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Morphological, Small Subunit rRNA, and Physiological Characterization of *Trimyema minutum* (Kahl, 1931), an Anaerobic Ciliate from Submarine Hydrothermal Vents Growing from 28 °C to 52 °C

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ABSTRACT. A thermophilic strain of *Trimyema minutum* was isolated from the hydrothermally heated sea floor at Vulcano Island (Italy) and cultivated monoxenically on *Marinobacter* sp. and *Methanococcus thermolithotrophicus*. It can be propagated strictly anaerobically and is sensitive to oxygen: if exposed to air at 48 °C all cells die within 60 min. It grows from 0.45–7.2% (w/v) salt and at pH 6.0–8.0. The isolate is the most extreme thermophilic ciliate which ever has been cultivated, exhibiting an optimal growth temperature of 48 °C (doubling time 6 h). Growth occurs between 28 °C and 52 °C. *Trimyema minutum* is redescribed using live observation and silver impregnation. Its morphology and the small subunit ribosomal RNA sequence is distinctly different from that of *T. compressum*, but morphology is highly similar to that of *T. shoalsia* Nerad et al. 1995, which is thus probably a junior synonym of *T. minutum*. To stabilize the bewildering species taxonomy in *Trimyema*, we suggest to recognize our population as a neotype of *T. minutum*.

Key Words. Marine, monoxenic culture, physiology, taxonomy, thermophilic, Vulcano Island, 18S rRNA.

IN our search for the upper temperature limit of microbial life, we investigated terrestrial and submarine high temperature environments, which turned out to harbor hyperthermophilic archaea able to grow at temperatures up to 113 °C (Blöchl et al. 1997; Stetter 1996). During investigations of hot vent sites in the Tyrrhenian Sea, we recognized a minute ciliate, possibly a thermophile.

Little is known about protozoans living at hydrothermal environments. Recently, ubiquitous flagellates have been found in deep sea hydrothermal vent sites with original temperatures of up to 30 °C (Atkins et al. 2000). Direct microscopic inspections of hot hydrothermal freshwater springs revealed the occurrence of amoebas, ciliates, and flagellates (Kahan 1969; for review, see Tansey and Brock 1978). The only protozoans from hot springs which were cultivated and studied in detail, have been the ciliates *Tetrahymena pyriformis* and *Cyclidium citrullus*: Phelps (1961) obtained *T. pyriformis* from various thermal sources. One strain isolated from Mimbres Hot Springs in Mexico was able to grow at temperatures up to 41.2 °C. Kahan (1972) cultivated *C. citrullus* from the Tiberias Hot Springs (Israel). Based on its wide growth temperature range (18–47 °C; T_{opt} = 43 °C) he designated this species as ''eurythermic'' (Kahan 1972; Kahan and Sharon 1976). Further, syngen 1 of *T. pyriformis* exhibited a unique tolerance for high temperature (up to 40 °C), and is described as a distinct species, *T. thermophila* Nanney & McCoy, 1976.

We identified the ciliate from the Tyrrhenian Sea as *Trimyema minutum* (Kahl, 1931), a poorly known and rare ciliate (Augustin et al. 1987). Thus, it is redescribed using classical and modern methods. Members of the genus are known to live anaerobically in sapropelic habitats (for review, see Augustin et al. 1987). The strictly mesophilic type species, *T. compressum* has been cultivated monoxenically (Goosen et al. 1990a; Wagener and Pfennig 1987; Yamada et al. 1994) and axenically (Broers et al. 1991). Its growth rates were determined (Goosen et al. 1990a) and its metabolism was studied (Goosen et al. 1990b; Holler and Pfennig 1991; Holler et al. 1994; Yamada et al. 1994). In order to investigate the heat tolerance and growth temperature range from the Tyrrhenian Sea isolate, we cultured it and studied it physiologically.

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MATERIALS AND METHODS

Sampling and enrichment. Sampling was carried out at the beach of Vulcano Island (Italy) north of Porto di Levante (38° 25′ 5″ N, 14° 57′ 14″ E). In a water depth between 0 and 2 meters, the sandy sea floor is heated by extended submarine hot springs and fumaroles, which exhibit a strong odor of H_2S. Within the heated sediments there are very steep temperature gradients ranging from about 25 °C (ambient sea water temperature) to 100 °C.

Sample EV10 was taken in a water depth of 1.5 meters. It consisted of strongly gassed sediment with temperatures up to 70 °C and a pH of about 6. The sediment was collected by a syringe (50 ml) equipped with an enlarged inlet (Stetter 1982). The water-containing sediment was transferred into a sterile 250-ml storage bottle until it was filled completely. Then it was sealed by a rubber stopper. The sample was carried to the laboratory at ambient temperature.

The following synthetic basic culture medium was used (Stetter et al. 1983; modified): NaCl 13.85 g/liter, $MgSO_4 \cdot 7 H_2O$ 3.5 g/liter, $MgCl_2 \cdot 6 H_2O$ 2.75 g/liter, KCl 325 mg/liter, NaBr 50 mg/liter, H_3BO_3 15 mg/liter, $SrCl_2 \cdot 6 H_2O$ 7.5 mg/liter, KJ 0.025 mg/liter and $CaCl_2 \cdot 2 H_2O$ 0.75 g/liter buffered with 25 mM BisTris/HCl at pH 7. This medium had a salinity of 1.8% (w/v) and resembled 1/2 diluted sea water in its salt composition. All media were sterilized by autoclaving (200 kPa, 20 min, 121 °C).

For ciliate enrichment, 50 ml of basic culture medium in a 250-ml storage bottle were supplemented with 0.2% (v/v) glycerol and 0.1% (w/v) yeast extract to support growth of bacteria and inoculated with about 100 ml of sample. Then, the bottle was closed tightly and incubated in a hot air incubator at 48 °C without shaking. Due to intensive bacterial growth, the enrichment became turbid within 12 h. After 3 d, small ciliates could be observed.

Monoxenisation. To isolate food bacteria, the enrichment culture was streaked onto plates of basic culture medium, which contained 0.4% (w/v) yeast extract and 1% (v/v) glycerol and were solidified by 1.8% (w/v) agar. The plates were incubated at 48 °C aerobically or under low oxygen conditions in a N_2/CO_2 (50:50 v/v) atmosphere in a polyacrylate chamber. Under low oxygen conditions only colonies of spore formers could be obtained, which were not suitable to support growth of the ciliate. Therefore, from the aerobic plate a single colony of the prevalent non-sporulating bacterial species was picked and grown in liquid basic culture medium with 0.4% (w/v) yeast extract and 1% (v/v) glycerol at 48 °C in shaken Erlenmeyer

flasks (120 rpm). This bacterial strain turned out to be an excellent food bacterium for the ciliate. Based on SSU rRNA analysis it was identified as *Marinobacter* sp. (data not shown; EMBL Accession No. AJ292528), which was rod-shaped, strictly aerobic and formed cream-colored colonies on agar plates at 48 °C. This bacterium grew at temperatures of 20–55 °C, but not at 10 °C and 60 °C, and tolerated salt concentrations from fresh water up to 10.8% (w/v).

For monoxenic cultivation, bacteria-free ciliates were obtained by lysozyme treatment and serial washing as described by Wagener and Pfennig (1987). Cultures of food bacteria were pre-grown for 12 h at 48 °C without shaking in 20-ml screw-capped vials containing 5 ml basic culture medium supplemented with 0.2% (v/v) glycerol and 0.1% (w/v) yeast extract, to use up oxygen and provide almost oxygen-free conditions for the ciliates. These pre-grown bacterial cultures were inoculated with single bacteria-free ciliates and further incubated. After 6 d, the dense monoxenic culture was examined for bacterial purity by microscopy and subcultured twice weekly by transferring 0.5 ml culture to new vials with pre-grown bacteria.

Cytological methods. *Trimyema minutum* was studied in vivo using bright field and interference contrast microscopy. Cytological details were revealed with protargol and the silver nitrate method of Chatton-Lwoff, as described in Foissner (1991), and the silver carbonate technique of Fernandez-Galiano, as described in Augustin et al. (1984). To remove the salt, which disturbs silver carbonate impregnation, 3 ml of the culture were fixed and washed in fixation solution (diluted formalin), using a centrifuge (1,500 *g*). All other steps, including the preparation of permanent slides, followed Augustin et al. (1984).

Counts and measurements on silvered specimens were performed at a magnification of 1,000×. In vivo measurements were made at magnifications of 40–1,000×. While the latter measurements provide only rough estimates, it is worth giving such data as specimens usually shrink in preparations or contract during fixation. Illustrations of live specimens were based on freehand sketches and micrographs; those of impregnated cells were made with a camera lucida. All figures were oriented with the anterior end of the organism directed to the top of the page.

Determination of growth requirements. For physiological studies ciliates were cultivated in serum bottles containing suspensions of aerobically grown bacteria under a N_2/CO_2 atmosphere. Ten milliliters of basic culture medium were transferred into serum bottles (120 ml) and tightly sealed by rubber stoppers. Prior to autoclaving, the air in the head space was replaced by a mixture of N_2/CO_2 (50:50 v/v; 200 kPa). Aerobically grown *Marinobacter* sp. cells were harvested by centrifugation (12,000 *g*), resuspended, and transferred by syringe to the medium-containing serum bottles to a final concentration of 5×10^9 bacteria/ml. This suspension of bacteria was inoculated with ciliates and incubated at 48 °C or the temperature stated. For cultures growing up to one week, no further feeding was necessary. New bacteria were added to more slowly growing cultures every week to maintain a final concentration of 1×10^9 bacteria/ml. All experiments were carried out at least in duplicate and one representative result is shown. If not stated otherwise, cultures were transferred at least once under the same conditions before growth curves were established.

For determination of salt tolerance, basic culture medium was diluted or concentrated as required. Growth in fresh water was tested in modified ALLEN-medium (Allen 1959; Brock et al. 1972), which was buffered at pH 7 by 25 mM BisTris/HCl. The pH-range of growth was examined by buffering the basic culture medium with 25 mM BisTris/HCl (pH 5.0, 6.0, 7.0) or 25 mM Tris/HCl (pH 7.0, 8.0, 9.0), respectively. Temperature experiments were carried out in water baths. The temperature was adjusted using a calibrated thermometer. Ciliate growth was followed by counting in a Fuchs-Rosenthal counting chamber (Brand, Wertheim, Germany). Doubling times during the logarithmic growth phase were calculated from the slopes of the growth curves.

Ciliates were surveyed at temperatures above their upper growth limit using a Zeiss Axiovert 135 Inverse Microscope, which was situated inside a heatable polyacrylate chamber (Horn et al. 1999) with a temperature-controlled atmosphere (25–70 °C; ± 0.3 °C). A growing ciliate culture was transferred to a tightly sealed observation chamber, which was placed under the microscope. In this and all further experiments only ciliates which showed motility were regarded as viable.

Strictly anaerobic cultivation. The anaerobic technique described by Balch and Wolfe (1976) was employed. *Methanococcus thermolithotrophicus* DSM 2095 (Huber et al. 1982), which was obtained from the Lehrstuhl für Mikrobiologie, Regensburg culture collection, served as food organism. For culturing *M. thermolithotrophicus*, the basic culture medium was supplemented with $K_2HPO_4 \cdot 3\ H_2O$ 0.2g/liter, NH_4Cl 0.25 g/liter, and 10 ml trace elements (Balch et al. 1979). Resazurin (1 mg/liter) was added as redox indicator. The medium was flushed with N_2 for 30 min (1 liter N_2/min). Oxygen was reduced with 0.5 g/liter $Na_2S \cdot 9\ H_2O$ whereby the resazurin was decolorized. In an anaerobic chamber (Aranki and Freter 1972) 10-ml portions were transferred into serum bottles, which were then stoppered and pressurized with H_2/CO_2 (80:20 v/v; 300 kPa). After autoclaving, the serum bottles were inoculated with 0.2 ml *M. thermolithotrophicus* culture and incubated at 60 °C under shaking. After 6 h, ciliates were transferred to the growing culture and the mixture was further incubated at 48 °C. After 2 d, most gas was consumed by the methanogens, and therefore the bottle was repressurized with H_2/CO_2 (80:20 v/v; 200 kPa).

Determination of oxygen tolerance. From an anaerobically grown ciliate culture feeding on *Methanococcus* (about 5,000 ciliates/ml), 2 ml were transferred to new serum bottles with air or an oxygen-free atmosphere (N_2/CO_2 50:50 v/v). The bottles were incubated at 20 °C or 48 °C under shaking (60 rpm) to ensure equilibrium of the gas and the liquid phase. Every 30 min a sample was withdrawn by syringe and the viable ciliates were determined microscopically based on their motility. The experiment was continued until no living cells could be observed in the aerobic bottles.

DNA extraction and analysis of the small subunit (SSU) rRNA gene sequence. Approximately 10^6 ciliates (about 10 ml of culture) were harvested by centrifugation (1,000 *g*). The ciliates were resuspended in a buffer containing 100 mM NaCl and 300 mM Tris/HCl, pH 7.5 and lysed by addition of 2% SDS (w/v) and 1 mg/ml proteinase K. The lysate was incubated at 65 °C for 30 min and then extracted once with phenol and once with chloroform:isoamylalcohol 25:1 (v/v). After precipitation with ethanol the DNA was sedimented by centrifugation and the dried pellet was resuspended in double-distilled water. Coding regions for SSU rDNA were amplified by PCR with a standard protocol using primers specific for eukaryotes (Medlin et al. 1988). The PCR product was sequenced completely (1,721 nucleotides) in both orientations using primers listed by Elwood et al. (1985). No ambiguous positions were detected in the PCR product.

The sequence was aligned with SSU rRNA gene sequences of reference ciliates obtained from EMBL and GenBank by using the automated tools of the ARB software package (Ludwig and Strunk 1997). The data set consisted of 32 taxa and

Table 1. Morphometric data on *Trimyema minutum*.

Characteristics[a]	ME[b]	x̄	M	SD	SE	CV	Min	Max	n
Body, length	IV	24.9	25	3.8	1.2	15.1	20	30	10
Body, length	CL	22.4	22	1.7	0.4	7.6	20	27	17
Body, maximum width	IV	15.7	16	2.7	0.8	17.0	12	20	10
Body, maximum width	CL	15.7	15	1	0.5	13.3	13	19	17
End of ciliary girdle 3 to posterior body end, distance	CL	6.4	7	0.9	0.2	14.7	5	8	17
Macronucleus, length	SC	5.3	5	0	0.2	18.6	4	7	17
Macronucleus, width	SC	4.7	5	1.1	0.3	2.2	3	7	17
Micronucleus, diameter	SC	2.0	2	3	0.1	5.2	1.5	3	17
Macronuclei, number	SC	1.0	1	0	0.0	0.0	1	1	17
Micronuclei, number	SC	1.2	1	—	—	—	1	2	17
Oral ciliary rows, number	SC	2.0	2	0.0	0.0	0.0	2	2	17
Kinetids in oral ciliary row 1	SC	20.7	21	1.2	0.3	5.9	19	22	17
Ciliary girdles on body, number	SC	3.0	3	0.0	0.0	0.0	3	3	17
Upper somatic ciliary girdle, number of kinetids	SC	18.0	18	1.4	0.3	7.6	15	20	17
Middle somatic ciliary girdle, number of kinetids	SC	18.4	18	1.7	0.4	9.2	16	22	17
Lower somatic ciliary girdle, number of kinetids	SC	18.1	18	1.9	0.5	10.7	16	22	17
Caudal cilia, number	SC	1.0	1	0.0	0.0	0.0	1	1	17

[a] Data from cultivated specimens, as described. Measurements in μm. CV—coefficient of variation in %, M—median, Max—maximum, Min—minimum, n—number of individuals investigated, SD—standard deviation, SE—standard error, x̄—arithmetic mean.

[b] Methods: CL—Chatton-Lowff silver nitrate impregnation as described by Foissner (1991), IV—in vivo, SC–permanent silver carbonate slides as described by Augustin et al. (1987).

1,413 unambiguously aligned positions. All phylogenetic analyses were computed with PAUP version 4.0b3a (Swofford 1999). The distance matrix tree was estimated using the Kimura two-parameter model (Kimura 1980) and the Fitch-Margoliash algorithm (Fitch and Margoliash 1967). The maximum likelihood analysis was run using the Tamura-Nei model (Tamura and Nei 1993) with the base frequencies and optimal substitution rate ratio being estimated based on the parsimony tree. In all analyses the heuristic search method was invoked with ten random stepwise additions of sequences. Distance and parsimony data were bootstrap resampled 100 times.

RESULTS

Redescription of *Trimyema minutum* (Kahl, 1931) Augustin et al. 1987 (Fig. 2–35, Table 1)

Improved Diagnosis. Size about 25 × 16 μm in vivo; ovoidal. On average 18 longitudinal somatic ciliary rows, forming 3 oblique somatic ciliary girdles; last girdle extends to posterior third of cell. Oral ciliature composed of 1 (silver carbonate impregnation) or 2 (protargol impregnation) semicircular kineties; 1 short kinety along left half of semicircular kinety(ies); and 3 scattered dikinetids at and near right end of semicircular kinety 1.

Neotype location. Beach north of Porto di Levante at Vulcano Island, Italy (38° 25′ 5″ N, 14° 57′ 14″ E).

Neotype material. Four slides each with protargol, Chatton-Lwoff silver nitrate, and silver carbonate-impregnated, cultivated specimens have been deposited in the Oberösterreichische Landesmuseum in Linz (LI) (Accession Nos. 79/2001–91/2001). Relevant specimens and those shown in the figures are marked by black-ink circles on the cover glass. The SSU rDNA sequence is found as EMBL Accession No. AJ292526.

Terminology. Serrano et al. (1988) and Gärtner-Schür (1990) showed that the oral structures of *Trimyema* are likely derived from prostome ciliates. However, our attempt to include these data in the descriptive terminology of *T. minutum* was abandoned by a reviewer and the Editor. Thus, we use the purely descriptive terminology of Augustin et al. (1987). Likely, ''oral kineties 1 and 2'' are circumoral kineties; ''oral kinety 3'' is a paroral membrane; and the scattered dikinetids near the right end of the oral kineties are adoral organelles.

Description of neotype population from Vulcano Island. All observations are from enrichment cultures as described. Size in vivo 20–30 × 12–20 μm, on average 25 × 16 μm; slightly shrunken in silver nitrate preparations (Table 1); minute (about 10 μm) and deformed individuals occur in old cultures. Shape (lateral outline) highly variable (broadly fusiform, semicircular, almost globular), usually ovoid with anterior end bluntly pointed and posterior broadly rounded (Fig. 3–5, 16–20, 28). Cells often wrinkled by more or less distinct, irregular ridges and grooves and up to 2:1 flattened laterally (Fig. 4, 5, 19, 20). Macronucleus and micronucleus in middle third of cell, globular; micronucleus usually attached to, rarely widely distant from macronucleus (Table 1; Fig. 3, 12, 16, 26, 30, 31). Contractile vacuole subterminal on ventral side, does not contract; excretory pore not recognizable in silver preparations (Fig. 3, 5). Cytopyge in posterior pole center slightly dorsad of caudal cilium, recognizable only in silver preparations, where it appears as a short, thick silverline or as an irregular, granulated area (Fig. 10, 34). Mucocysts invisible in vivo, but occasionally very distinct in silver carbonate and silver nitrate preparations showing a similar or the same pattern as the silverlines, appear as heavily argyrophilic granules 1–2 × 0.5–1 μm in size (Fig. 6, 22, 24, 26, 33). Cytoplasm colorless, contains many lipid droplets 1–3 μm across and up to 6 μm-sized food vacuoles with globular and rod-shaped bacteria (Fig. 3, 16–20). Movement very conspicuous because never swimming like an ordinary ciliate but shivering on the spot and slowly creeping between organic debris.

Somatic cilia 7–8 μm long in vivo, arranged in about 18 longitudinal rows forming three oblique girdles and a semicircular oral kinety (Fig. 2, 3, 7, 9–15, 21–25, 27–32). Ordinary somatic kinetids composed of a large anterior granule (very likely a parasomal sac; Detcheva et al. 1981) and a small, ciliated posterior granule in silver nitrate preparations (Fig. 35); after protargol and silver carbonate impregnation, kinetids usually appear as a single granule having attached, at right, a conspicuous fibre (very likely a kinetodesma) extending anteriorly and, at left, a minute structure extending laterally (Fig. 8, 23, 30). Distance between anterior and middle ciliary girdle slightly smaller than between middle and posterior girdle. Ciliary girdles commence ventrolaterally on right anterior surface and ex-

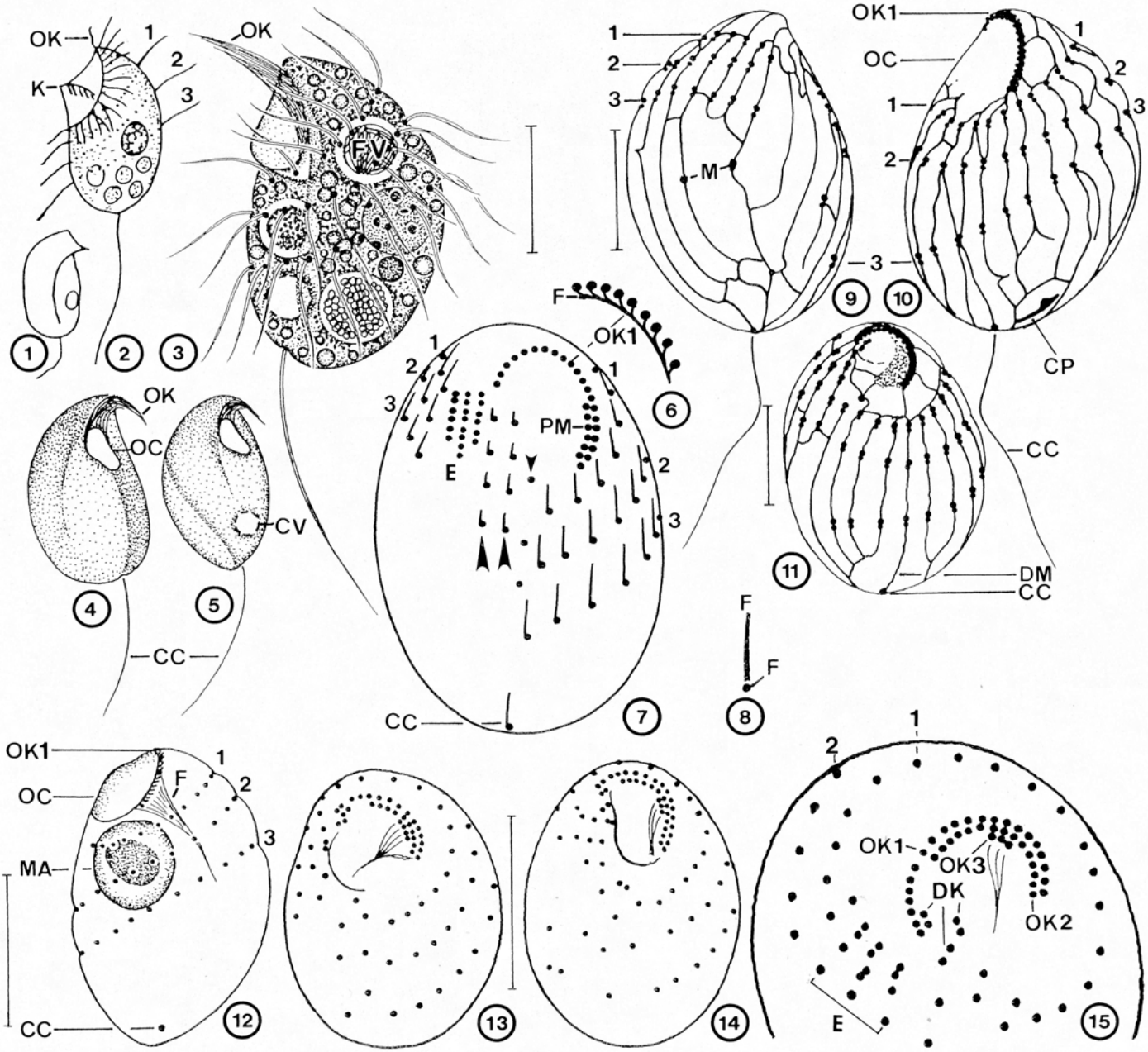

Fig. **1–15**. *Trimyema minutum* from life (2–5), after silver carbonate impregnation (6–8), silver nitrate impregnation (9–11), and protargol impregnation (12–15). **1, 2.** Left and right side view of German type specimens, length 20 μm (from Kahl 1931). **3.** Left lateral view of a representative specimen from Italian neotype population, length 25 μm. The specimen is packed with small lipid droplets and food vacuoles containing coccal and rod-shaped bacteria. **4, 5.** Right side view of shape variants showing cortical ridges and the bright oral cavity. **6, 7.** Diagram of somatic and oral ciliary pattern according to silver carbonate preparations. Large arrowheads mark two postoral rows with short fibrillar associates; small arrowhead denotes an inconspicuous row of basal bodies, possibly ''kinety n''. Oral kinety 1 gives rise to a fibre extending into the pharynx (6). **8.** Somatic kinetids have two associated fibres (?). **9, 10.** Right and left lateral view of same specimen showing ciliary pattern and silverline system. **11.** Ciliary pattern and silverline system of ventral side. **12.** Left side view. **13–15.** Ventral views and anterior polar view showing details and variability of oral and somatic ciliary pattern. CC—caudal cilium, CP—cytopyge, CV—contractile vacuole, DK—oral dikinetids, DM—director meridian, E—epaulet, F—fibres, FV—food vacuole, K—keel (ridge), M—mucocysts, MA—macronucleus, OC—oral cavity, OK—oral kinety(ies), OK1, OK2, OK3—oral kineties, 1,2,3—somatic ciliary girdles. Scale bars = 10 μm.

tend to ventral posterior body fourth, describing an almost full turn (~ 330°; Table 1); this leaves blank a rather wide postoral area containing three kinds of kinetids after silver carbonate impregnation (from right to left; Fig. 7, 22, 27, 29–31): (i) three to four short rows of narrowly spaced mono- and dikinetids forming the so-called epaulet, first shown by Nerad et al. (1995) in *T. shoalsia*; (ii) two, rarely three, short rows each consisting of 3–5 basal bodies having associated a minute fibre at right; and (iii) a short row composed of 3–4 widely spaced kinetids without associates, possibly an ''oblique kinety'' (Augustin et al. 1987) or a ''kinety n'' (Serrano et al. 1988). Details are very difficult to recognize in this region, especially in protargol prep-

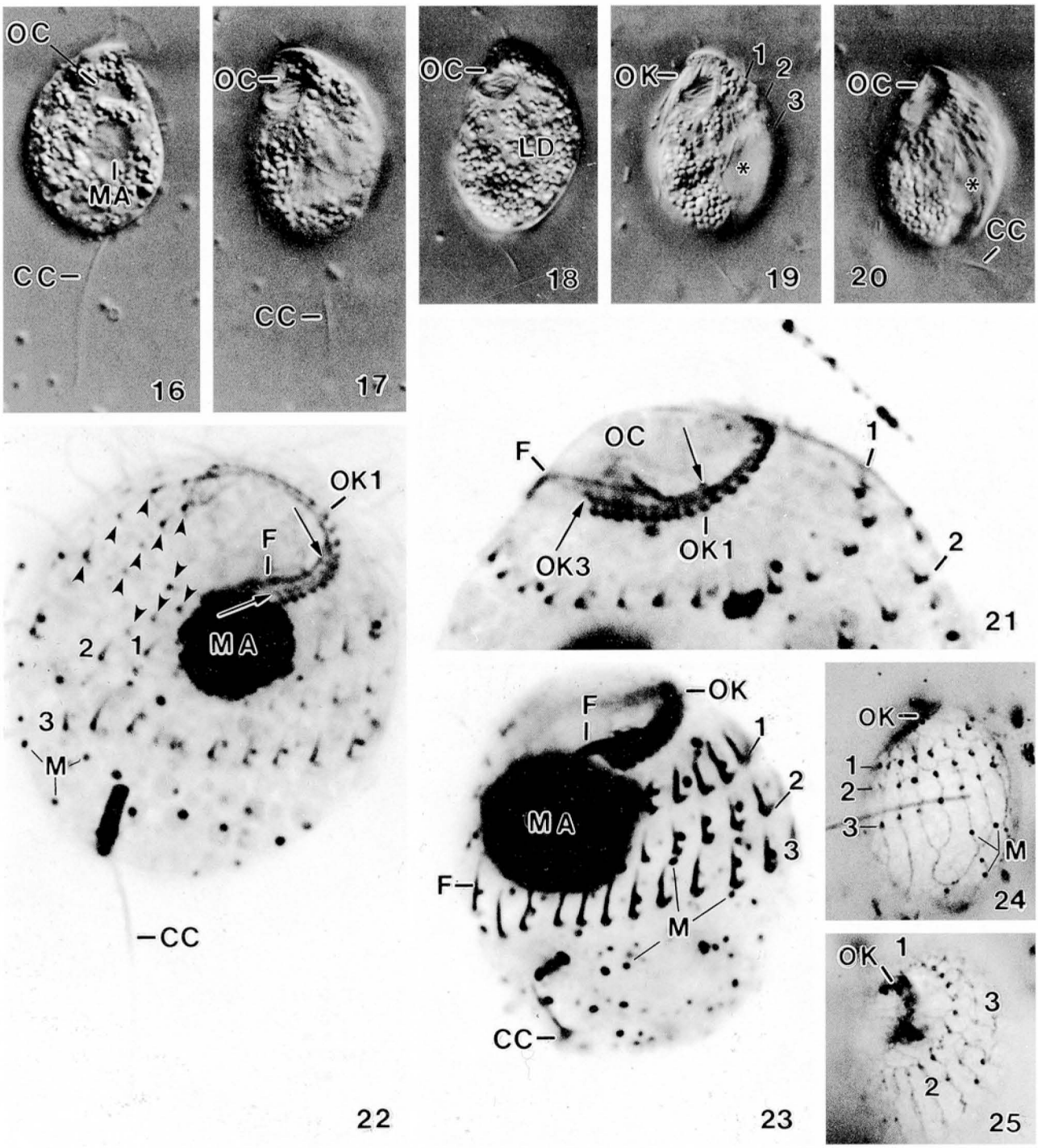

Fig. **16–25**. *Trimyema minutum* from life (16–20), after silver carbonate impregnation (21–23), and silver nitrate impregnation (24, 25). **16, 18.** Right and left lateral optical section of slightly squeezed specimens showing main cell organelles. **17, 19, 20.** Left lateral surface views of freely motile specimens showing main cell organelles and a broad postoral groove (asterisk). **21.** Anterior body portion showing oral kinety 1, faintly impregnated oral kinety 3 (bordered by arrows), and fibres extending into pharynx. **22, 23.** Ciliary pattern and other details of ventral and left side. Arrows denote faintly impregnated oral kinety 3. Arrowheads mark oral and postoral kinetids. Note that mucocysts and cytoplasmic inclusions have the same size as basal bodies making interpretation of structures difficult. **24, 25.** Ciliary pattern and silverline system of left side and anterior pole area. CC—caudal cilium, F—fibres, LD—lipid droplets, M—mucocysts, MA—macronucleus, OC—oral cavity, OK—oral kinety(ies), OK1, OK3—oral kineties 1,3, 1,2,3—somatic ciliary girdles.

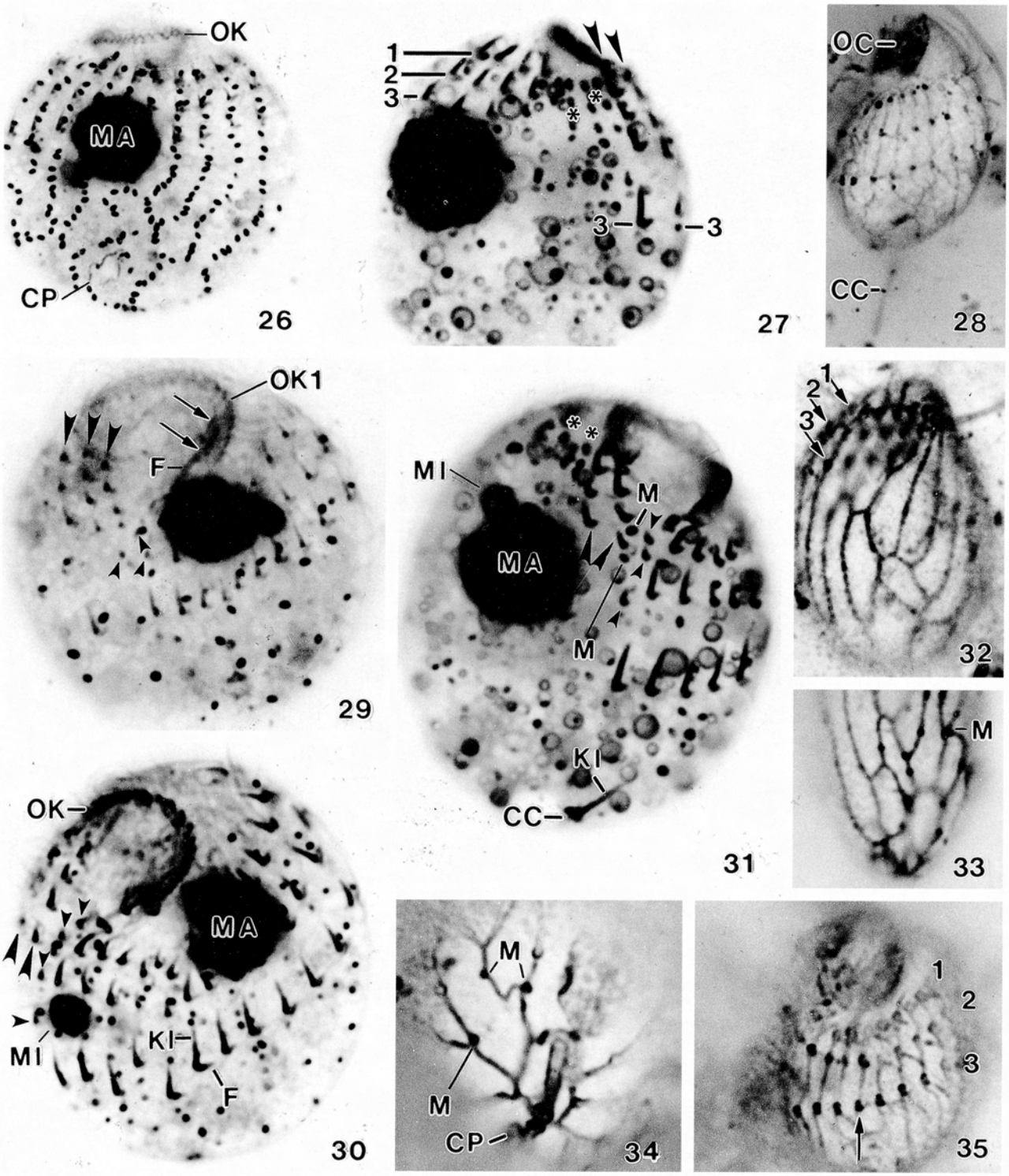

Fig. **26–35**. *Trimyema minutum* after silver carbonate (26, 27, 29–31) and silver nitrate (28, 32–35) impregnation. **26.** Mucocyst pattern. **27, 29–31.** Left lateral (27) and ventral views. Large arrowheads mark two to three specialised ciliary rows with short kinetodesma. Small arrowheads mark a short row (kinety ''n''?) of kinetids without associates. Asterisks denote the epaulet, a field of densely spaced (di?)kinetids. **28, 32.** Ciliary pattern and silverline system of left and right side. The cilia form about 18 longitudinal and 3 oblique rows. **32, 33.** Silverline system of right side and posterior region. **34.** Posterior polar view showing cytopyge. **35.** The somatic kinetids are usually composed of a large anterior and a small posterior granule, occasionally vice versa (arrow). CC—caudal cilium, CP—cytopyge, F—fibres, KI—kinetodesma, M—mucocysts, MA—macronucleus, MI—micronucleus, OC—oral cavity, OK—oral kinety(ies), OK1—oral kinety 1, 1,2,3—oblique ciliary girdles.

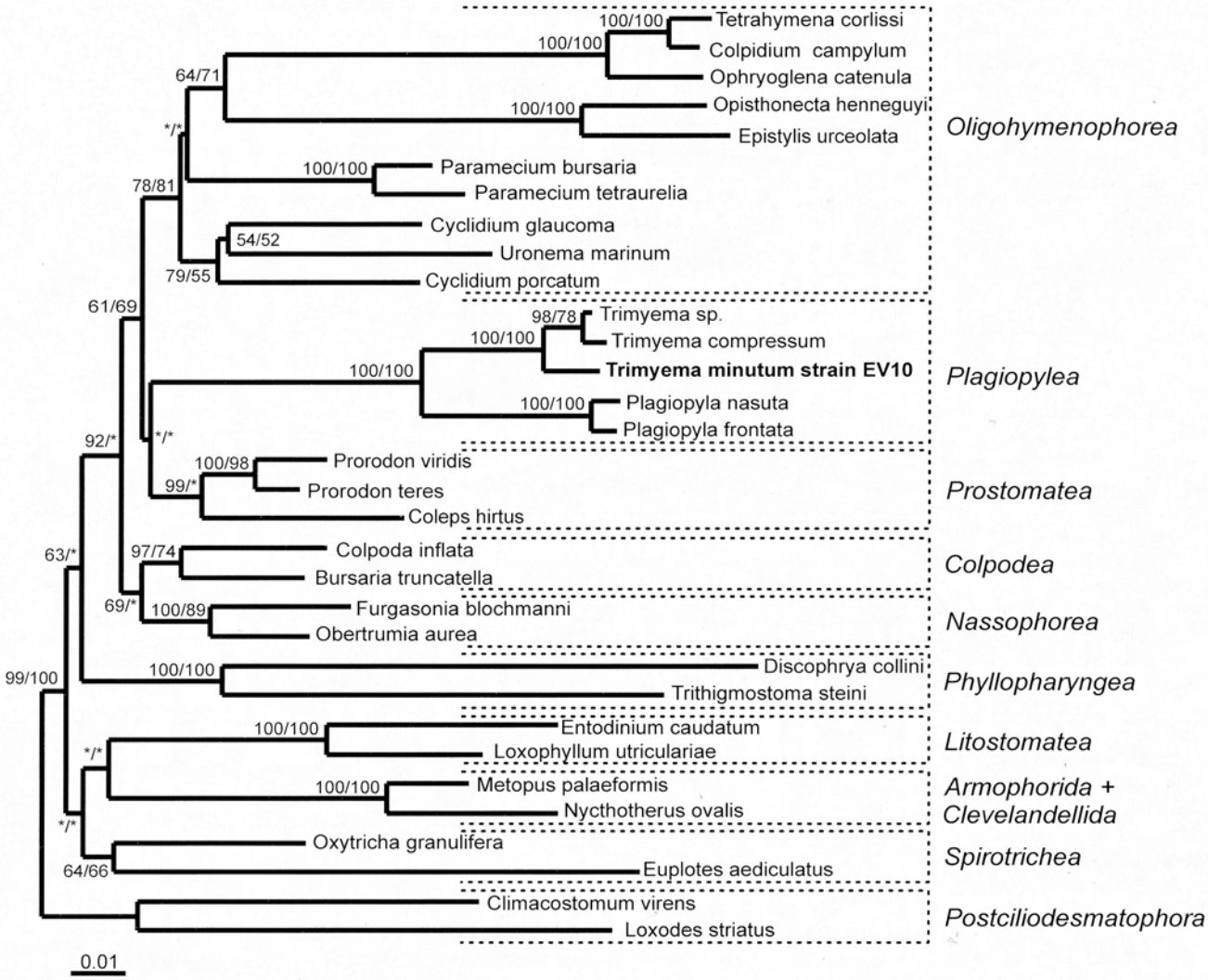

Fig. **36**. Distance matrix tree of *Trimyema minutum* strain EV10 and reference ciliates derived from small subunit ribosomal RNA sequences. The tree was calculated using the Kimura two-parameter model (Kimura 1980) and the Fitch-Margoliash algorithm (Fitch and Margoliash 1967). The scale bar corresponds to 5 estimated changes per 100 nucleotides. The distance bootstrap percentage is followed by the parsimony bootstrap value. Asterisks indicate bootstrap values below 50%.

arations, due to the minute size of the organism and the mucocysts, which often impregnate more or less distinctly and are easily mistaken as basal bodies. Caudal cilium very motile and usually obliquely spread dorsad, about as long as cell; inserted slightly ventral to pole centre right of cytopyge, originates from a complex of three granules in silver nitrate preparations and possesses a conspicuous, anteriorly directed kinetodesma after silver carbonate impregnation (Fig. 1–5, 7, 11, 12, 22, 23, 31).

Oral apparatus subapically on ventral side, oral opening ovoidal, oral cavity broadly obconical and deep approaching dorsal side of cell and thus appearing as highly characteristic, bright spot at middle magnification (200—600×; Fig. 1–5, 16–20). Right oral wall pocket-like projecting at posterior vertex in swirling specimens; left wall irregularly dentate, bears single, semicircular oral kinety composed of an average of 21 cilia producing conspicuous tuft, reminiscent of urotrichid oral flaps (Foissner et al. 1999; Fig. 3–5, 16, 19). Details of oral infraciliature considerably different in silver carbonate and protargol preparations. Thus, both aspects will be described separately. Protargol impregnation (Fig. 12–15): Dorsal and lateral margin of oral cavity surrounded by oral kineties 1 and 2, proximal row 2 shortened at right and possibly unciliated. Oral kinety 3 composed of only three to four, likely unciliated, basal bodies close to left half of oral kinety 2. At right end of oral kinety 1 and right margin of oral opening three scattered groups of dikinetids: group 1 at distal (right) end of oral kinety 1, composed of four basal bodies; groups 2 and 3 along right margin of oral entrance, each composed of two basal bodies. Silver carbonate impregnation (Fig. 6, 7, 21–23, 27, 29–31): Dorsal and lateral margin of oral cavity surrounded by oral kinety 1, which is associated with a fibre bundle composed of very fine fibres originating from individual basal bodies. Oral kinety 2 not recognizable. Oral kinety 3 composed of 5–10, possibly unciliated, basal bodies, difficult to recognize because faintly impregnated and extending close to proximal portion of kinety 1. Scattered dikinetids at right end of oral kinety 1 and right margin of oral opening not unequivocally to identify, but likely present.

Silverline system basically as described by Augustin et al. (1987) in *T. compressum*, but with three remarkable specialisations (Fig. 9–11, 32–35): (i) there are hardly any cross-connectives between the longitudinal silverlines; (ii) the silverline meshes are comparatively large and often have sharp angles in

Table 2. Uncorrected pairwise distances (changes per 100 nucleotides) between small subunit ribosomal RNA sequences of *Trimyema minutum* strain EV10 and related ciliates.

	Trimyema compressum	*Trimyema* sp.	*Plagiopyla frontata*	*Plagiopyla nasuta*
Trimyema minutum	4.4	4.8	11.8	12.1
Trimyema compressum		2.6	11.8	12.7
Trimyema sp.			11.8	12.5
Plagiopyla frontata				3.4

the unciliated right lateral and posterior portion; and (iii) a ''director meridian'' extends from the left end of oral kinety 1 to the caudal cilium.

SSU rRNA phylogeny. Maximum likelihood, parsimony and distance matrix analyses gave similar tree topologies. All three methods supported the monophyly of each ciliate class (except Prostomatea in parsimony trees), but varied in the groupings of the ciliate classes. In all trees isolate EV10 was placed in the class Plagiopylea, with 100% bootstrap support in distance and parsimony analyses (Fig. 36). Isolate EV10 was specifically related to *Trimyema compressum* and *Trimyema* sp., with uncorrected evolutionary distances of 4.4% and 4.8%, respectively (Table 2), while members of the genus *Plagiopyla* were more distantly related to isolate EV10.

Physiological characterization of *T. minutum* strain EV10. *Influence of oxygen.* In cultures of ciliates that had been exposed to air at 48 °C, the concentration of viable ciliates decreased from 4,100 cells/ml to 3,100 cells/ml within 30 min. After 60 min, all ciliates had died. No decrease in viability could be observed in the anaerobic controls after the same time. In aerobic bottles at 20 °C, the concentration of viable ciliates slowly diminished. Even after 90 min, about ¼ of the initial ciliates was still motile, but after 120 min no living ciliates were left. In the anaerobic control bottles, 40% of the ciliates also died within 30 min, but then the number of living cells remained the same. The initial cell death was probably caused by the temperature shift from 48 °C to 20 °C.

Isolate EV10 grew well in screw-capped tubes containing cultures of *Marinobacter* sp., but if the tubes were opened frequently, growth rates of the ciliates varied depending on the oxygen input. Reproducible growth rates were achieved in tightly stoppered serum bottles with N_2/CO_2 atmosphere with suspensions of the aerobically pre-cultured *Marinobacter* sp. as food. Under these conditions, doubling time was 6 h at 48 °C (Fig. 37). Under strictly anaerobic conditions, the ciliate could be propagated in growing cultures of the archaeon *Methanococcus thermolithotrophicus*. In this case, doubling time was 12 h at 48 °C (Fig. 38). The isolate has been grown for over 6 mo on *M. thermolithotrophicus* as sole food source. The ingested methanogens in the food vacuoles could be visualized by fluorescence microscopy (Fig. 39), but no methanogenic endosymbionts could be detected.

Salt tolerance. The ciliate grew from 7.2% (w/v) salt (2 times concentrated sea water) to 0.45% (w/v) salt (8 times diluted sea water). No growth was observed in 3 times concentrated sea water (10.8% w/v salt) or in fresh water. Transfer of cultures grown at 1.8% (w/v) salt (Fig. 40, triangles) into medium with a salinity of 7.2% (w/v) resulted in a lag time of about 36 h (Fig. 40, empty circles). On the other hand, no significant lag phase could be observed when the ciliates were transferred to 3.6% (w/v) salt (Fig. 40, full circles). The doubling times were rather similar and independent of the external salt concentrations (Fig. 40). Inoculation of cultures grown at

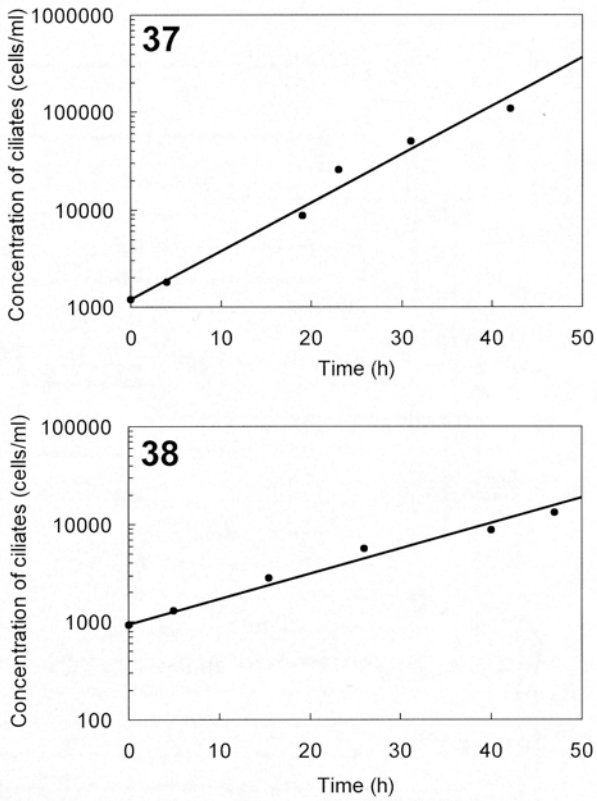

Fig. **37, 38**. Growth curves of *Trimyema minutum* strain EV10 at 48 °C. **37.** On a suspension of *Marinobacter* sp. under a N_2/CO_2 atmosphere. **38.** On *Methanococcus thermolithotrophicus* under anaerobic conditions.

a salinity of 1.8% (w/v) to medium with 0.9% (w/v) and 0.45% (w/v; 4 and 8 times diluted sea water) yielded lag times of 3 and 6 d, respectively (data not shown). During these periods of time no ciliates could be detected microscopically, indicating lysis of most of the inoculated cells.

Influence of pH. Isolate EV10 grew in medium adjusted to pH 6.0, pH 7.0, and pH 8.0 with similar rates (not shown). No growth was observed at pH 5.0 and pH 9.0.

Growth at different temperatures. Isolate EV10 could duplicate in a temperature range of 28–52 °C. Ciliates grown at 48 °C could be successfully transferred to 30 °C and 50 °C. Cultures grown at 30 °C and 50 °C served as successful inocula for cultivation at 28 °C and 52 °C, respectively. The doubling

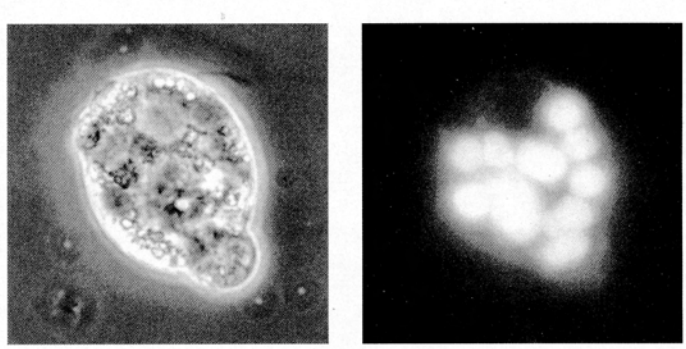

Fig. **39**. Slightly flattened ciliate with ingested *Methanococcus thermolithotrophicus* cells in the food vacuoles in (left) phase contrast and (right) epifluorescence (excitation at 436 nm).

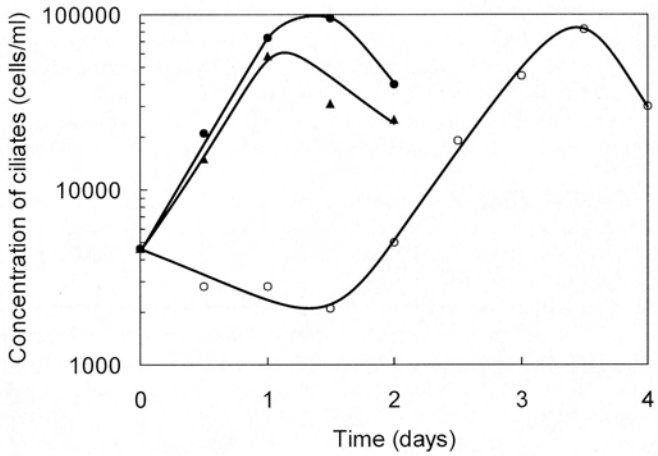

Fig. **40**. Growth curves of *Trimyema minutum* strain EV10 at different salt concentrations: –▲– 1.8% (w/v), –●– 3.6% (w/v), –○– 7.2% (w/v).

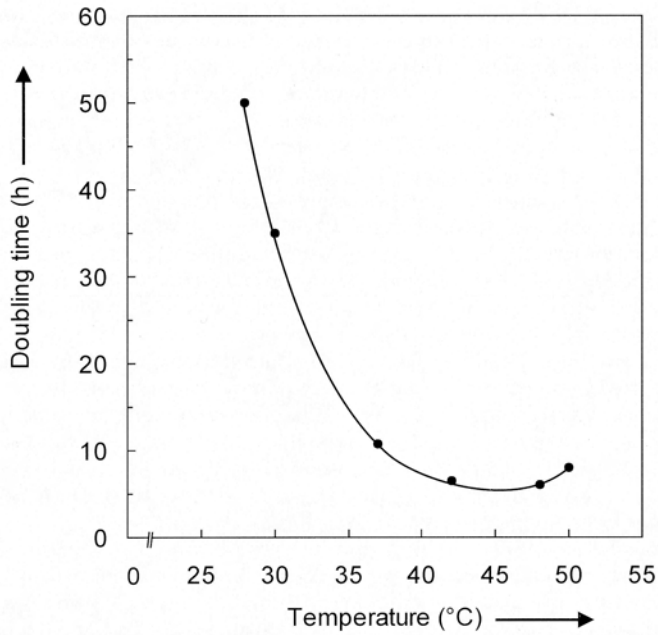

Fig. **41**. Effect of temperature on doubling time of *Trimyema minutum* strain EV10. No growth was observed below 28 °C and above 52 °C.

times during the exponential growth phase were determined at various temperatures. Optimal growth was observed between 40 °C and 50 °C with the shortest doubling time of 6 h at 48 °C (Fig. 41). At 52 °C, if at all, very weak growth could be detected and dividing cells were observed. No growth was observed at 55 °C and at 25 °C.

Survival at extreme temperatures. A culture of the new ciliate, which had been pre-grown at 48 °C was shifted to 55 °C under the heated microscope. After 2 h cells moved more slowly and some of them became spherical, containing a huge vacuole. After 4 h at 55 °C some motile cells were still visible. In agreement with this result, such cultures could still be successfully transferred to 48 °C. However, after 8 h at 55 °C only some immotile cells remained and no successful transfer was possible. If a culture grown at 48 °C was shifted to 60 °C, ciliates became very active in the beginning. After 5 min however, the first cells already stopped moving. After 10 min at 60 °C, all ciliates were immotile and sank down to the bottom of the observation chamber. No successful transfer to 48 °C was possible, indicating that all cells had died within 10 min. In a control chamber kept at 48 °C, ciliates remained viable until the food was exhausted after 2 d.

When a ciliate culture grown at 48 °C (containing 30,000 ciliates/ml) was stored at 25 °C, cell numbers remained constant for about 1 wk although motility of the cells clearly slowed. After 1 wk several cells had a deformed appearance or contained huge vacuoles. After 2 wk the ciliate concentration had already decreased by 50%. After 4 wk only 5% of the initial ciliate number could be counted. When this culture was shifted to 48 °C in the heatable microscope, all remaining cells began to move very quickly for about 15 min. Then, about 90% of the cells, including deformed cells slowed down and finally stopped moving. Seven hours later only very active cells and obviously dead cells could be detected. After 24 h, several dividing cells could be observed. In agreement, cultures stored for 4 wk at 25 °C, served as a successful inoculum for cultures at 48 °C. At 30 °C, ciliates have been grown and subcultured for 6 mo. Even ciliates from this acclimated lineage were not able to grow at 25 °C and storage at 10 °C resulted in a dramatic loss of viability: after 12 h, only very rarely could slowly moving cells be observed. During this phase of storage, cultures could still serve as successful inoculum, while this was not the case after 48 h at 10 °C. When cultures grown at 30 °C were stored at 4 °C, all cells were killed within 12 h.

DISCUSSION

Identification. The original description of *T. minutum* is very brief, incomplete, and lacks type material (Kahl 1931; Fig. 1, 2). Thus, the species requires deposition of neotype slides in an acknowledged repository. We believe our specimens possess all the characteristics of this species mentioned by Kahl (1931), namely: (i) size about 20 μm (20–30 μm in our material); (ii) posterior end broadly rounded (as in most of our specimens); (iii) surface with distinct cortical ridges and a beak-like projection at the oral opening (ridges and furrows are common also in our specimens; the beak is probably the pocket-like projection of the oral vertex or the protruding ciliary tuft formed by the oral kinety); (iv) relatively long and stiff cilia (as in our specimens, which hardly swim like ordinary ciliates); and (v) three somatic ciliary girdles (as in our specimens). The only significant difference is the habitat: Kahl (1931) discovered *T. minutum* in a freshwater ditch polluted by liquid manure. However, Wenzel (1961) and Tucolesco (1961) recorded *T. minutum* from marine and brackish habitats, indicating that it is euryhaline.

The only other species similar to *T. minutum* and our isolate is *T. shoalsia*, a marine species recently described by Nerad et al. (1995). Nerad et al. (1995) separated *T. shoalsia* from *T. minutum* by the ''lack of a beaked vestibulum and dorsal keel (ridge)''. Unfortunately, they do not support this statement by appropriate micrographs and a thorough description of live specimens. Thus, a detailed comparison is impossible. However, our neotype of *T. minutum* does differ from *T. shoalsia*, whose ciliary girdles are longer (at least 450° vs. 330°) and restricted to the anterior body half (vs. second third of body).

Comparison with related species. Minute ciliates, such as *T. minutum* and *T. shoalsia*, pose problems of observation and preparation, which must be considered when results from different methods and authors are compared. Thus, small differences should not be used to distinguish species, unless they are substantiated by transmission electron microscopy. An example is the suggestion by Nerad et al. (1995; Table 2) that Augustin

et al. (1987) and Serrano et al. (1988) investigated different species, that is, only one of the two is *T. compressum*. However, all distinguishing features used by Nerad et al. (1995) are either minute (e.g. 3–4 vs. 6 kinetosomes) or dependent on the preparation method (protargol vs. silver carbonate): for instance, cell size which is double in Serrano et al. (1988) because they measured heavily squashed and/or inflated specimens.

Our comparative analysis emphasizes that details of the somatic and oral infraciliature depend highly on the preparation method and thus hardly can be used to define species. The semicircular oral kinety 1, for instance, is composed of a single row of granules (basal bodies) in silver carbonate preparations, while two granule rows (kineties) are recognizable after protargol impregnation (Fig. 7, 15). Furthermore, results may be different even with modifications of the same method: in *T. compressum* each of the two oral rows appears composed of granule pairs in the silver carbonate preparations of Augustin et al. (1987), while they consist of single granules in the silver carbonate preparations of Serrano et al. (1988); the latter aspect has been supported by transmission electron microscopy (Gärtner-Schür 1990). In silver nitrate preparations, the somatic kinetids of *Trimyema* appear as double granules (''dikinetids''), but only the posterior granule is ciliated, while the anterior represents a parasomal sac (Augustin et al. 1987; Detcheva et al. 1981; Fig. 9, 10, 35).

With this knowledge, it is likely that the oral structures of *T. compressum, T. minutum*, and *T. shoalsia* are highly similar (as is usual in congeners), differing mainly in morphometric features: *T. compressum* has at least twice the number of somatic and oral kinetids as *T. minutum* and *T. shoalsia*, which are highly similar both qualitatively and quantitatively. Nerad et al. (1995) state that *T. shoalsia* lacks oral kinety 3. However, the micrographs provided do not show the ''critical'' area and are thus not entirely convincing in this respect. Furthermore, it is highly unlikely that such a conservative structure is variable within a genus.

Generic assignment and systematic position. Both morphology and SSU rRNA sequences unequivocally relate our thermophilic isolate to the genus *Trimyema* and show that it is different from *T. compressum* (Fig. 36).

Trimyema was considered as a trichostome ciliate (Augustin et al. 1987; Corliss 1979). Serrano et al. (1988) then suggested a prostome relationship because they discovered a minute ''prostomatid oral apparatus'' during cell division. This was sustained by electron microscopical data (Gärtner-Schür 1990). Later, Nerad et al. (1995) proposed that *Trimyema* might belong to the class Oligohymenophora, but did not provide any reasons. However, an oligohymenophoran / prostomatid relationship is now strongly supported by gene sequence data (Stechmann et al. 1998). Our observations also revealed several oligohymenophoran features: namely, a ''caudal cilium complex'' composed of three argyrophilic granules and a striated silverline pattern with a ''director meridian'' extending from the oral kinety1 to the caudal cilium (Fig. 11). These features are typical for many tetrahymenine oligohymenophorans, as is the oral apparatus, which shows a tetrahymenine configuration in the middle ontogenetic stages (Serrano et al. 1988).

Distribution. *Trimyema minutum* is possibly a rare species because it has been reported only thrice. However, the incidence might be higher because such small species are often overlooked or not identified in routine investigations. The habitats where it was retrieved were remarkably different: Kahl (1931) discovered *T. minutum* in a ditch contaminated with liquid manure. Wenzel (1961) observed it in the sponge *Halichondria panicea* from the Gulf of Naples, about 300 km from the site where we rediscovered it. Tucolesco (1961) recorded *T. minutum* twice from old, mixed infusions of the para-marine Roumanian Lake Tekirghol. This indicates that *T. minutum* can adapt to different environments. In the hydrothermal areas at the beach of Vulcano Island *T. minutum* is very common (data not shown). Possibly, thermophilic strains can be found in different heated environments but no studies have been carried out yet.

General culture requirements. Similar to the other cultivated species of *Trimyema*, *T. compressum* and *T. shoalsia*, the *T. minutum* strain EV10 requires anaerobic or at least low oxygen conditions (Goosen et al. 1990a; Nerad et al. 1995; Wagener and Pfennig 1987). In enrichment cultures of *T. minutum* strain EV10, oxygen consumption by the concomitantly growing mixture of aerobic heterotrophic bacteria created the anaerobic conditions required for the ciliate. Free-living anaerobic ciliates had been reported to exhibit variable oxygen sensitivity, some of them even surviving full atmospheric oxygen tension up to 2 d (Fenchel and Finlay 1990). At ambient temperature *T. minutum* strain EV10 survives aerobic conditions up to 90 min, long enough to stand the washing procedure. In contrast, at its optimal growth temperature (48 °C) similar to anaerobic thermophilic prokaryotes the adverse effect of oxygen was much stronger (Stetter 1995). In spite of its anaerobic mode of life, no methanogenic endosymbionts were detected in *T. minutum* strain EV10. Inspection of original samples revealed that they were already missing from the beginning as it has been reported for other anaerobic ciliates (Fenchel and Finlay 1990).

For physiological experiments suspensions of the aerobic *Marinobacter* sp. under a N_2/CO_2 atmosphere were used to exclude the influence of oxygen on growth rates of the ciliates. For unknown reasons the doubling time on suspensions of the *Marinobacter* sp. is two times less than on the strictly anaerobic *Methanococcus thermolithotrophicus* and the cell yield is ten times higher. Dependence of the velocity of growth on the food bacterium had been reported previously for *T. compressum* (Holler et al. 1994; Yamada et al. 1994). The culture conditions of ciliates feeding on *Methanococcus* are defined and easily controllable with what facilitates mass-culturing. Recently, we were successful in growing *T. minutum* in a 300-liter fermentor (10 g cell mass; data not shown).

Trimyema minutum strain EV10 exhibits a broad range of salt tolerance in the laboratory, which is consistent with previous reports that it is a euryhaline species. The pH required for growth of *T. minutum* strain EV10 is from slightly acidic to slightly alkaline corresponding well to that of the marine original sample.

Specific temperature demands of growth and survival. By its optimal (fastest) growth at 48 °C and upper temperature limit of 52 °C, *T. minutum* strain EV10 appears as the most extremely thermophilic ciliate that ever has been cultivated. In the heatable microscope, *T. minutum* cells died rapidly when the temperature was raised to 60 °C. This shows that the cells had not adapted to the 70 °C as measured in the environment. Very likely, this discrepancy is due to temperature micro-inhomogeneities in the hydrothermal vent system, which were not detected by our temperature probe. Kahan (1972) , who isolated the ciliate *C. citrullus* from the Tiberias Hot Springs, also measured a temperature at the sampling site 10 °C above the upper growth temperature of the ciliate. Therefore, one cannot deduce growth temperatures simply from in situ temperature measurements (e. g. Dombrowski 1961, Pax 1951).

In contrast to *C. citrullus*, the ciliate with the highest growth temperature so far (Kahan 1972, Kahan and Sharon 1976) *T. minutum* strain EV 10 is not only unable to adapt and propagate, but even is killed by temperatures below 28 °C. However, so far uncultivated ''strains'' of *T. minutum* apparently thrive

at temperatures below 28 °C (Kahl 1931; Tucolesco 1961; Wenzel 1961).

The discovery of *T. minutum* strain EV 10 extends the upper temperature limit of ciliates. Further, even more extreme ciliates may exist even in hotter environments.

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