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Isosphaera pallida, gen. and comb. nov., a gliding, budding eubacterium from hot springs

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Abstract. An unusual filamentous, budding bacterium was isolated from several North American hot springs and named *Isosphaera pallida*. Filaments are composed of spherical cells 2.5–3.0 μm in diameter, with cell growth and division occurring by formation of intercalary buds. These obligately aerobic, heterotrophic isolates closely resemble *Isocystis pallida* Woronichin, which has been previously described as a cyanobacterium, and later as a yeast, based on collected specimens.

Isolates were salmon-colored due to the presence of carotenoids and contained gas vesicles. Growth occurred at temperatures up to 55° C in defined media using 0.025% glucose or lactate as carbon sources. Glucose concentrations of 0.05% or higher inhibited growth of the culture.

Ultrathin sections observed by TEM revealed an unusual tri-laminar wall structure. Pit-like ultrastructural features were found in the cell wall. Growth of cultures was not inhibited by penicillin G, and the Gram reaction gave variable results.

Cells formed motile, macroscopic aggregates ("comets") when harvested from liquid cultures and plated on media containing Gelrite (Kelco Co.) as a solidifying agent. Aggregation and motility were observed in both the light and the dark. However, comets were strongly phototactic. Negative stains revealed numerous pili, but not flagella.

We propose that this highly unusual prokaryote be placed in a new genus.

Key words: *Isosphaera pallida* – Thermophile – Hot spring – Phototaxis – Gliding motility – Gas vesicles – Budding – Cell wall

We have isolated an unusual gliding, budding bacterium from North American hot springs. The morphology of these strains is distinctive: spherical cells are arranged in filaments, with growth and division occurring by formation of intercalary buds. This organism, which is readily recognized because of its distinctive morphology, is a familiar component of the warm spring microflora in Europe and the United States, where it is found in neutral and alkaline springs between 35 and 55° C.

This organism has previously been known as *Isocystis pallida* Woronichin, based on collected specimens. We propose naming it *Isosphaera pallida*, thereby assigning it to a new genus.

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In addition to its unusual morphology, *Isosphaera pallida* possesses a combination of unusual phenotypic traits which has not been seen in other prokaryotes. It is the only budding microorganism known to move by "gliding", and it is the only heterotrophic prokaryote known to be phototactic. Muramic acid and diaminopimelic acid, essential components of peptidoglycan, are absent from the cell wall of *Isosphaera pallida* (Giovannoni, unpublished work).

Phylogenetic analyses based on sequence comparisons of 5S rRNAs (Bomar, unpublished work) and 16S rRNAs (Giovannoni, unpublished work) have demonstrated that *Isosphaera pallida* is related to members of the *Planctomyces* group of budding bacteria, which also lack peptidoglycan walls (König et al. 1984).

Here we present the results of initial studies on the ultrastructure and physiology of this organism.

Materials and methods

Isolation and culture conditions. Unless otherwise stated, strain IS1B, isolated from Kah-nee-tah Hot Springs in Oregon, was used in all experiments. Strains were isolated by streaking plates of medium IM containing 1.5% Bacto-Agar. Routine cultivation used medium IMC. Large batch cultures were raised in 1000 ml "bubbler" vessels (Nelson and Castenholz 1982, for a description). Bubbler cultures were sparged with 5% CO_2/95% air. Small batch cultures (50 ml) were grown in 125 ml Erlenmeyer flasks equipped with a Hungate-type septum screw cap, and purged with 5% CO_2/95% air. Cultures on plates or in tubes (shakes or slants) were incubated in Baltimore Biological Laboratories "Gas-Paks". A single "Alka-Seltzer Gold" tablet was added to a flask of water in the "Gas-Paks" before closure. One "Alka-Seltzer Gold" tablet evolves 109 ml of gaseous CO_2, producing a final concentration of 5.3% CO_2 in the "Gas-Pak". Unless otherwise specified, cultures were incubated at 42° C and cells were harvested in late exponential growth phase (0.5–0.6 OD_{650} nm).

Medium IM was prepared as follows: 250 ml of solution A was added to 650 ml distilled water (15 g/l agar was added for plates), autoclaved, and cooled to 50° C. 100 ml of solution B was then added to the sterile medium. Solution A contained (per liter H_2O): $CaCl_2 \cdot 2H_2O$ 320 mg, $MgSO_4 \cdot 7H_2O$ 400 mg, KCl 500 mg, NaCl 1 g, $(NH_4)_2SO_4$ 500 mg, KH_2PO_4 300 mg, $FeCl_3$ 0.292 mg, micronutrient solution SL7 (Pfennig and Trüper 1981): 10 ml, vitamin B_{12}: 0.005 mg. The solution was brought to pH 7.6 with 2 M NaOH, and filtered through Whatman no 1 filter paper to

remove the precipitate. Solution B contained 0.05 M $NaHCO_3$ which had been sterilized by autoclaving, followed by vigorous sparging with sterile CO_2 for 1 h.

Medium IMC was prepared by adding 0.025% D-glucose (filter sterilized), 0.025% casamino acids (filter sterilized) and 0.5 ml/l of vitamin solution to medium IM. This vitamin solution contained: nicotinic acid: 2 mg/ml, thiamine HCl: 1 mg/ml, p-aminobenzoic acid: 0.2 mg/ml, and biotin: 0.02 mg/ml.

Medium IM containing 0.4% agarose Type I (Sigma) was used for the preparation of shake cultures. After the addition of sodium bicarbonate, the medium was bubbled with sterile 5% CO_2/95% N_2 for 30 min prior to pouring, to bring the CO_2/bicarbonate buffer system to pH equilibrium. Tubes incubated in the light received 3.5×10^{15} quanta/cm^2 · s of coolwhite fluorescent light. Tubes were incubated for 14 days at 45° C.

Carbon source utilization. All carbon sources used in nutritional experiments were filter sterilized and added to medium IM following autoclaving. Either 0.5% agarose (Sigma, type I), or "Gelrite" (Kelco Co.) was used as a solidifying agent. 20×50 mm dishes containing 50 ml of medium were used. Carbon sources were added to a concentration of 0.025%. Control plates contained either no carbon source or 0.025% of the carbon source being tested in addition to 0.025% D-glucose and 0.025% casamino acids. One loopful of cells from a fresh plate of medium IMC was resuspended in 1 ml of medium IM as an inoculum. 10 µl of this suspension was spotted onto plates and incubated for 2 weeks, followed by scoring.

Electron microscopy. Exponential growth phase cells were resuspended in 50 mM sodium cacodylate buffer containing 2% glutaraldehyde and fixed on ice for 1.5 h. Equal volumes of fixed cells were then added to a warm solution of 0.8% agarose in 50 mM cacodylate buffer, and drawn up in the tips of Pasteur pipettes. The agarose containing the cells was then extruded from the pipettes and fixed in 1% OsO_4 for 1 h at room temperature. After osmium fixation, the agarose strands were washed in several changes of buffer, dehydrated in an ethanol series, and embedded in Epon/Araldite. Sections were cut using a Reichert OM-U2 ultramicrotome, placed on copper grids, and poststained in 1 or 2% uranyl acetate. Samples were examined and photographed using a Philips 300 transmission electron microscope. Pili were visualized by negative staining with 2% uranyl acetate or by rotary shadowing with platinum.

Oxygen uptake measurements. All O_2 uptake measurements were carried out using a polarographic oxygen electrode mounted in a temperature-controlled glass cuvette equipped with a magnetic stirrer. Inhibitors were added through a glass capillary using a 100 µl Hamilton syringe.

Optical absorbance spectra. A Cary 14R spectrophotometer was used for measurement of optical absorbance. Cell-free extracts were prepared by sonicating cell suspensions in 50 mM pH 7.5 potassium phosphate buffer, followed by centrifugation at 12,000 *g* for 10 min to remove unbroken cells. Samples were oxidized by adding hydrogen peroxide (final concentration ca. 0.03%). "Reduced" samples were stored in a sealed Hungate tube under nitrogen. Protein was measured by the dye binding method of Bradfield (1976).

Table 1. List of *Isosphaera pallida* strains

Strain	Locality	Collection temperature (°C)
IS1B	Kah-nee-tah Hot Springs, Oregon, USA	44
IS2	Painted Pool, Mammoth Hot Springs, Yellowstone Nat. Park, USA	40
IS4	"Rabbit Creek Spouter", Midway Geyser Basin, Yellowstone Nat. Park, USA	35–38
IS5	Bath Lake, Mammoth Hot Springs, Yellowstone Nat. Park, USA	44
IS6	White Sulfur Spring, Thermopolis, Wyoming, USA	51
IS9	Big Spring, Thermopolis, Wyoming, USA	36

DNA base composition. DNA was purified by the method of Marmur (1961). The mole percent guanine plus cytosine base ratio was determined by buoyant density centrifugation in a cesium chloride density gradient.

Results

Isolation and cultivation. Isosphaera pallida was isolated from hot springs by streaking samples directly onto agar plates of medium IM and incubating the plates for two weeks in darkness at 45° C. After incubation *Isosphaera pallida* colonies were usually the most conspicuous on the plate. Since medium IM contains no organic constituents except vitamin B_{12}, growth of *Isosphaera pallida* under these conditions appeared to be occurring on organic contaminants of agar. Isolation was accomplished by re-streaking, followed by transfer to medium IMC for routine cultivation.

A list of strains is shown in Table 1. Unless otherwise noted, the experiments described here were done with strain IS1B, a variant of strain IS1 which forms short (ca. 2–6 cell) chains, rather than the usual filaments of indefinite length.

Colony and cell morphology. Wild-type *Isosphaera pallida* forms small, pink, convex colonies of firm consistency when grown on Bacto-agar. Colonies may migrate on wet agar plates or Gelrite plates. Migrating colonies are phototactic and often comet-shaped (Fig. 1).

The cells are spherical in shape and from 2.5 to 3.0 µm in diameter (Fig. 2). Wild-type cells form chains of indefinite length. Cell growth and division occur by formation of intercalary buds (Fig. 2). Bud formation always occurs on the chains axis; branching was never observed. Budding begins with the formation of a small protuberance connected to the mother cell by a narrow constriction (Fig. 3a). Buds enlarge until they reach the approximate size of the mother cell, at which time "pinching off" occurs (Fig. 3b–d). Cytoplasmic connections are not visible between mature cells of a chain.

Gas vesicles are often present in recent isolates of *Isosphaera pallida*, but disappear during extended cultivation. When observed by phase-contrast microscopy, *Isosphaera* often appears to have a capsule. In fact, light

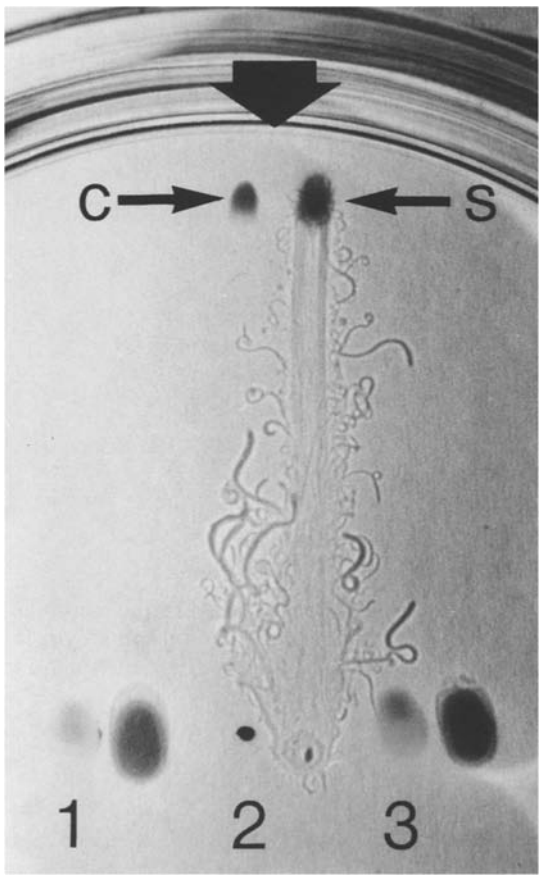

Fig. 1. Formation of phototactic aggregates ('comets'). *Arrow* indicates the direction of the light source (3.5×10^{15} quanta $cm^{-2} \cdot s^{-1}$, coolwhite fluorescent light). Incubation time: 72 h at 42° C. A point light source was used to illuminate the plates for photography, revealing comets (*c*) and shadows of comet tracks (*s*). Strain IS1B (cell suspension plated at positions *1* and *3*) showed little response. Wid-type strain IS9 (plated at position *2*) was motile in the light and the dark (not shown), and was positively phototactic

scattering by a dense layer of pili surrounding the cell causes this effect.

Ultrastructure. Ultrathin sections viewed by TEM reveal a cell wall having two electron dense layers separated by an electron transparent layer (Fig. 4b). The total wall thickness is 11 nm. Neither a peptidoglycan layer nor an outer membrane layer were evident. A 40 nm thick layer resembling the glycocalyx of Gram negative bacteria was apparent.

Ring-like structures with a diameter of 27 nm are evenly spaced over the surface of negatively stained cells (Figs. 4a, 5c).

Negative stains and platinum shadows showed that *Isosphaera* is covered with pili which appear to be evenly distributed over the surface of the cell (Fig. 5a, b). The pili have a diameter of 4 nm and are up to 4 μm in length. Flagella were not observed.

Motility and phototaxis. Cells are not motile on plates containing 1.5% Bacto-agar. When 0.4% agarose or 0.5% Gelrite was used as a solid support, gliding motility occurred (Fig. 1). Wet slide-mounted *Isosphaera pallida* filaments glide on slides at 0.0037 to 0.005 μm/s. Intact colonies (comet-shaped aggregates) glide at an average rate of 0.053 μm/s. Colonies migrate in uniform light or in darkness at the same rate, but are positively phototactic in the light.

Effect of temperature on growth. Isosphaera grows at temperatures from 34 to 55° C, with the maximum rate of growth occurring at 41° C ($\mu = 0.042\ h^{-1}$) (Fig. 6).

Absorbance spectra. Optical absorbance spectra (350–1000 nm) of crude cell extracts showed absorbance peaks at 417, 478, 503, and 536 nm (Fig. 7). No peaks corresponding to the absorption maxima of bacteriochlorophylls or bacteriorhodopsin were observed. The peaks at 478, 503, and 536 nm correspond to the absorption maxima of carotenoids (Karin Schmidt, personal communication). Reduced minus oxidized difference spectra of cell-free extracts confirmed that the major peak at 417 nm was due to Soret band absorption by cytochromes, indicating a high cytochrome content (Fig. 8).

Absorption spectra of acetone-methanol (1:1) extracts of *Isosphaera* showed peaks at 370, 386, 489 and 520 nm, which are attributed to carotenoid absorption (Fig. 7).

In order to determine if conditions of lowered O_2 concentration might induce formation of photosynthetic pigments, the sparging of "bubbler" cultures in exponential growth phase was stopped for 24 h. The dissolved oxygen content of the cultures dropped rapidly with the cessation of sparging, as measured by an oxygen electrode. Acetone-methanol extracts revealed no peaks from 300 to 1100 nm in addition to those in aerobic controls (data not shown).

Physiology. Isosphaera pallida is an obligately aerobic chemoheterotroph. Growth did not occur below the surface in agar shakes of medium IMC or in Gas-Paks under anaerobic conditions. Cell-free extracts of *Isosphaera* consumed O_2 at 0.14 μg O_2 $(min)^{-1} \cdot (mg\ protein)^{-1}$. Sodium cyanide (660 μM) completely inhibited oxygen uptake.

Substrate utilization and nutritional requirements. Isosphaera pallida is capable of growth in defined media containing glucose as the sole carbon source. Of 14 substrates tested, only glucose and lactate served as sole sources of carbon (Table 2). Growth of all strains was inhibited by glucose concentrations of 0.05%, and some strains were inhibited by other carbon sources as wall (Table 2). In batch culture, growth on glucose was efficient, with 103 g (dry weight) of cells produced per mole of glucose initially present in the medium.

Antibiotic sensitivity. Growth of *Isosphaera pallida* was not inhibited by 1670 units/ml of penicillin G.

DNA base composition. The mole% guanine + cytosine content of *Isosphaera pallida* DNA was 62.2%.

Discussion

Isosphaera pallida is an obligately aerobic chemoheterotroph with a limited nutritional range and a slow growth rate. Growth inhibition by some carbon sources, including glucose, at concentrations of 0.05%, suggests that it is adapted to low nutrient habitats.

I. pallida occurs in hot spring habitats either as plankton or as a component of algal mats dominated by cyanobac-

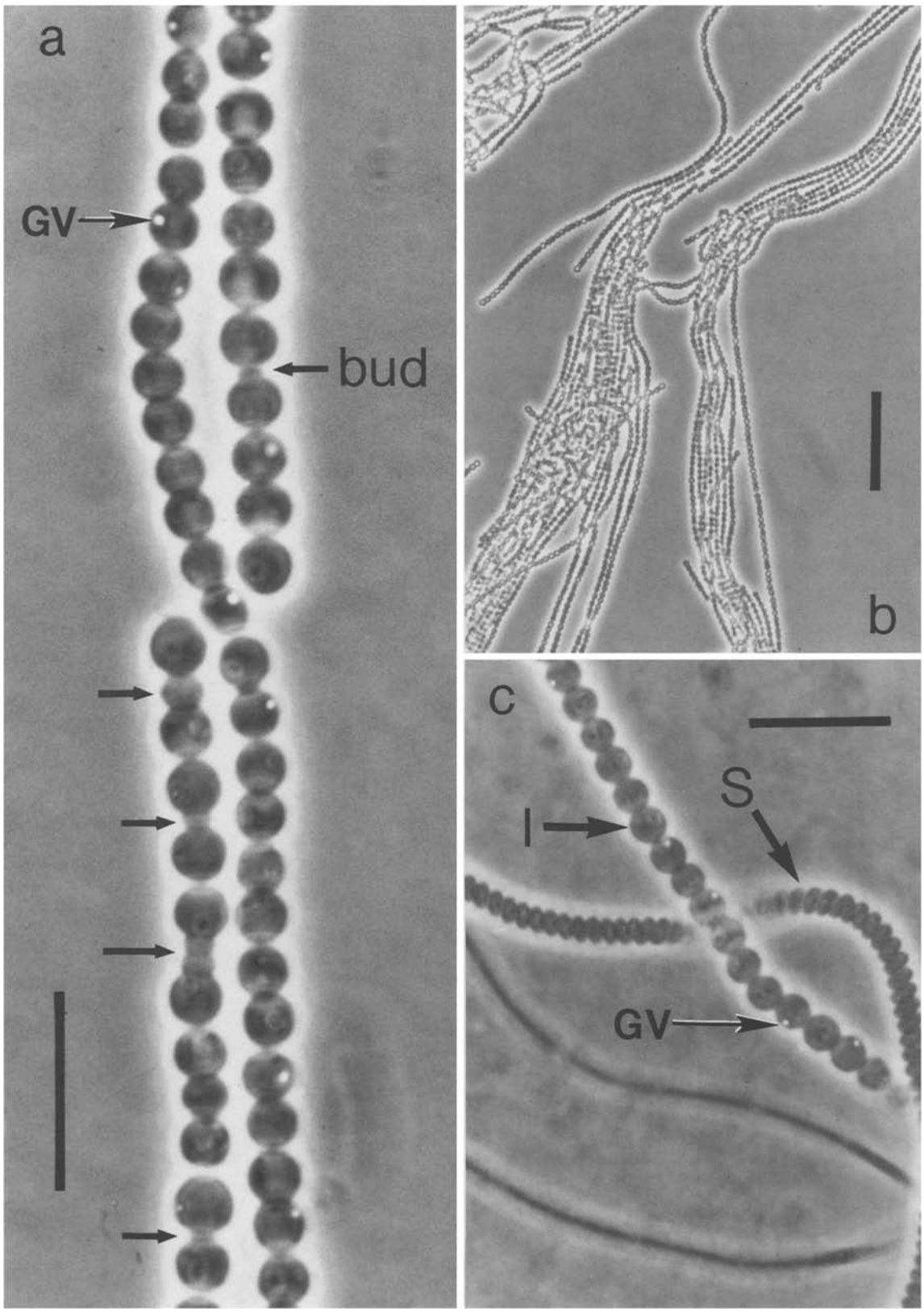

Fig. 2 a – c. Phase contrast micrographs of *Isosphaera pallida*: **a** from culture in exponential growth phase (strain IS1), 1000 ×, bar = 10 µm; **b** exponential phase culture, (strain IS1), 100 ×, bar = 50 µm; **c** a natural population, Mammoth Hot Springs, Yellowstone, USA, showing *Isosphaera pallida* (I) and *Spirulina labrinthiformis* (*S*), bar = 10 µm. *Arrows* indicate buds and gas vacoules (*GV*)

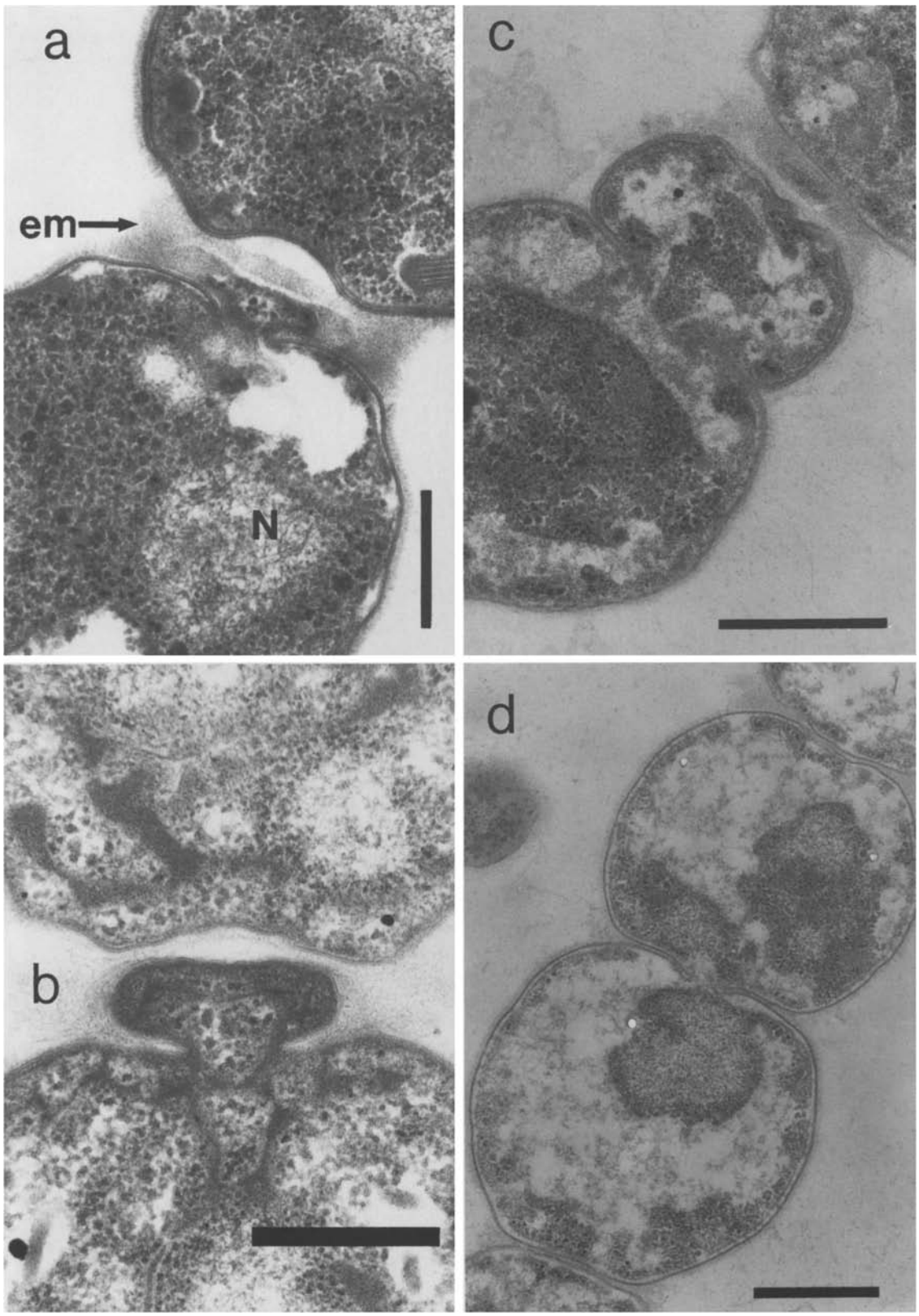

Fig. 3 a – d. Thin sections showing stages of budding (**a – d**); bars = 1 μm. *N* nucleosome, *em* extracellular material

teria. Two adaptations, gas vacuoles and gliding motility coupled with a positive phototactic response, may enable *I. pallida* to control its position along the vertical axis. The selective value of these motility responses remains unclear. The capacity of this organism for efficient heterotrophic growth on glucose could adapt it for growth in proximity to cyanobacteria, which may excrete glucose during periods of high light intensity. *I. pallida* was unable to grow using

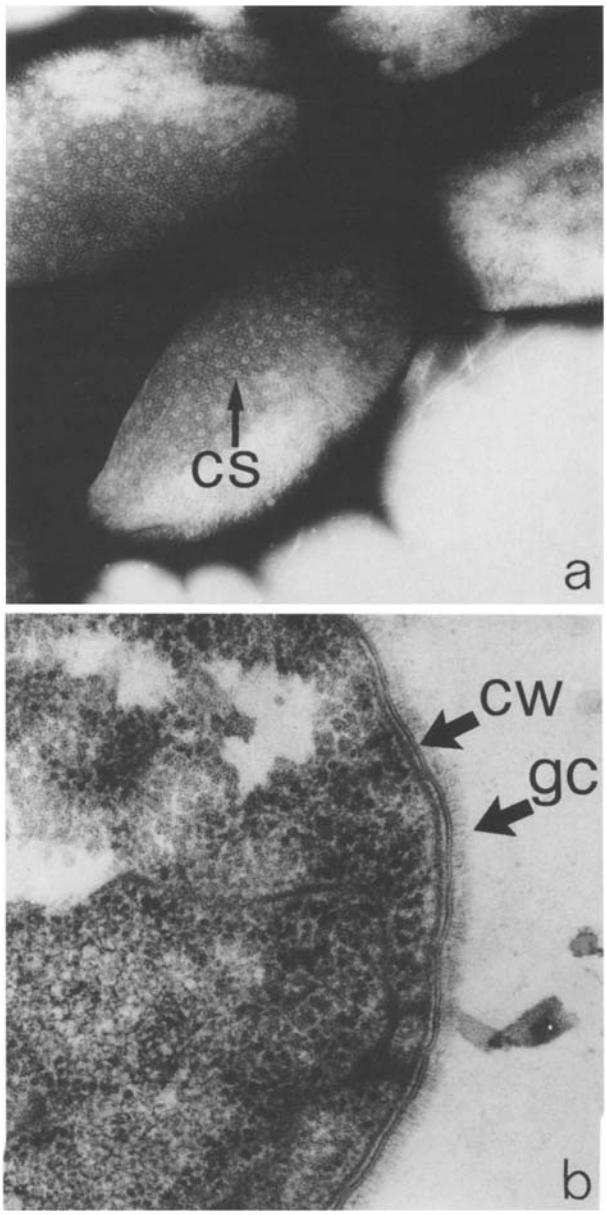

Fig. 4. **a** Surface of negatively stained cell showing crateriform structures *CS*, bar = 1 μm; **b** Thin section showing tri-laminar structure of cell wall *CW* and "glycocalyx" *gc*, bar = 1 μm

glycolate, another by-product of photosynthetic metabolism, as a sole source of carbon.

This is the first report of strictly phototactic behavior in a heterotrophic prokaryote. Photo-responses other than phototaxis have been observed in some non-photosynthetic prokaryotes: brief exposures to high intensity blue light have been shown to induce tumbling in *Salmonella typhimurium* and *Escherichia coli* (Macnab and Koshland 1974; Taylor and Koshland 1975), and a step-up photophobic response, with an action spectrum peaking at 425 nm, was detected in *Beggiatoa* sp. (Nelson and Castenholz 1982). However, these are simple photoresponses. The phototactic response observed in *I. pallida* involves orientation to the direction of a light source, and is thus more complex. The random migration of colonies in the dark or in uniform light proves that this response is not caused by photokinesis, a non-directional increase in motility rate in the light. Because *I. pallida* is not phototrophic it presents a unique opportunity for the study of phototactic mechanism in a prokaryote where phototaxis and photosynthetic metabolism need not be experimentally differentiated.

The formation of comet-like motile aggregates of cells has been reported previously in a cyanobacterium (*Pseudanabaena* sp., Castenholz 1982). *Pseudanabaena* "comets" are also phototactic. Although *Pseudanabaena* sp. are somewhat similar to *I. pallida* in morphology, these organisms do not appear to be phylogenetically related (Giovannoni, unpublished work).

The budding mode of cellular division is widely distributed among diverse prokaryotes; hence, it is of limited use as a taxonomic marker. Nonetheless, organisms belonging to the *Planctomyces* group of budding bacteria form a phylogenetically coherent taxon (Stackebrandt et al. 1984) which is phenotypically distinct. All members of the *Planctomyces* group examined so far lack peptidoglycan walls, a feature which is also found in the genus *Chlamydia* (Barbour et al. 1982; Garrett et al. 1974).

Distinctive ultrastructural features of the cell wall, termed crateriform structures (Schmidt and Starr 1981), are also characteristic of the *Planctomyces* group. We observed similar structures of ring-like appearance on the surface of negatively stained *I. pallida* cells. In *I. pallida* these structures were uniformly distributed over the cell surface, as they are in *Pirella*. In *Planctomyces* spp. crateriform structures are arranged around the cell pole. Unlike *Pirella* and *Planctomyces*, *I. pallida* does not form holdfasts or flagella. In ultrastructure the cell wall of *I. pallida* resembles those of *Planctomyces*, *Pirella* and *Blastocaulis*; two electron dense layers, separated by an electron transparent layer, are evident, but a discrete peptidoglycan layer cannot be visualized (König et al. 1984; Schmidt and Starr 1982).

The mole % guanine plus cytosine content of *I. pallida* DNA (62.2%) was close to that of other budding bacteria, e.g. *Hyphomicrobium* (59.29 – 64.69), *Pedomicrobium* (62.27 – 65.00), *Planctomyces* (50.5 – 54.4), and *Pirella* (56.4 – 59.0) (Gebers et al. 1985).

Field collected and cultured *Isosphaera pallida* is identical in morphology to *Isocystis pallida* Woronichin (1927), which was based on field material collected from Eurasian hot springs. Later Geitler (1955, 1963) argued that all species of *Isocystis* should be regarded as yeasts (*Torulopsidosira*) because of the budding type of cell division. It has since become apparent that these organisms and, specifically, *Isocystis pallida*, are prokaryotes (Anagnostidis and Rathsack-Kuzenbach 1967). The original assignment of the genus *Isocystis* to the blue-green algae (cyanobacteria) was based entirely on morphological criteria. No species have been cultured previously. Most species bear a superficial resemblance to Nostocacean cyanobacteria, and most occur in the photic zone associated with cyanobacteria.

Since the original description of the genus (*Isocystis messanensis*, Borzi, 1878 ex Bornet and Flahault, 1888 – based on the morphology of feral cells), the genus *Isocystis* has been ignored by many authors of cyanobacterial taxonomies, with notable exceptions (e.g. Geitler 1932; Elenkin 1949; Starmach 1966). Members of the genus *Isocystis* other than *I. pallida* have not been cultured. They include some species, *I. filamentosa* and *I. salina*, which are very similar in appearance to *I. pallida* and could be closely

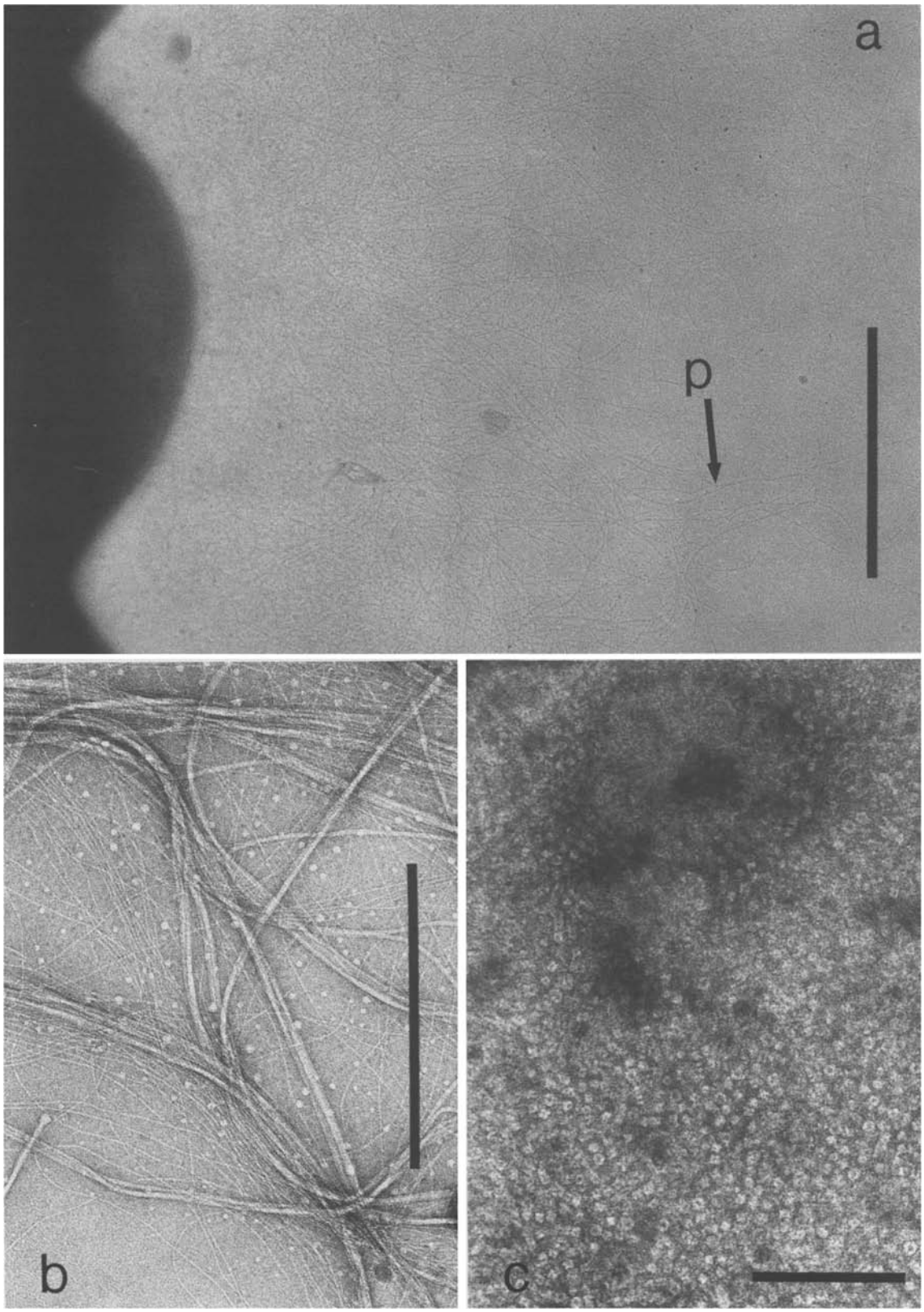

Fig. 5 a–c. Pili, transmission electron micrographs: **a** platinum shadow, bar = 0.1 μm *p*; **b** platinum shadow, bar = 0.1 μm; **c** negative stain showing small rings (diameter = 4 nm) on cell surface, bar = 0.25 μm

related. Although it appears that the genus *Isocystis* may, in time, be abandoned altogether as a cyanobacterial genus, in keeping with the rules of systematic nomenclature we have used a new generic name for *I. pallida*.

The colorful history of this organism in the taxonomic literature serves as a reminder of the importance of physiological and phylogenetic studies using axenic microbial cultures.

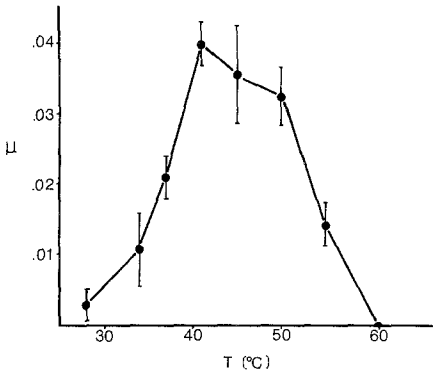

Fig. 6. Growth rate (growth rate constant μ, h^{-1}) as a function of temperature. *Isosphaera pallida* strain IS1B was grown on medium IMC in sealed flasks incubated in shaking water baths. Growth was measured as optical density at 650 nm. Bars indicate standard deviation of μ about the regression line

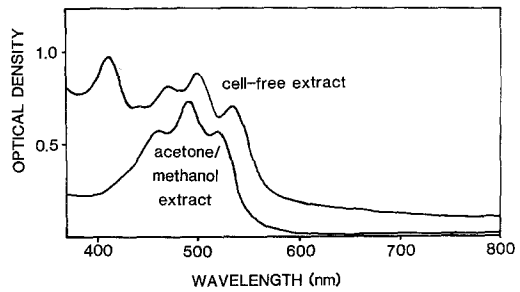

Fig. 7. Absorbance spectra of cell-free extract and acetone/methanol (1/1) extract

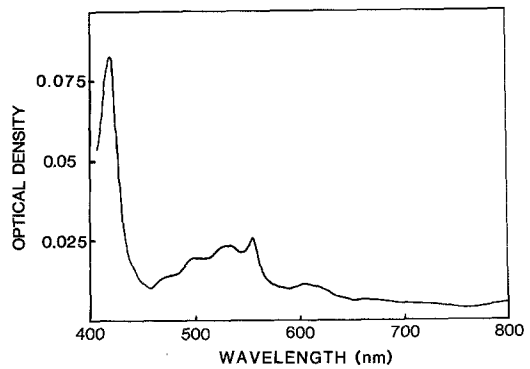

Fig. 8. Reduced minus oxidized difference spectrum of a cell-free extract (1.12 mg protein/ml)

Conclusion

Isosphaera gen. nov. (I.so.sphaer'. a. Gr. adjective *iso* equal, L. fem. noun *Sphaera* ball or sphere; *pallida* L. adjective, pale).

Spherical cells, 2.5–3.0 μm, forming chains. Chain length indefinite, often over 100 cells. Cell division by budding. Buds intercalary or at chain ends. Chain branching does not occur. Capsules not formed. Gas vesicles usually present in recent isolates. Resting stages are not known. Motile by gliding; phototactic. Pili, but no flagella, present. Gram variable. Rigid cell wall of proteinaceous composition, lacking peptidoglycan. Wall contains pit-like structures (crateriform structures), evenly spaced.

Strictly aerobic. Temperature range 40–55° C. Optimal pH range 7.8–8.8. Chemoheterotrophic with respiratory metabolism. Glucose or lactate only known carbon source. Carotenoids, cytochromes present. Mol % G + C 62.2% for one strain (IS1B).

Table 2. Carbon source utilization by *I. pallida*. Carbon sources were present at 0.025% (w/v). +, growth; 0, no growth. Control plates contained carbon source being tested plus 0.025% D-glucose and 0.025% casamino acids. An "I" indicates growth inhibition on the control plate

Carbon Source	Strain:			
	IS1B	IS2	IS4	IS9
D-Glucose	+,I	+,I	+	+,I
D-Ribose	0	+,I	0	+,I
Maltose	0	0,I	+	+
Mannitol	0	0	0	0
Fructose	0	0,I	0	0,I
Glycerol	0	0	0	0
Aspartate	0	0	0	0
Glutamine	0	0	0	0
Citrate	0	0	0	0
Malate	0	0	0	0
Acetate	0	0	0	0
Glycolate	0	0,I	0	0
Palmitate	0	0	0	0
Lactate	+	+	+	+

Type species. *Isosphaera pallida* (Woronichin) Giovannoni and Castenholz. (pallida L. adjective, pale).

Synonyms. Isocystis pallida Woronichin 1927 – as blue green alga (cyanobacterium), *Torulopsidosira pallida* (Woronichin) Geitler 1963 – as a yeast. The type species for *Isocystis* is *I. messanensis* Borzi 1878 (Bornet and Flahault 1888).

Source. Kah-nee-ta Hot Springs, Warm Springs Indian Reservation, Warm Springs, Oregon, USA.

Type strain. IS1B is designated as holotype. It is deposited in the culture collection of thermophilic microorganisms, Dept. of Biology, Univ. of Oregon, Eugene, Oregon, USA and the ATCC.

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