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Ectothiorhodospira halochloris sp. nov., a New Extremely Halophilic Phototrophic Bacterium Containing Bacteriochlorophyll b*

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Abstract. A new bacteriochlorophyll *b* containing phototrophic bacterium was isolated from extremely saline and alkaline soda lakes in Egypt. Enrichment and isolation were performed using a synthetic medium with high contents of sodium carbonate, sodium sulfate and sodium chloride. Photoautotrophic growth occurred with hydrogen sulfide as photosynthetic electron donor. During oxidation of sulfide to sulfate extracellular elemental sulfur globules appeared in the medium. Cells were also capable to grow under photoheterotrophic conditions with acetate, propionate, pyruvate, succinate, fumarate or malate as carbon sources and electron donors. Under these conditions sulfate was assimilated. Optimal growth under the applied experimental conditions occurred at a total salinity of 14–27‰, a pH-range between 8.1 and 9.1 and a temperature between 47° C and 50° C. The cells were 0.5–0.6 µm wide and, depending on cultural conditions, 2.5–8.0 µm long; they were spiral shaped, multiplied by binary fission and were motile by means of bipolar flagella. Intercytoplasmic photosynthetic membranes were present as stacks. Bacteriochlorophyll *b* was the main photosynthetic pigment; small amounts of carotenoids were mainly present as glucosides of rhodopin and its methoxy derivative. The new organism is described as *Ectothiorhodospira halochloris*.

Key words: Anaerobic phototrophic bacteria – *Ectothiorhodospira halochloris* – Halophilic bacteria – Alkaliphilic bacteria – Sulfide oxidation – Bacteriochlorophyll *b*.

The first reports on phototrophic bacteria from concentrated brines were given by Baas Becking (1928) and van Niel (1931). Both described red colored spirilla, and possibly these bacteria were identical with the later isolated *Ectothiorhodopsora halophila* (Raymond and Sistrom, 1967, 1969). In 1957 Jannasch made some enrichments with brine samples from a Wadi Natrun lake. He observed several red and green colored bacteria with sulfur globules inside the cells and also with deposition of elemental sulfur outside the cells, but he did not isolate them. Hence so far *E. halophila* was the only phototrophic bacterium isolated from an extreme saline environment and growing in media containing 9–30‰ sodium chloride.

During our present studies on the microbial ecology of the soda lakes in the Wadi Natrun, Egypt, we were able to isolate a second extremenly halophilic phototrophic bacterium. Several morphological and physiological properties of the new isolate justify to place it into the genus *Ectothiorhodospira* Pelsh and to describe it as the new species *Ectothiorhodospira halochloris*.

MATERIAL AND METHODS

Media for Enrichment and Isolation

Based upon data from Jannasch (1957) about the ionic composition of a lake in the Wadi Natrun, for enrichment cultures and isolation procedures the following medium was composed: 1 ml trace element solution ("SLA"), 1 ml vitamin solution ("VA"), 0.05 g $CaCl_2 \cdot 2\,H_2O$, 0.5 g KH_2PO_4, 0.8 g NH_4Cl, 0.1 g $MgCl_2 \cdot 6\,H_2O$, 180 g NaCl, 20 g Na_2SO_4, 20 g Na_2CO_3 were dissolved in 1 l of distilled water. As supplements to this basal growth medium 0.05% yeast extract, 0.1% sodium succinate and 0.1% $Na_2S \cdot 9\,H_2O$ were added. For the isolation initial pH was adjusted to 9.6, later on to 8.5. In the first enrichment cultures the total salinity was 35‰.

Trace Element Solution. 1.8 g $FeCl_2 \cdot 4\,H_2O$, 250 mg $CoCl_2 \cdot 6\,H_2O$, 10 mg $NiCl_2 \cdot 6\,H_2O$, 10 mg $CuCl_2 \cdot 2\,H_2O$, 70 mg $MnCl_2 \cdot 4\,H_2O$, 100 mg $ZnCl_2$, 500 mg H_3BO_3, 30 mg $Na_2MoO_4 \cdot 2\,H_2O$ and 10 mg $Na_2SeO_3 \cdot 5\,H_2O$ were dissolved in 1 l of bidistilled water.

Vitamin Solution. 10 mg biotin, 35 mg nicotinamide, 30 mg thiamine dichloride, 20 mg p-aminobenzoic acid, 10 mg pyridoxal chloride, 10 mg Ca-pantothenate and 5 mg vitamin B_{12} were dissolved in 100 ml bidistilled water.

* Dedicated to Professor C. B. van Niel on the occasion of his 80th birthday

Isolation

Ectothiorhodospira halochloris was isolated by repeated agar shake dilution according to Pfennig (1965). The procedure was performed using the described medium and a final agar concentration of 0.6%. In sulfide free media anaerobic conditions were obtained by the addition of 0.05% sodium ascorbate. Test tubes were incubated at 30° C and illuminated with a light intensity of about 1000 lux. After purity was achieved one single colony was transferred into liquid culture medium and grown under the same conditions.

Growth Experiments

For the determination of the pH optima a salinity of the medium of 25% was used. The ratio of Na_2CO_3 to $NaHCO_3$ was varied to obtain the right pH-values; if necessary the pH was adjusted with NaOH or H_2SO_4. Salt optima were determined at pH 8.5 with a constant ratio of $NaCl/Na_2CO_3/Na_2SO_4$ = 8/1/1 (w/w/w). In all experiments growth was followed at least over three passages in the same medium. To examine the utilization of carbon, sulfur and nitrogen compounds two parallels were inoculated for each substrate. The temperature optimum was measured in a temperature controlled light-fermentation vessel with optimal pH-value and salt concentration. This experiment was started at 30° C and the temperature was than stepwise increased to 55° C using an appreciable amount of the previous culture as inoculum for the next step. Growth was measured as counts of total cells.

Preparation of Cells for Light and Electron Microscopy

For photomicrographs a 2% solution of washed agar was distributed in 2 ml portions on slides and completely dried. In order to achieve that cells were properly fixed between agar and cover glass, but not squeezed different volumes of cell suspensions were used (5–15 µl). For electron micrographs cells were sedimented on a pioloform F coated copper slide, stained with a 2% solution of phosphotungstic acid with the addition of 0.1% glycerol (pH 6.7). All electron micrographs were taken with a Phillips EM 200 electron microscope.

Characterization of Photosynthetic Pigments

Absorption spectra were measured with a Zeiss DMR 21 and a Perkin Elmer 124 spectrophotometer. Extraction of bacteriochlorophylls and separation on silicagel was done as described by Gloe et al. (1975). The identification of the bacteriochlorophyll was carried out by cochromatography of the bacteriopheophytins of *E. halochloris* strain BN 9850, *Rhodopseudomonas viridis* strain BN 170 and *Rhodospirillum rubrum* strain Ha; the absorption spectrum of the separated bacteriopheophytin was measured. Carotenoid extraction and identification was done in the usual manner (Jensen, 1962; Hager and Stransky, 1970).

Determination of DNA Base Composition

Deoxyribonucleic acid was isolated after Meyer and Schleifer (1975) using cetyltrimethylammoniumbromide (CTAB) for the precipitation. Purity was checked spectroscopically after Ulitzur (1972). If necessary an additional precipitation step with isopropanol was carried out. The base ratio expressed as moles-% guanine plus cytosine was calculated from the melting temperature according to Marmur and Doty (1962); this work was kindly done by Dr. H. Hippe (Göttingen).

RESULTS

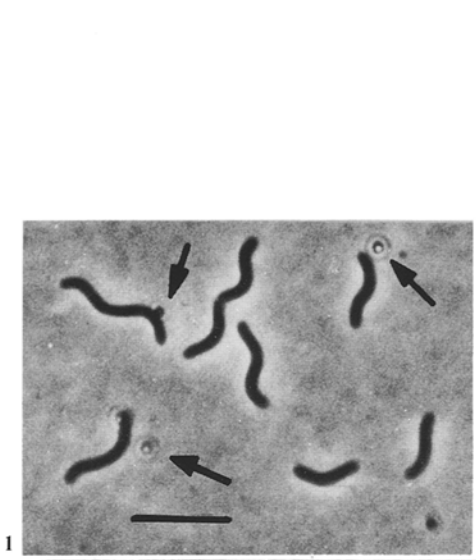
Natural Habitat and Isolation

Ectothiorhodospira halochloris is living in several extremely saline lakes of the Wadi Natrun (Egypt). This location is a depression in the Libyan Desert with several lakes, which contain concentrated brines with total salinities of up to 40%. In most of the lakes a layer of crystalline salt deposits exists. High concentrations of sulfate and organic matter in the sediments and the mud surrounding the lakes favor the development of sulfate-reducing bacteria and lead to the evolution of considerable amounts of hydrogen sulfide and carbon dioxide (Abd el Malek and Rizk, 1963). With its high salinities, pH-values of about 11.0, and anaerobic conditions due to its content of hydrogen sulfide, this environment has unique properties.

Using a medium with high contents of sodium carbonate, sodium sulfate and sodium chloride *E. halochloris* was isolated from mud and water samples of several pools in the Wadi Natrun. Agar shake dilution series inoculated with water from small pools or water from the sediment-water interface of the lakes Abu Gabara, Zugm, Hamra, Gaar, Muluk and Rizunia yielded 10^8 to 10^9 viable cells per liter of both *E. halochloris* and *E. halophila*. Probably *E. halochloris* is very common in environments similar to the investigated lakes of the Wadi Natrun.

Morphology

Cells of *E. halochloris* are spiral shaped (Figs. 1 and 2) and usually show one or two complete turns under favorable conditions. One complete turn measures 3.5–5.0 µm. Whole cells are 0.5–0.6 µm wide and – depending on cultural conditions – 2.5–8.0 µm long, under unfavorable conditions even 15 µm or more. *E. halochloris* is bipolarly flagellated possessing two or three flagella at each cellular pole (Fig. 2). Cell division occurred by binary fission. Intracytoplasmic membranes occur as stacks (Figs. 2 and 3) as in the brown colored *Rhodospirillum* species, *R. fulvum*, *R. molischianum*, *R. photometricum* (Gibbs et al., 1965) and the species of the genus *Ectothiorhodospira*, *E. shaposhnikovii* (Cherni et al., 1969), *E. mobilis* (Trüper, 1968; Remsen et al., 1968; Holt et al., 1968) and *E. halophila* (Raymond and Sistrom, 1967, 1969). The stacks can be observed under high magnification with the light microscope as areas of stronger light absorption, in pressed cells even as dark inclusions. Negative stains viewed with the electron microscope revealed a more detailed structure. Cells grown with light intensities below 1000 lux were almost filled up with stacks, which appeared as spherical bodies under low magnification. One single stack is composed



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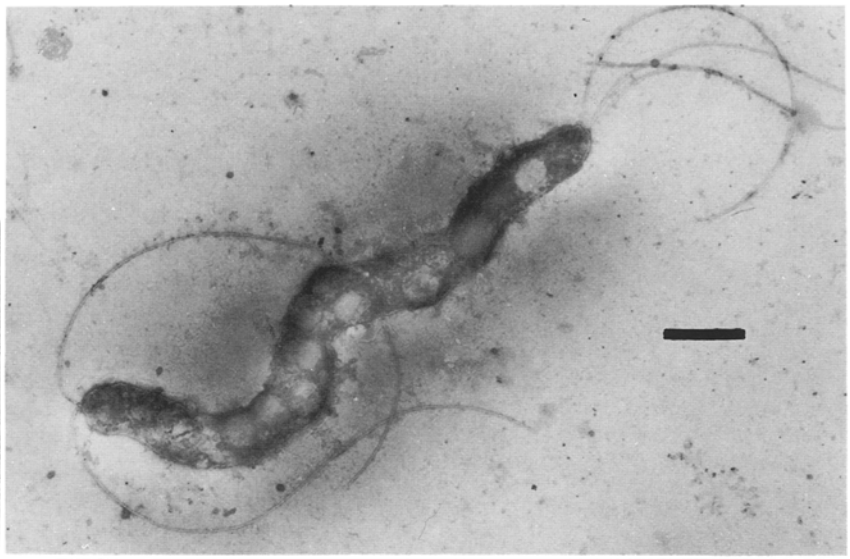

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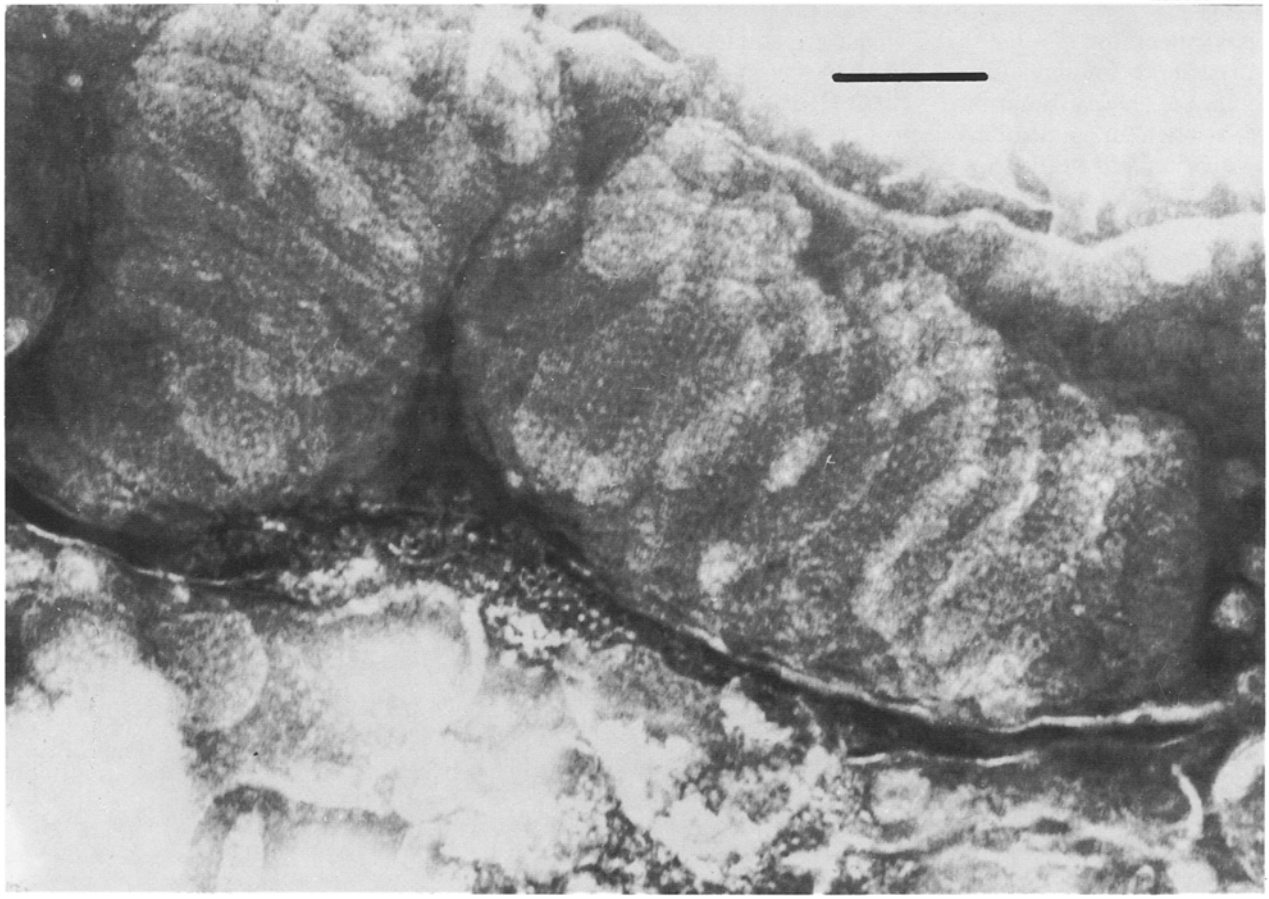

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Fig. 1. Phase contrast photomicrograph of *Ectothiorhodospira halochloris* strain BN 9850 grown autotrophically on sulfide as electron donor. *Arrows* indicate globules of elemental sulfur. Bar indicates 5 μm

Fig. 2. Electron micrograph of a negatively stained cell of *Ectothiorhodospira halochloris* strain BN 9850 nearly filled up with membrane stacks. The cell is fixed in liquid medium in a phase of active movement. Bar indicates 1.0 μm

Fig. 3. Top view of two membrane stacks within a negatively stained cell of *Ectothiorhodospira halochloris* strain BN 9850, revealing a granulated surface structure of the membranes with an average diameter of the granules of about 10 nm. Bar indicates 200 nm

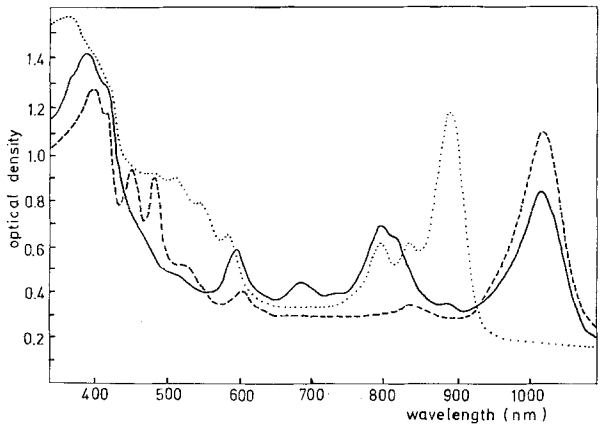


Fig. 4. Absorption spectra of chromatophore suspensions of *Ectothiorhodospira halochloris* strain BN 9850 (——), *Ectothiorhodospira halophila* strain BN 9910 (------) and *Rhodopseudomonas sulfoviridis* strain P_1 (– – –)

of several flat membrane discs, which are connected with each other and possibly also with the cytoplasmic membrane as was found in *E. mobilis* (Remsen et al., 1968). High magnification electron micrographs of negatively stained cells showed a regularly granulated structure of the membrane surfaces, with an average granule diameter of about 10 nm (Fig. 3). The same structure was also seen in cell free fractions of isolated chromatophores of *E. halochloris*.

Photosynthetic Pigments

Young cultures of *E. halochloris* have a pale green color like that of gooseberry fruits, whereas dense populations get a brownish shade. The absorption maxima at 389, 598 and 1018 nm of whole cells and cell free suspensions indicate the presence of bacteriochlorophyll *b* (see Fig. 4). In addition peaks at 374, 796, and 884 nm occur, which were not observed in cell free suspensions of *Rps. viridis* and *Rps. sulfoviridis*; they are present in extracts of *E. halophila* (Fig. 4) and the other phototrophic bacteria with bacteriochlorophyll *a* as the main photosynthetic pigment. Therefore, the presence of a second bacteriochlorophyll is suggested. This may be bacteriochlorophyll *a* or a very similar compound. In *Rps. viridis* and *Rps. sulfoviridis* (Fig. 4), which show almost identical spectra and also in *Thiocapsa pfennigii* (Eimhjellen et al., 1967) the maxima of bacteriochlorophyll *b* appear at 398, 602, and 1020 nm. The slight shift of the absorption maxima to shorter wavelength in *E. halochloris* is possibly due to the presence of the second bacteriochlorophyll compound. Cochromatography of the extracted bacteriopheophytins of *E. halochloris* and *Rps. viridis* showed identical R_f-values. The brown color of the bacteriopheophytin *b* was clearly visible. Though bacteriopheophytin *a* has nearly the same R_f-value, it is easily distinguishable from bacteriopheophytin *b* by its intensive violet color. We did not find bacteriopheophytin *a* in the chromatograms of *E. halochloris* extracts. The purified bacteriopheophytin *b* from *E. halochloris* showed absorption maxima at 368, 399, 526, 619, 628, and 775 nm in acetone. The same spectrum was reported for the bacteriopheophytins of *Rps. viridis* (Drews and Giesbrecht, 1965; Gloe, unpublished results) and *Thiocapsa pfenigii* (Eimhjellen et al., 1967).

As the in vivo absorption maxima between 450 and 550 nm are rather low (Fig. 4), the total carotenoid content is low compared to that of the other phototrophic bacteria. According to preliminary results (K. Schmidt, personal communication) the major carotenoids of *E. halochloris* are glucosides of rhodopin (20%) and its methoxy derivative (60%). About 10% of each rhodopin and lycopene are present as free carotenoids.

Optimal Growth Conditions

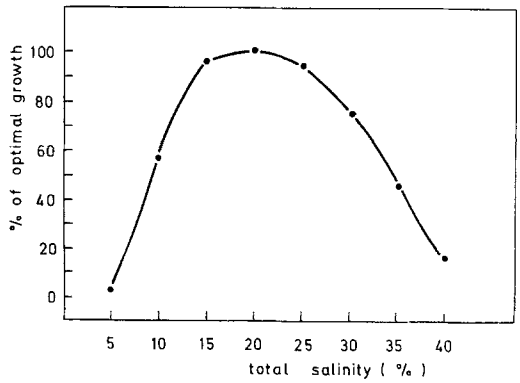
E. halochloris is an extremely halophilic bacterium. It shows adaptation to a wide range of different salt concentrations, even if growing very slowly at the extremes. This broad salt optimum is presented in Figure 5: More than 50% of the optimal growth yield, expressed as optical density at 650 nm, occurred between 10% and 34% total salinity and more than 90% between 14% and 27%; optimum salinity was 20%.

Best growth was observed at pH 8.5; more than 50% of the maximum growth yield was obtained between pH 7.5 and 10.0 and more than 90% between 8.1 and 9.1 (Fig. 6).

E. halochloris is slightly thermophilic. Below 33° C growth was very slow with a generation time of 30 h at 30° C. From 33–50° C a slow decrease in the generation time from 20 h to 10 h was observed (Fig. 7). Best growth occurred between 47° C and 50° C with generation times of 10.9 and 10.0 h, respectively. At 50° C cells became unusually thick, and further increase of temperature led to lysis of the cells.

Physiology

E. halochloris is able to grow under anaerobic, photolithoautotrophic conditions with hydrogen sulfide as electron donor and carbon dioxide as sole carbon source. Under these conditions sulfide is first oxidized to extracellular elemental sulfur, which is further oxidized to sulfate when sulfide becomes depleted. Globules of elemental sulfur appeared in the medium, some-



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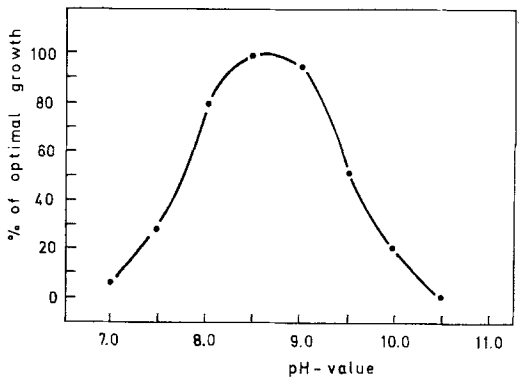

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Fig. 5. Salt optimum graph of *Ectothiorhodospira halochloris* strain BN 9850; each *point* represents the average value of four succeeding passages; growth was measured as optical density at 650 nm

Fig. 6. pH optimum graph of *Ectothiorhodospira halochloris* strain BN 9850. Each point represents the average value of three succeeding passages at the same pH; growth was measured as optical density at 650 nm

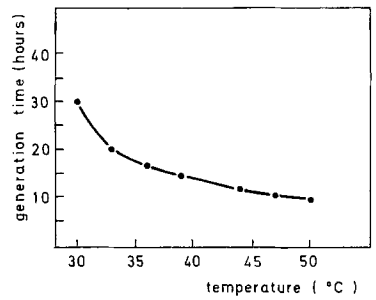

Fig. 7. Temperature dependence of the generation time of *Ectothiorhodospira halochloris* strain BN 9850; above 50° C no growth was obtained

Table 1. Utilization of organic carbon sources by *Ectothiorhodospira halochloris*, strain BN 9850 in mineral salts medium with 0.1% sodium sulfide

fructose	–	propanol	–	malate	+
glucose	–	acetate	++	propionate	++
saccharose	–	fumarate	+	butyrate	–
sorbose	–	succinate	+	valerate	–
galactose	–	pyruvate	++	caproate	–
mannitol	–	citrate	–	caprylate	–
sorbitol	–	tartrate	–	pelargonate	–
glycerol	–	ascorbate	–	benzoate	–
methanol	–	casamino		lactate	–
ethanol	–	acids	+	formate	–

– = Growth as in the control without added substrate; + = additional growth, OD at 650 nm below 0.3; ++ = additional growth, OD at 650 nm 0.3 to 0.9
Final substrate concentration 0.1% in the first and second and 0.05% in the third column

times closely attached to the cells, but were never observed inside the cells (Fig. 1). More abundant growth occurred under photoheterotrophic conditions with acetate, propionate, pyruvate, succinate, malate or fumarate as electron donor and carbon source; reducing conditions were provided by the addition of 0.05% sodium ascorbate. In addition to the above mentioned electron donors, casamino acids supported growth in the presence of sulfide and carbonate; sugars, sugar alcohols and higher fatty acids as well as citrate, lactate and formate were not used (Table 1). Without carbonate growth did not occur with any of the substrates tested. No measurable growth was obtained under aerobic or anaerobic conditions in the dark. Microaerophilic growth of *E. halochloris* was observed in agar shake cultures in the light. Good growth occurred with ammonia or glutamine as nitrogen source; nitrate, nitrite, urea, glutamate and asparagine were not utilized. *E. halochloris* was capable of assimilatory sulfate reduction and growth with sulfate, thiosulfate, elemental sulfur, cysteine or sulfide as sulfur source was possible.

DISCUSSION

Thus far only three species of phototrophic bacteria containing bacteriochlorophyll *b* have been described, namely *Rhodopseudomonas viridis* (Drews and Giesbrecht, 1966), *Rhodopseudomonas sulfoviridis* (Keppen and Gorlenko, 1975) and *Thiocapsa pfennigii* (Eimhjellen et al., 1967; Eimhjellen, 1970). They all differ in cell shape and structure of the intracytoplasmic membranes from the new isolate. While *Rps. viridis* and *Rps. sulfoviridis* are small rods multiplying by budding and possessing photosynthetic membranes underlying the cytoplasmic membranes (Drews and Giesbrecht, 1965, 1966; Whittenbury and McLee, 1967; Keppen and Gorlenko, 1975), *Thiocapsa pfennigii* is a coccus with tubular intracytoplasmic membranes (Eimhjellen et al., 1967).

E. halochloris has spiral shaped cells and possesses several distinct membrane stacks carrying the photo-

Table 2
Comparison of the major characteristics of *Ectothiorhodospira halochloris* with those of the other species of the genus *Ectothiorhodospira*

	E. halochloris	*E. halophila*[a]	*E. mobilis*[a]	*E. shaposhnikovii*[a]
Cell shape	spiral	spiral	spiral	spiral
Cell diameter	0.5–0.6 µm	0.8 µm	0.7–1.0 µm	0.8–0.9 µm
Flagellation	bipolar	bipolar	polar tuft	polar tuft
Internal membrane system	stacks	stacks	stacks	stacks
bchl	b(a)	a	a	a
Major carotenoid	rhodopin[b]	spirilloxanthin	spirilloxanthin	spirilloxanthin
Rhodopin content[c]	about 90	< 1.0	10–20	10–20
Optimal pH	8.1–9.1	7.4–7.9	7.6–8.0	8.0–8.5
Optimal salinity	14–27%	11–22%	2–3%	< 1%
Optimal temperature	48° C	47° C	25–29° C	30–35° C
Sulfate assimilated	+	–	+	+
Sulfide oxidized	+	+	+	+
Extracellular sulfur formed	+	+	+	+
DNA-base ratio	52.9	68.4	67.3–69.9	61.2–62.8

[a] References in the text
[b] Present as rhodopin, rhodopin glucoside and methoxy rhodopin glucoside
[c] Values in % of the total carotenoid content

synthetic pigments; in this respect it closely resembles the species of the genus *Ectothiorhodospira* and the brown colored *Rhodospirillum* species, but differs from the bacteriochlorophyll *b* containing bacteria mentioned above.

The growth of *Rps. viridis*, belonging to the Rhodospirillaceae is suppressed by rather low concentrations of sulfide (Drews and Giesbrecht, 1966). *Rps. sulfoviridis* differs from *Rps. viridis* by its obligate dependence on reduced sulfur compounds such as sulfide or cysteine (Keppen and Gorlenko, 1975). *Thiocapsa pfennigii* on the other hand belongs to the Chromatiaceae. Growing with sulfide as electron donor and sole sulfur source it accumulates elemental sulfur inside the cells (Eimhjellen et al., 1967).

E. halochloris deposits elemental sulfur outside the cells as is characteristic for the genus *Ectothiorhodospira* and the phototrophic green bacteria. The latter, however, differ markedly in structure and pigment content from the phototrophic purple bacteria (Pfennig and Trüper, 1974). Cell shape, fine structure and the pattern of sulfide oxidation together justify the allocation of the new species within the genus *Ectothiorhodospira*.

Within the genus *Ectothiorhodospira*, *E. halochloris* is characterized by the possession of bacteriochlorophyll *b* while the other species contain bacteriochlorophyll *a* as the main photosynthetic pigment. *E. mobilis*, *E. shaposhnikovii* and *E. halophila* have similar proportions of carotenoids of the spirilloxanthin series with spirilloxanthin as the main component (Schmidt and Trüper, 1971). *E. halochloris* possesses only small amounts of carotenoids; mainly glucosides of rhodopin and its methoxy derivative are present. The situation is comparable to that between *Rps. viridis* and *Rps. palustris*; both show similar cell shape and fine structure, but *Rps. viridis* possesses bacteriochlorophyll *b* and *Rps. palustris* bacteriochlorophyll *a*. They also have different carotenoids.

Optimal growth conditions of *E. halochloris* differ from those of the other species; they are most similar to the growth conditions of *E. halophila*. A summary of some characteristic properties of the *Ectothiorhodospira* species is given in Table 2. *E. halochloris* shows a requirement for high amounts of salts and does not grow with less than 10% total salts. The same requirement is reported for *E. halophila* by Raymond and Sistrom (1967); *E. mobilis* needs only 2–3% sodium chloride (Trüper, 1968) and *E. shaposhnikovii* has no requirement for sodium chloride. As is shown in Table 2, the optimal pH-range is more alkaline for *E. halochloris* and the temperature optimum is as high as for *E. halophila*, being about 47–48° C. The extreme growth conditions together with morphological observations and characteristic absorption spectra of living cells allow a relatively easy recognition of *E. halochloris*.

SPECIES DESCRIPTION

Ectothiorhodospira halochloris sp.nov. ha.lo.chlo'ris. hals Gr.n. salt, chloros Gr. adj. green, halochloris M. L. adj. green colored and salt loving.

Morphology. Cells spiral shaped. Width 0.5–0.6 µm; length depending on cultural conditions, normally 2.5–8.0 µm. Motile by means of bipolar flagella. Multiplying by binary fission. Intracytoplasmic membranes present as large lamellar stacks.

Culture. Phototrophic growth under anaerobic conditions; photoautotrophic and photoheterotrophic growth is possible. Optimal pH-range 8.1–9.1. Optimal salinity 14–27% total salts, no growth below 10%. Optimal growth temperature 48° C. Color of cell suspensions pale green to green, in dense populations with a brownish shade. In solid media green lens-shaped colonies are formed. If grown on sulfide, young colonies are surrounded by a whitish halo of elemental sulfur, which dissappears during further growth. Capable of assimilatory sulfate reduction.

Photosynthetic Pigments. Bacteriochlorophyll *b* is the main photosynthetic pigment. Small amounts of carotenoids are present mainly as glucosides of rhodopin and its methoxy derivative. Maxima of in vivo absorption spectra at 374, 796, and 884 nm indicate the presence of a second bacteriochlorophyll compound, which may be similar or identical with bacteriochlorophyll *a*.

Photosynthetic Electron Donors. Sulfide, elemental sulfur, acetate, propionate, pyruvate, succinate, malate, fumarate.

DNA Base Composition. 52.9 moles-% guanine plus cytosine (thermal denaturation).

Source. Hydrogen sulfide containing, extremely saline, alkaline soda lakes, small pools and upper sediment layers of salt lakes.

Holotype. Strain BN 9850 of the culture collection of the Institut für Mikrobiologie at the University of Bonn; also deposited with the German Collection of Microorganisms in Göttingen, number DSM 1059.

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