Sulfur-inhibited *Thermosphaera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences

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Recently, a new procedure was developed which allowed for the first time the isolation of a hyperthermophilic archaeum tracked by 16S rRNA analysis from a terrestrial hot solfataric spring ('Obsidian Pool', Yellowstone National Park, WY, USA). This novel isolate is characterized here. Cells are round cocci with a diameter of 0.2–0.8 µm, occurring singly, in pairs, short chains and in grape-like aggregates. The aggregates exhibit a weak bluish-green fluorescence under UV radiation at 420 nm. The new isolate is an anaerobic obligate heterotroph, using preferentially yeast extract for growth. The metabolic products include CO₂, H₂, acetate and isovalerate. Growth is observed between 65 and 90 °C (optimum: 85 °C), from pH 5.0 to 7.0 (optimum: 6.5) and up to 0.7 % NaCl. The apparent activation energy for growth is about 149 kJ mol⁻¹. Elemental sulfur or hydrogen inhibits growth. The core lipids consist mainly of acyclic and cyclic glycerol diphytanyl tetraethers. The cell envelope contains a cytoplasmic membrane covered by an amorphous layer of unknown composition; there is no evidence for a regularly arrayed surface-layer protein. The G+C content is 46 mol %. On the basis of 16S rRNA sequence comparisons in combination with morphological, physiological and biochemical properties, the isolate represents a new genus within the *Desulfurococcaceae*, which has been named *Thermosphaera*. The type species is *Thermosphaera aggregans*, the type strain is isolate M11TL^T (= DSM 11486^T).

Keywords: *Thermosphaera aggregans* sp. nov., archaea, hyperthermophile, 16S rRNA sequences

INTRODUCTION

Members of the *Desulfurococcaceae* are hyperthermophilic, anaerobic, coccoid to disk-shaped cells, belonging to the *Crenarchaeota* branch within the *Archaea* domain. They grow either chemolithoautotrophically by sulfur reduction or strictly heterotrophically by sulfur respiration or fermentation (12, 46, 52, 54, 55). The family *Desulfurococcaceae* includes the genera '*Thermodiscus*', '*Igneococcus*', *Desulfurococcus* and *Staphylothermus* (12). Along with the type species *Desulfurococcus mucosus* (55), the genus *Desulfurococcus* (12, 55) also includes *Desulfurococcus*

mobilis (55), '*Desulfurococcus saccharovorans*' (48), '*Desulfurococcus amylolyticus*' (9) and two as-yet-unnamed isolates S and SY (29). Members of *Desulfurococcus* were found in continental solfatara fields (9, 49, 55) and hydrothermal vent sites (29). The genus *Staphylothermus* is currently only represented by the type species *Staphylothermus marinus* (15), which has been isolated from both shallow and abyssal marine hydrothermal systems (15).

Cells of *Desulfurococcus* are coccoid and occur singly and in pairs (55), whereas *Staphylothermus* grows mainly in grape-like aggregates (15). They thrive under anaerobic conditions preferentially on complex organic substrates such as yeast extract, meat extract, bacterial and archaeal cell extracts, peptone, casein, starch or glycogen (9, 15, 19, 55). As metabolic

The EMBL accession number for the M11TL^T 16S rRNA gene sequence reported in this paper is X99556.

products, CO₂, H₂, acetate, isovalerate, isobutyrate and H₂S (in sulfur-containing media) were identified (15, 19, 55). Members of the genus *Desulfurococcus* are not strictly sulfur-dependent but growth is stimulated by sulfur (9, 55). On the other hand, *Staphylothermus marinus* is strictly sulfur-dependent when grown as pure culture (15). However, in the absence of sulfur, it can be grown in syntrophic culture together with H₂-consuming organisms (10). In contrast, inhibition of growth by sulfur was found recently in the hyperthermophiles *Pyrobaculum aerophilum* (51) and *Pyrobolus fumarii* (6), both growing either by microaerophilic or by anaerobic nitrate respiration.

A great phylogenetic variety of cultured hyperthermophiles is already known (47). However, *in situ* 16S rRNA gene sequence analyses of samples from 'Obsidian Pool' (Yellowstone National Park, WY, USA) revealed many unknown archaeal sequences, indicating the existence of many as-yet-uncultivated species (3, 4). Recently, a new procedure was developed, which allowed for the first time the isolation of a hyperthermophilic archaeum (M11TL^T) from this natural environment by the combination of *in situ* 16S rRNA analyses, specific cell hybridization within enrichment cultures and 'selected cell isolation' by the use of a laser microscope ('optical tweezers'; 4, 21). In an independent approach, Kane *et al.* (30) enriched and isolated a sulfate-reducing bacterium from an anaerobic laboratory bioreactor, originally identified as a molecular isolate phylogenetically related to *Desulfovibrio vulgaris*.

Here we characterize the novel isolate M11TL^T, the 16S rRNA sequence of which is identical to that of clone pSL91, known from 'Obsidian Pool' (4). It represents a new genus within the *Desulfurococcaceae*, the type species of which we name *Thermosphaera aggregans*.

METHODS

Collection of samples, enrichment and isolation. Sampling, enrichment and isolation of the new organism was described recently by Huber *et al.* (21).

Strains and culture conditions. For stock maintenance and experimental studies, the new isolate M11TL^T was cultivated in a medium containing the following components (l⁻¹): 2.2 g MgCl₂·6 H₂O, 1.0 g NaHCO₃, 0.9 g NaCl, 17 mg KCl, 12.5 mg NH₄Cl, 7.0 mg CaCl₂·2 H₂O, 7.0 mg K₂HPO₄·3 H₂O, 0.05 mg Fe(III)Cl₃, 10 ml vitamin solution (2), 1.0 g Bacto yeast extract (Difco), 1.0 g Bacto peptone (Difco), 0.02 mg resazurine. The medium was reduced by the addition of 0.5 g Na₂S·9H₂O. The pH was adjusted to 6.5 with HCl. Strictly anaerobic culture media were prepared according to the anaerobic technique of Balch & Wolfe (1). Unless stated otherwise, the gas phase consisted of 300 kPa N₂/CO₂ (80:20). Isolate M11TL^T was routinely grown at 85 °C and approximately 60 r.p.m. in 28 ml serum tubes (borate silicate glass, Schott) containing 10 ml medium. For the determination of pH dependence of growth, cultures were grown in 100 ml flasks (Schott) in the presence of 200 kPa N₂. When organic compounds other than yeast extract or

peptone were tested as substrates, the yeast extract and peptone concentration was reduced to 0.005% (w/v). To determine the optimal NaCl concentration for growth, only the medium NaCl was varied, while the concentration of all other salts remained the same.

Determination of growth. Cell densities were determined by direct cell counting using a Thoma chamber (depth 0.02 mm).

Effect of temperature on growth rate. Influence of temperature on growth was assayed by the quantitative determination of the whole cell protein concentration as described (34, 42). For this experiment, M11TL^T was grown at different temperatures in 1 l culture bottles containing 250 ml medium. During growth, 20 ml samples were taken at different time intervals and the cells were collected by centrifugation (Beckman J2-21 centrifuge, 30 min, 4 °C; rotor: JA20). To solubilize the cells, the centrifuged cell sediment was resuspended in 100 µl SDS (5%) followed by incubation at 100 °C for 15 min. For protein determination, 100 µl solubilized cell material was incubated with 500 µl reagent A' and 4 ml reagent B of the Bio Rad DC Protein assay (Bio Rad). The protein concentration was determined spectrophotometrically (750 nm; Beckman). A BSA standard containing 0.03–1.5 mg protein ml⁻¹ was used. The protein concentration was used to determine the respective growth rate constant k. The logarithm of k was plotted against the reciprocal of the absolute temperature and the apparent activation energy for growth was calculated from an Arrhenius plot (18).

Light and electron microscopy. Cells were observed with a Zeiss standard phase-contrast microscope (Oberkochen) equipped with an oil-immersion objective (×100/1.3). Micrographs were taken on a Zeiss Axiovert 35 inverse phase-contrast microscope with a Olympus OM3 camera. To bring the cells into a horizontal position, the organisms were allowed to settle down onto the cover slide for approximately 30 min. Electron microscopy was carried out as described (24). For ultrathin sections, cells, fixed with 1% glutaraldehyde in the culture medium, were freeze-substituted in diethyl ether (containing molecular sieve, 0.4 nm).

Detection of metabolic products. Acetate and isovalerate were determined by HPLC (25), H₂ and CO₂ by GC according to the methods of Huber *et al.* (26, 28). Methane was determined with a Hewlett-Packard 5890 gas chromatograph equipped with a Molecular Sieve 5A column (Supelco) and a flame ionization detector (oven temperature: 140 °C; detector and injector temperature: 170 °C). H₂S was detected qualitatively by addition of 20 µl saturated lead acetate solution to 0.5 ml samples. A dark brown precipitate demonstrated its presence.

Following growth on heat-treated xylan (121 °C, 20 min), the reducing sugars formed by M11TL^T were determined by the 3,5-dinitrosalicylic acid (DNSA) method, according to Miller (38). DNSA solution (750 µl) was added to a 500 µl sample, incubated for 20 min at 90 °C, and the colour reaction was measured spectrophotometrically at 595 nm.

Lipid analysis. Lipids were extracted from cell mass as described by Huber *et al.* (25). The core lipids were identified by TLC (50). As reference substances, extracted core lipids of *Thermococcus stetteri* (39) and *Pyrobaculum organotrophum* (23) were used.

DNA isolation and base composition. DNA was isolated as described by Lauerer *et al.* (33). The G+C content was

determined by melting point analysis (36), with calf thymus DNA (42 mol % G + C) as a reference.

DNA-DNA hybridization. DNA-DNA hybridization was carried out after radioactive *in vitro* labelling of the DNA (31) with a nick translation reagent kit (BRL) using the filter technique as described previously (16, 32). The hybridization took place for 20 h at 66 °C in $3 \times \text{SSC}$ (25 °C below T_m = 'optimal' conditions; 7, 35, 37), where $1 \times \text{SSC}$ is 0.15 M sodium chloride plus 0.015 M sodium citrate. The amount of radioactivity bound (in c.p.m.) in the homologous filters (for example, 396 000 c.p.m. for M11TL^T) was set as 100 % homology and compared with the activity of the heterologous filters.

16S rRNA analysis. 16S rDNA was amplified by PCR (40, 43, 44). The primers employed in the amplification had the sequences CTY GGT TGA TCC TGC C and CGG HTA CCT TGT TAC GAC TT and corresponded to position 8–23 and 1511–1492 respectively in the 16S rRNA (*Escherichia coli* numbering, 11). Both strands of the PCR product were sequenced (1414 positions determined) and the analysis of the sequence data was performed according to the methods of Burggraf *et al.* (12).

RESULTS

Morphology

Cells of the isolate M11TL^T were regular cocci. During the exponential growth phase, they grew predominantly in grape-like aggregates consisting of five to several hundred individuals. Under the UV microscope at 420 nm, these aggregates exhibited a weak bluish-green fluorescence which faded rapidly under UV radiation. The aggregates with diameters up to 10 µm could not be disintegrated into single cells by supersonic treatment, mechanical stirring, enzyme treatment (trypsin, proteinase K, cellulase) or periodic acid (0.7 %, w/v). In the late-logarithmic and stationary growth phases, smaller aggregates, single cells, duplex forms and short chains with up to four cocci were visible (Figs 1, 2a). The diameter of the cells varied between 0.2 and 0.8 µm. Morphology was influenced by culture conditions: at the upper temperature limit of growth (90 °C) or in the presence of sulfur (e.g. 0.05 %, final concn), single cells up to

3.5 µm in diameter containing one or two dark granules became visible.

Grape-like cell aggregates possessed several flagella (Fig. 2a), single cells possessed up to eight flagella. Ultrathin sections of freeze-substituted cells revealed a cytoplasmic membrane (4 nm), plus an amorphous layer, approximately 10–15 nm thick (Fig. 2b). In a micrograph of a freeze-etched cell aggregate, no regular lattice, indicative of a surface-layer protein, was detected (Fig. 2c). The fracture face revealed a fine granular cytoplasm (Fig. 2c).

Physiological characterization

M11TL^T grew from 67 to 90 °C with an optimal doubling time of 110 min at 85 °C (Fig. 3). No growth occurred at 65 or 92 °C. The Arrhenius plot yielded a straight line between growth temperatures of 75 and 85 °C. The apparent activation energy for growth calculated from the mid-zone was about 149 kJ mol⁻¹.

Growth of isolate M11TL^T occurred between pH 5.0 and 7.0 with an optimum of 6.5. No growth was observed at pH 4.5 or 7.5. M11TL^T grew when between 0 and 0.7 % NaCl was added to the medium. To adapt cultures to NaCl concentrations above 0.4 %, the NaCl concentration was increased stepwise by 0.1 % during each culture transfer. Growth was optimal in the absence of exogenous NaCl.

Metabolism

In closed serum tubes, isolate M11TL^T grew strictly heterotrophically with yeast extract (final concentration 0.1 %, w/v) and peptone (0.1 %) under anaerobic culture conditions up to cell titres of about 2×10^8 cells ml⁻¹. With yeast extract as single substrate, titres of about 1×10^8 cells ml⁻¹ were obtained. Maximal cell densities of M11TL^T with up to 4×10^8 cells ml⁻¹ were obtained by flushing exponentially growing cultures periodically with N₂/CO₂ (80:20) to remove hydrogen, a product of fermentation (see below) that is a potent growth inhibitor for this species. No growth occurred, when cultures were pressurized with hydrogen-containing gas (200 kPa N₂/CO₂ = 80:20 + 100 kPa H₂/CO₂ = 80:20). This inhibition was reversible, for growth resumed after gas exchange (300 kPa N₂/CO₂ = 80:20).

No growth was observed on native xylan (final concentration 0.2 %, w/v) or xylose (0.1 %). However, growth took place on heat-treated xylan (0.2 %) with cell densities of about 7×10^7 cells ml⁻¹. After growth, an increase of reducing sugars in the culture medium was detected. Furthermore, growth was observed on gelatin (0.2 %), on a defined mixture of amino acids (0.04 %) and on glucose (0.1 %), yielding cell densities of only $2\text{--}5 \times 10^7$ cells ml⁻¹. No growth occurred on meat extract, bovine heart infusion, peptone, amylose, glycogen, cellulose, cellobiose, maltose, raffinose, pyruvate and acetate (final concentration 0.1 %, w/v).

Fig. 1. Phase-contrast micrograph of cells of *Thermosphaera aggregans* during the late-exponential growth phase. Bar, 5 µm.

Fig. 2. (a). Electron micrograph of a grape-like cell aggregate of *Thermosphaera aggregans* (air-dried and platinum-shadowed). (b) Ultrathin section of a freeze-substituted cell aggregate of *Thermosphaera aggregans*. Arrows point to the cytoplasmic membrane (CM) and to the amorphous layer (AL). (c) Electron micrograph of a freeze-etched cell aggregate of *Thermosphaera aggregans*. Arrows point to the cell surface (CS) and to the cytoplasm (CY) of a fractured cell. Bars, 0.5 µm.

Elemental sulfur was a potent inhibitor of growth. Even at the very low concentration of 0.05% (w/v), only about 10% of the final cell titres were determined

compared to cultures without sulfur. When 0.05% sulfur was added to exponentially growing cultures, cell growth stopped. During further incubation, H₂S

Fig. 3. Effect of temperature on growth of *Thermosphaera aggregans*.

was formed in large amounts and cells began to lyse. Similar observations have been reported for methanogens and hyperthermophilic sulfate-reducers (13, 56). Sulfide did not inhibit growth of M11TL^T, when added to the medium in the same concentration as sulfur.

When M11TL^T was cultivated on yeast extract and peptone, isovalerate, acetate, CO₂ and H₂ were identified as fermentation products.

Lipid composition

Thin-layer chromatograms of the core lipids of M11TL^T showed mainly acyclic (*R_f* value 0.43) and cyclic glycerol diphytanyl tetraethers with one to four pentacyclic rings (*R_f* values 0.4, 0.39, 0.37, 0.35, respectively). Traces of glycerol diphytanyl diethers (*R_f* 0.75) were detected in addition.

DNA base composition, DNA–DNA hybridization and 16S rRNA phylogeny

The G + C content of isolate M11TL^T was 46 mol %, as determined by melting point analysis (36).

In DNA–DNA hybridization studies, DNA of isolate M11TL^T showed no hybridization signal with the isolated DNA of *Staphylothermus marinus* and *Desulfurococcus mobilis*.

By 16S rRNA sequence comparisons, isolate M11TL^T was identified as a member of the *Crenarchaeota* within the domain *Archaea*. The closest relatives of M11TL^T are members of the genera *Desulfurococcus* and *Staphylothermus*, as indicated by an estimated exchange of 3.5 and 3.9 nucleotides per 100 positions, respectively (12). Fig. 4 shows the phylogenetic relationship of M11TL^T with other members of the *Crenarchaeota*.

DISCUSSION

Based on 16S rRNA sequence comparisons, the novel isolate M11TL^T was identified as a member of the *Crenarchaeota*. Within this branch, M11TL^T was clearly affiliated to the *Desulfurococcaceae* with closest relationship to *Staphylothermus* and *Desulfurococcus* (12, 15, 55). However, M11TL^T differs in several important phenotypical properties from *Staphylothermus* and *Desulfurococcus*. A considerable difference to these genera is obvious with respect to the utilization of sulfur. In contrast to the sulfur-respiring *Desulfurococcus* and *Staphylothermus*, growth of M11TL^T is prevented in the presence of elemental sulfur. This sulfur inhibition was unexpected, because all other genera within the *Desulfurococcaceae* respire sulfur (12). However, within other main phylogenetic branches of the *Crenarchaeota*, the type of metabolism also varies. Within the otherwise strictly anaerobic,

Fig. 4. Phylogenetic tree of *Thermosphaera aggregans* and various members of the *Crenarchaeota* derived from 16S rDNA sequence data, using the neighbour-joining method for calculation. *Methanopyrus kandleri* belongs to the *Euryarchaeota* and *Aquifex pyrophilus* to the *Bacteria*. The scale bar indicates 10 estimated changes per 100 nucleotides.

sulfur-respiring *Thermoproteales* and *Pyrodictiaceae* (12), *Pyrobaculum aerophilum* (51) and *Pyrolobus fumarii* (6) utilize aerobic or anaerobic respiration with nitrate. Furthermore, *Stygiolobus azoricus* (45), a strictly anaerobic member of the *Sulfolobales*, is the closest relative of the strict aerobe *Sulfolobus acidocaldarius* (8). In view of those unexpected properties, it is not always justified to infer the metabolic type based upon the metabolic properties of the phylogenetic relatives.

In contrast to *Desulfurococcus*, M11TL^T grows in large, grape-like cell aggregates similar to *Staphylothermus*. However, cell aggregates of M11TL^T exhibit a bluish-green fluorescence at 420 nm. The source of this fluorescence is unknown. A unique feature of M11TL^T is evident in its cell architecture, with no surface-layer protein being detectable: there is no evidence for a regular surface lattice like that in *Desulfurococcus* or any other members of the *Crenarchaeota* (12), nor for a structure like the tetra-brachion of *Staphylothermus* (Fig. 2c; 41, 53).

M11TL^T is also unique in the composition and structural features of its core lipids. In contrast to *Staphylothermus* and *Desulfurococcus*, where glycerol diphytanyl diether- and acyclic glycerol diphytanyl tetraether lipids have been identified, acyclic tetraethers and cyclic tetraethers with one to four pentacyclic rings are the predominant lipids in M11TL^T (14, 15). The occurrence of cyclic tetraether lipids in members of the *Desulfurococcaceae* was so far unknown and emphasizes again the uniqueness of M11TL^T within this family.

The evolutionary distances between isolate M11TL^T and its nearest relatives *Staphylothermus marinus* and *Desulfurococcus mobilis* are 3.9 and 3.5%, respectively (12). This divergence is similar to that between the genera *Staphylothermus* and *Desulfurococcus* or *Pyrobaculum* and *Thermoproteus* (12), which indicates that isolate M11TL^T represents a new genus. The significance of these differences is also evident in the branching of the 16S rRNA phylogenetic tree, which strengthens this criterion (Fig. 4) and is also reflected in hybridization studies by the lack of significant DNA homology.

Therefore, on the basis of 16S rRNA sequence comparisons as the main criterion, in combination with unique morphological, physiological and biochemical properties, we propose that M11TL^T represents a new genus, which we describe here as *Thermosphaera* ('the hot sphere'). The type species is *Thermosphaera aggregans*, type strain *Thermosphaera aggregans*, isolate M11TL^T (DSM 11486).

Growth of *Thermosphaera aggregans* occurs within a temperature range of 25 °C similar to many hyperthermophilic archaea (5). From 75 to 85 °C, *Thermosphaera aggregans* grows within the Arrhenius relation with an activation energy of 149 kJ mol⁻¹, which is significantly higher than that described for mesophilic and psychrophilic bacteria (17, 27).

Recently, we were able to isolate additional hyperthermophiles from the 'Obsidian Pool' (22); their presence was predicted from extraction and sequencing of DNA from this site (3, 4). One of the isolates is S10TFL (sequence identical to clone sequence pJP6; 3, 12), a strict anaerobe, which grows preferentially on archaeal cell extracts (22). S10TFL and *Thermosphaera aggregans* use different biopolymers for growth, and therefore together they act as degraders of organic matter in their biotope. *Thermosphaera aggregans* produces isovalerate, acetate, hydrogen and CO₂, and can provide growth substrates for other hyperthermophiles present in the 'Obsidian Pool'. One of these consumers is a new isolate S10L (sequence identical to clone sequence pSL23; 4), which represents a novel, hyperthermophilic sulfate-reducer (22).

Description of *Thermosphaera* gen. nov.

Thermosphaera (Ther.mo.sphae'ra. Gr. fem. n. *therme*, heat; L. fem. n. *sphaera*, sphere; L. fem. n. *Thermosphaera*, the hot sphere).

Cells are cocci, occurring singly, in pairs, in short chains and in aggregates. No spores formed. Cell envelope consists of a cytoplasmic membrane with an amorphous layer on top. Membrane contains acyclic and cyclic glycerol diphytanyl glycerol tetraethers. Growth up to 90 °C and up to 0.7% NaCl. Anaerobic. Strictly heterotrophic. Growth by fermentation. Sulfur or hydrogen inhibits growth. DNA base composition 46 mol% G + C. No significant DNA homology with *Staphylothermus marinus* and *Desulfurococcus mobilis*. By 16S rRNA analysis, *Thermosphaera* represents a new genus belonging to the *Desulfurococcaceae*. Habitat: solfataric hot spring.

The type species is *Thermosphaera aggregans*.

Description of *Thermosphaera aggregans* sp. nov.

Thermosphaera aggregans (ag'.gre.gans. L. v. *aggregare* to aggregate, referring to the ability of the cells to form grape-like aggregates).

Cells are cocci from 0.2 to 0.8 µm in diameter, occurring singly, in pairs, in short chains and in grape-like aggregates. During growth, aggregates up to 10 µm with several hundred individuals visible. Cell aggregates exhibit a bluish-green fluorescence at 420 nm. Spores are not formed. Flagella present. Cell envelope composed of a cytoplasmic membrane (width: 4 nm), covered by an amorphous layer, 10–15 nm thick. No evidence for a regular arrayed surface-layer protein. Core lipids consist mainly of acyclic and cyclic glycerol diphytanyl glycerol tetraethers with one to four pentacyclic rings. Growth between 67 and 90 °C (optimum: 85 °C), pH 5.0–7.0 (optimum: 6.5) and 0–0.7% NaCl (optimum: 0%). Optimal doubling time 110 min. Anaerobic. Heterotrophic growth with yeast extract. Peptone stimulates growth. CO₂, H₂, acetate and isovalerate formed as metabolic products. Growth inhibition by sulfur or hydrogen. G + C content 46 mol%. 3.9 and 3.5%

evolutionary distance to *Staphylothermus marinus* and *Desulfurococcus mobilis*, respectively.

The type strain is isolate M11TL^T, DSM 11486, Braunschweig, Germany (isolated from 'Obsidian Pool' in the Mud Volcano area of Yellowstone National Park, WY, USA).

ADDENDUM IN PROOF

After this work was submitted for publication, Hensel *et al.* (*Syst Appl Microbiol* **20**, 102–110, 1997) published *Sulfophobococcus* as a new genus within the *Crenarchaeota* with closest relationship to *Desulfurococcus*. Comparison of 16S rRNA sequences and conserved bases in the 16S rRNA indicate no closer relationship between *Thermosphaera* and *Sulfophobococcus*.

ACKNOWLEDGEMENTS

We are grateful to K. O. Stetter for critical discussions. We wish to thank M. Bock, P. Hummel and K. Eichinger for excellent technical assistance. This work was supported by grants of the Deutsche Forschungsgemeinschaft (Ste297/10) and the Fonds der Chemischen Industrie.

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