from a deep subsurface gold mine in South Africa (Kieft et al. 1999); NMX2 A.1 was isolated from a hot spring in Jemez Springs in New Mexico (Hudson 1986); and VI-7 was isolated from a hot spring, the Caldas de Vizela, in Portugal (Santos et al. 1989). This distribution suggests that metal-reducing *T. scotoductus* exists in hot, subterranean waters worldwide, possibly because of its versatility with regard to the utilization of a wide range of electron acceptors. The observation of broad geographic distribution of *Thermus* genospecies is consistent with the conclusion of Williams et al. (1996) that the "concept of circumscribed geographical distribution of *Thermus* species may be more apparent than real."

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