Arch Microbiol (1983) 136:42–48

Archives of
Microbiology

Clostridium lortetii sp. nov., a halophilic obligatory anaerobic bacterium producing endospores with attached gas vacuoles

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Abstract. A strain of *Clostridium* was isolated from Dead Sea sediment, differing from the previously described *Clostridium* types in its halophilic character. It required NaCl concentrations between 1 and 2 M, and optimal growth was found in 1.4–1.5 M NaCl at 30° C and in 1.7 M NaCl at 45° C. In sporulating cells gas vacuoles developed, generally near the developing terminal endospore only, and these vacuoles remained attached to the mature endospore after degeneration of the vegetative cell. Fermentation products included acetate, butyrate and hydrogen. Glucose and a few other carbohydrates stimulated growth, though they were poorly utilized. A new species name has been proposed for the organism: *Clostridium lortetii*.

Key words: *Clostridium lortetii* – Endospores – Gas vacuoles – Butyric acid – Fermentation – Halophilic bacteria – Dead Sea

The first microorganisms that were ever isolated from the Dead Sea were anaerobic, sporeforming bacteria belonging to the genus *Clostridium*: mud samples from the Dead Sea bottom, inoculated in the early nineties of last century by Lortet into suitable media, gave rise to mass development of pathogenic clostridia, able to cause the symptoms of tetanus and gas gangrene (Lortet 1892). These clostridia are unable to multiply at the high salinities prevailing in the Dead Sea sediment, and probably survived in the mud as dormant endospores, able to germinate only upon transfer to growth media of low salinity. Thus they can not be considered as indigeneous to the Dead Sea.

About forty years later a varied indigeneous microflora was discovered in the Dead Sea during the pioneering work of Benjamin Elazari-Volcani, and the lake's sediment was found to be inhabited by various anaerobic halophilic bacteria (Elazari-Volcani 1943). Several later studies (Kaplan and Friedman 1970; Nissenbaum 1975) suggested the existence of sulfate-reducing, sporeforming bacteria in Dead Sea sediments, resembling *Desulfotomaculum nigrificans*, and unable to develop at salinities above 5%. The organism from the Dead Sea has never been grown in pure culture, and no cultures are extant today.

In the course of our attempts to isolate sulfate-reducing bacteria from Dead Sea sediments we isolated a novel anaerobic, halophilic, rod-shaped, endospore-forming bacterium. The strain, designated MD-2, did not perform dissimilatory sulfate reduction, and was therefore classified in the genus *Clostridium*. The strain proved unique by its high salt requirement (1–2 M), being the first obligately halophilic *Clostridium* we know of, and it produced gas vacuoles, a property shared by very few *Clostridium* strains only. In this work we describe and characterize the isolate. The strain was found to differ sufficiently from all other clostridia described to justify its designation as a new species.

Materials and methods

Isolation and culture methods. Dead Sea surface sediment was sampled in December 1979 from a depth of 60 m by means of a grab sampler, at a distance of about 2 km off the lake's western shore near Massada. A 5-g mud sample was incubated for three weeks at 30° C in a 150 ml glass stoppered bottle completely filled with medium containing (g/l): NH_4Cl, 1.0; K_2SO_4, 2.0; sodium lactate, 3.5; yeast extract (Difco), 1.0; KH_2PO_4, 0.14; ascorbic acid, 0.5, and thioglycolic acid, 0.1, in 80% (vol/vol) Dead Sea water, pH 6.5. Subsequently the contents of the bottle were mixed and tenfold dilutions were made in test tubes containing 10 ml molten agar medium, as above, supplemented with 15 g/l Bacto-agar and 0.5 g/l $FeSO_4 \cdot 7H_2O$, and containing 70% Dead Sea water instead of 80%. After solidification, the agar was overlayered with paraffin wax. After three weeks of incubation at 30° C, in addition to other types of colonies a number of black colonies developed, consisting of irregularly shaped cells with swollen ends. Colonies were transferred to liquid medium of similar composition, and the strain, designated MD-2, was finally purified by streaking on agar plates of the following composition (g/l): trypticase (BBL), 2.0; L-glutamic acid, 4.0; yeast extract, 4.0; NaCl, 145; $MgCl_2 \cdot 6H_2O$, 10.0; $CaCl_2 \cdot 2H_2O$, 3.7; K_2SO_4, 1.7; $FeSO_4 \cdot 7H_2O$, 0.002; resazurin, 0.001; Bacto-agar, 20; L-cysteine, 0.5; trace elements solution and vitamin solution (Balch et al. 1979), 1 ml/l of each. The pH was adjusted to 6.5 with NaOH. Media were prereduced by boiling under nitrogen, whereafter the cysteine was added, and anaerobic culture techniques as described by Balch et al. (1979) were used throughout.

During subsequent work another medium was developed which enabled better growth, and which was used in all experiments. Its composition was (g/l): casamino acids (Difco), 2.0; nutrient broth (Gibco), 2.0; yeast extract (Difco), 2.0; L-glutamic acid, 4.0; NaCl, 105; KCl, 0.75; $MgCl_2 \cdot 6H_2O$, 10.0; $CaCl_2 \cdot 2H_2O$, 3.7; $FeSO_4 \cdot 7H_2O$, 0.002; resazurin, 0.001; L-cysteine, 0.5; trace elements solution and vitamin solution, 10 ml/l of each, pH 6.5, adjusted

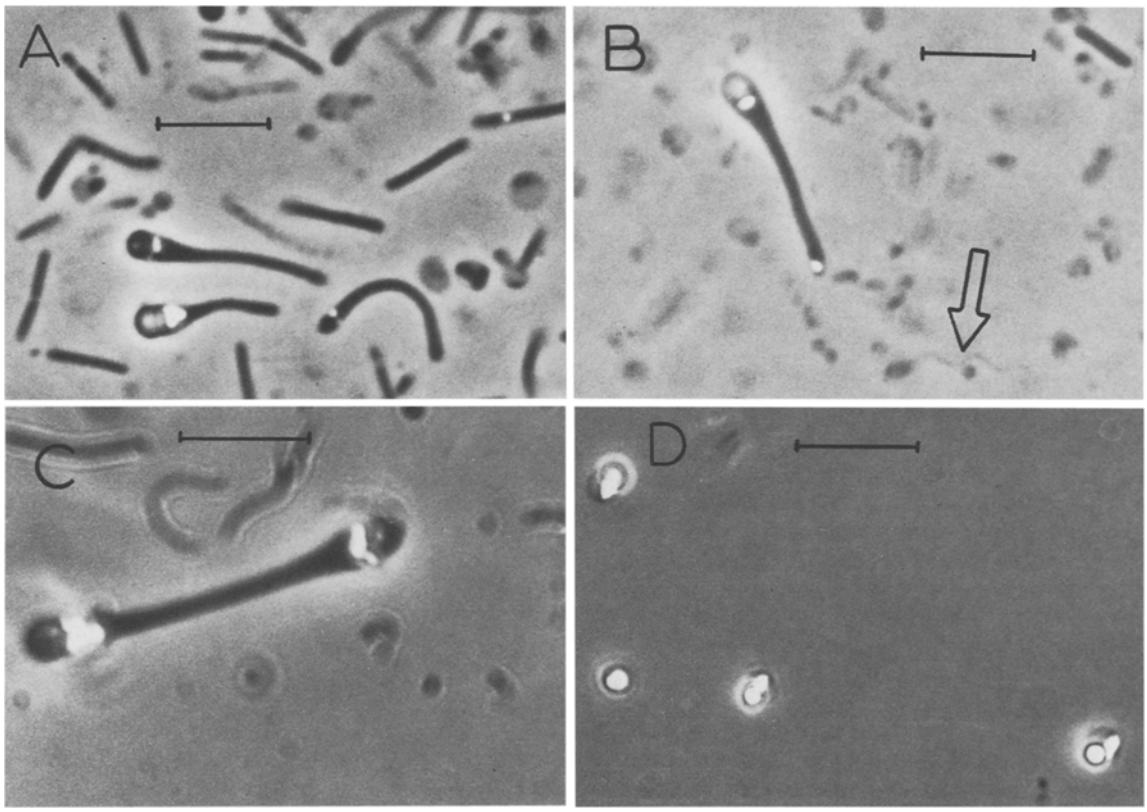

Fig. 1 A–D. Phase contrast micrographs of *Clostridium* strain MD-2. **A** different stages of development of endospores and gas vacuoles; **B** a cell with gas vacuoles at both ends. Note the flagellar bundle (*arrow*); **C** a cell with gas vacuoles and beginning endospore formation at both ends; **D** mature endospores. The bars represent 5 μm

with NaOH. The $MgCl_2$ and $CaCl_2$ were added from concentrated autoclaved anaerobic solutions to the autoclaved medium. For growth on plates, Bacto-agar (20 g/l), $CaCO_3$ (5 g/l) and soluble starch (2 g/l) were added to the medium. Cells were grown at 37° C on plates incubated in nitrogen-filled containers or in liquid medium in 25-ml stoppered serum tubes (Balch et al. 1979) containing 10 ml of growth medium under a gas phase of nitrogen. Modifications of this standard procedure were used with respect to incubation temperature, salt concentrations, and addition of sugars and other compounds to the medium as indicated in the experiments. Growth was followed by measuring the turbidity of the cultures at 660 nm.

Microscopy. Cultures were examined microscopically using a microscope with phase optics; for electron microscopical examination cells were fixed by adding glutaraldehyde to the growth medium to a final concentration of 4%, and staining with 1% uranyl acetate or 2% phosphotungstic acid. Ultrathin sections were prepared using standard dehydration, embedding and sectioning techniques, but for dehydration mixtures of 1.5 M NaCl and ethanol were used. Cells were embedded in Epon, and sections were stained with lead citrate and uranyl nitrate. All preparations were viewed in a JEOL JEM 100C electron microscope, operating at 60 or 80 kV.

Pressure relationships of gas vacuoles. Colonies of cells containing mature endospores were resuspended in growth medium, and spore suspensions in serum tubes were exposed to increasing pressures of nitrogen for 1 min. Then the suspensions were examined microscopically and the percentage of spores that has lost their attached refractile gas vacuoles was determined.

Analysis of fermentation products. Cells were grown either in the standard medium or in a medium containing 0.2% L-glutamic acid, 0.05% yeast extract, with inorganic salts, resazurin, cysteine and vitamins at concentrations identical to those in the standard growth medium. Volatile fermentation products were extracted with ether after acidification of the culture supernatants, and the presence of volatile fatty acids and alcohols in the extract was detected in a Basic model GC 9700 gas chromatograph, using a column of SP-100, 1% H_3PO_4 on Chromosorb W AW, operating at 155° C, using nitrogen as carrier gas, and a flame ionization detector. Lactate was assayed enzymatically (Dawes et al. 1971), the procedure being modified by replacing phosphate buffer by PIPES buffer [piperazine-N,N′-*bis* (2-ethanesulfonic acid)]. Hydrogen in the gas phase above the culture was detected using a Packard model 428 gas chromatograph, equipped with a thermoconductivity detector, and using a column of Carbosphere 80–100 no. 5682, operating at 120° C with nitrogen as carrier gas.

When carbohydrates were included in the growth medium, their concentration was followed during growth by assaying with anthrone reagent (Trevelyan and Harrison 1952), or, in the case of glucose, by using Glucostat reagent (Worthington).

Miscellaneous biochemical tests. Gram stains were performed using both heat-fixed smears, and smears fixed with acetic acid (Dussault 1955). Assays for production of indole (in

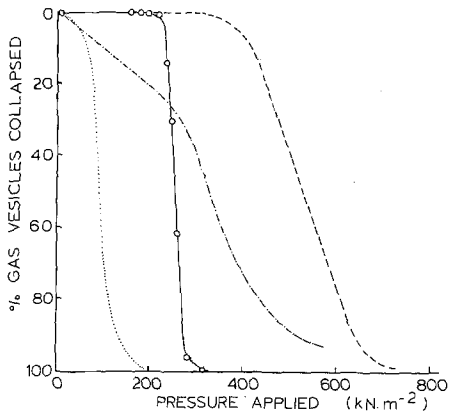

Fig. 2. Pressure relationship of gas vacuoles of *Clostridium* strain MD-2. Mature endospores with attached gas vacuoles were subjected to increasing pressure of nitrogen, whereafter the percentage of the spores that has lost the refractile gas vacuoles was determined microscopically (○). For comparison data were included on gas vacuoles of *Halobacterium* (....), *Amoebobacter* (–·–·–·–) and *Anabaena* (– – – –), derived from Walsby (1972)

tryptophan-enriched medium), gelatin hydrolysis, sulfide formation and catalase and oxidase tests were performed using standard techniques (Holding and Collee 1971). Sulfide was quantitated using a colorimetric assay according to Trüper and Schlegel (1964). The presence of cytochromes was tested using the procedure of Badziong et al. (1978), modified by suspending cell extracts in 1.5 M KCl + 50 mM Tris-HCl, pH 7.0. DNA was extracted and purified by the Marmur method (Marmur 1961), and its Guanine + Cytosine content determined by equilibrium density gradient centrifugation in CsCl (Mandel et al. 1968); *Escherichia coli* DNA (Sigma) served as standard.

Results

The organism isolated was a colorless, Gram-negative, rod-shaped bacterium (Fig. 1), displaying a considerable variation in cell size, from 0.5 × 2.5 µm to giant cells as big as 2 × 15 µm which were only occasionally observed. The mean cell size was 0.5–0.6 × 5–10 µm. Cells grown on solidified media tended to be longer than those grown in liquid media. Growing cells were often motile; in older cultures motility was lost, and within colonies on agar plates structures resembling flagellar bundles were often seen (Fig. 1B). Sporulation, though occasionally observed in liquid media, occurred much more readily on solidified medium as described upon incubation of the plates at 37° C for 2–3 weeks. We did not succeed in obtaining reproducible sporulation in any of the liquid media tested. Sporulation started with the appearance of refractile bodies at one end of the cell (Fig. 1A). These inclusions disappeared upon the application of pressure or upon high-speed centrifugation, and could therefore be identified as gas vacuoles (Walsby 1972). A pressure of about 225 kN/m^2 was found to be required to collapse half of the gas vacuoles present in mature spores (Fig. 2). Subsequent to the appearance of gas vacuoles, the side of the cell containing the vacuoles started to swell, and within the swollen part, distally from the gas vacuoles, the round terminal endospore started to develop. Occasionally gas vacuoles were also found in other parts of the cell (Fig. 1B), and on very rare occasions cells with gas vacuoles and beginning endospore formation at both ends were seen (Fig. 1C). Finally the vegetative cell degenerated and the mature endospores retained their attached gas vacuoles (Fig. 1D). Negatively stained cells showed peritrichous flagella; sometimes large numbers of flagella were concentrated in a bundle at one pole of the cell (Fig. 3A). Thin sections of vegetative cells (Fig. 3B) showed a Gram-negative type of cell wall. Negatively stained preparations of mature spores (Fig. 3C) revealed four to ten gas vacuoles near every spore, resembling those of *Halobacterium halobium* (Houwink 1956). The endospores themselves were invisible with the type of sample preparation used. A thin section through a mature endospore (Fig. 3D) showed a large number of gas vesicles in the spore appendage. The endospore was found to be surrounded by a thick cortex and a sporecoat showing five to six electron-dense layers (Fig. 3E).

The strain showed a high salt requirement: no growth was found below 0.7 M and above 2.5 M NaCl, and optimal growth was obtained in 1.4–1.5 M NaCl at 30° C and in 1.7 M NaCl at 45° C (Fig. 4A); the minimal doubling time measured was 8 h. Divalent cation concentrations of above 20 mM were required for optimal growth and magnesium concentrations of up to 0.5 M (additionally to 1.8 M NaCl present in the medium) were tolerated well (Fig. 4B). Figure 4B also shows a marked stimulation of growth when glucose was added to the medium (see below). The optimal growth temperature was 37–45° C, and no growth was observed above 55° C (Fig. 5).

The organism was found to be an obligate anaerobe, unable to grow when exposed to air. It grew well in the standard growth medium containing glutamate and other amino acids as organic nutrients. Fermentation products in a seven-day old culture grown in standard medium were acetate (14 mM), propionate (0.9 mM), isobutyrate (0.3 mM), n-butyrate (1 mM) and isovalerate (0.4 mM). Hydrogen was detected in the gas phase of the culture. In culture medium containing 0.2% glutamic acid and 0.05% yeast extract we detected after 2 days of incubation at 37° C 9.5 mM acetate and 0.45 mM butyrate. Lactate was neither detected among the fermentation products, nor was it consumed when added to the standard growth medium at a concentration of 2 mM (less than 20% decrease in the lactate concentration was found after 2 days of growth). Glucose, though highly stimulatory when added to the growth medium, was poorly utilized during growth, a significant decrease in free glucose content of the medium was observed during the stationary phase of growth only (Fig. 6). The stimulatory effect of glucose was expressed in increased growth rates as well as final growth yields. When glucose (0.2%) was added to liquid growth medium, final cell yields were increased by more than 50%. Similar yield increases were enabled by 0.2% fructose, maltose and starch, but not by lactose, cellobiose, galactose and mannose. None of the above sugars was found to be degraded in significant amounts during the exponential phase of growth .

Strain MD-2 was found to be oxidase and catalase negative and did not contain cytochromes. Up to 1 mM sulfide was detected in the culture supernatant of cells grown in the standard growth medium. The source of the sulfide appeared to be cysteine: when the cysteine in the medium was replaced by 0.05% L-ascorbic acid no sulfide was formed, while growth rates and yields were not significantly altered. Addition of 0.2% Na_2SO_4 to the standard growth medium did not significantly increase the amount of sulfide formed.

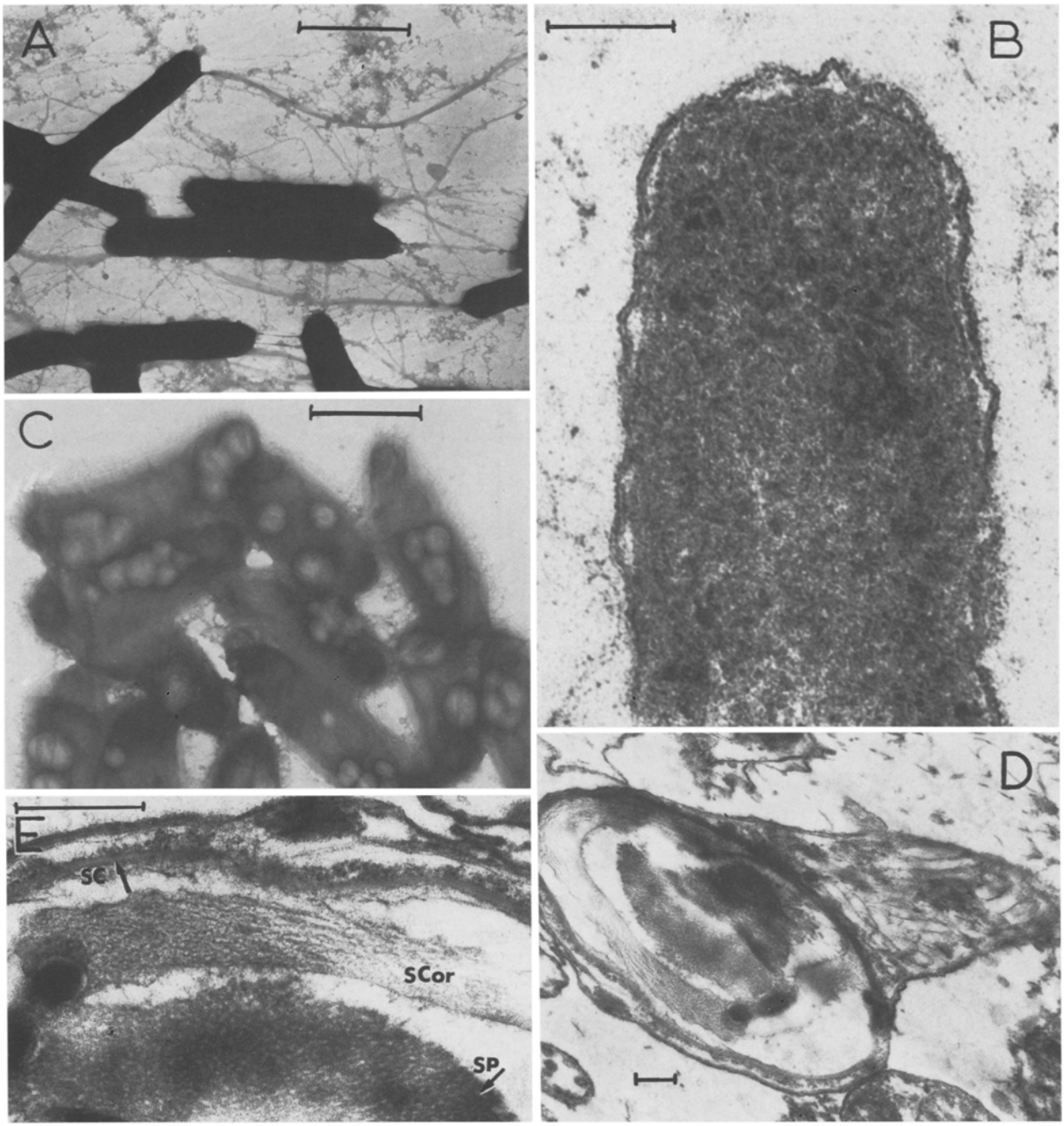

Fig. 3 A–E. Electron micrographs of *Clostridium* strain MD-2. **A** young cells from a colony on an agar plate, negatively stained with 2% phosphotungstic acid. The bar represents 1 μm; **B** thin section through a young cell, showing wall structure. The bar represents 0.1 μm; **C** mature endospores with gas vacuoles, negatively stained with 1% uranyl acetate. The bar represents 1 μm; **D** thin section through a mature endospore with attached gas vacuoles. The bar represents 0.2 μm; **E** enlargement of part of **D**, showing spore coat (*SC*), spore cortex (*SCor*) and spore protoplast (*SP*). The bar represents 0.1 μm

The strain produced indole in growth medium supplemented with 0.1% tryptophan. Gelatin was not hydrolyzed. The buoyant density of its DNA was found to be 1.6909 g/ml, corresponding with a G + C content of 31.5 mol%.

Discussion

This work describes the isolation of a new type of *Clostridium*, possessing two properties distinguishing it from the other known representatives of the genus: its high salt requirement and the formation of gas vacuoles by sporulating cells. Though clostridia can readily be isolated from marine environments, most marine isolates belong to species commonly found in freshwater and soil (Smith 1968), and a typical marine strain such as *C. oceanicum*, able to grow in the presence of 4% salt (but not in 8%), readily grows in freshwater media (Smith 1970); the latest edition of Bergey's manual contained no description of obligately halophilic

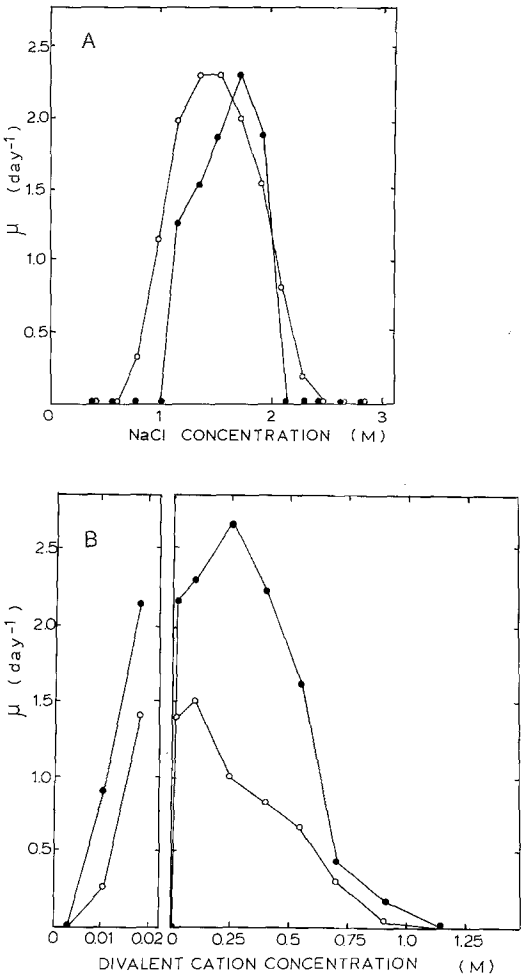

Fig. 4A, B. Effect of salt concentration on the growth of *Clostridium* strain MD-2. **A** effect of NaCl: cells were grown at 30° C (○) or at 45° C (●) in medium containing different NaCl concentrations, and in the presence of 100 mM $MgCl_2$, 50 mM $CaCl_2$ and 10 mM KCl. After different incubation periods the turbidity of the cultures was measured and the growth rates were calculated from the slope of the exponential part of the growth curves; **B** effect of $MgCl_2$: cells were grown at 30° C in the presence of 1.8 M NaCl and 10 mM KCl, with (●) and without (○) 0.2% glucose, and in the presence of varying $MgCl_2$ concentrations

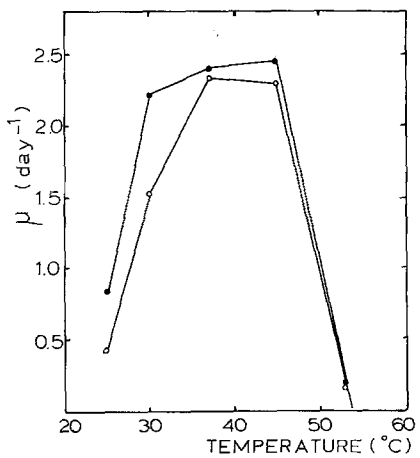

Fig. 5. Effect of temperature on the growth of *Clostridium* strain MD-2. Cells were grown at different temperatures in standard growth medium, with (●) and without (○) 0.2% glucose

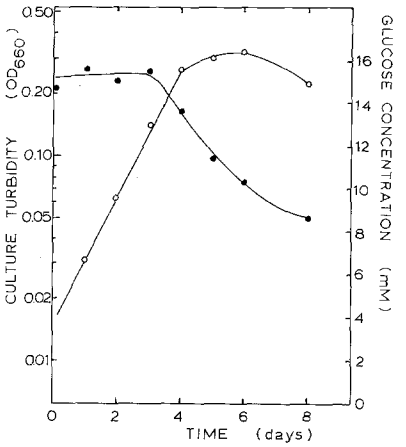

Fig. 6. Metabolism of glucose during growth of *Clostridium* strain MD-2. Cells were grown at 30° C in standard growth medium supplemented with 15 mM glucose. After different incubation periods the culture's turbidity (○) and the glucose concentration in the culture supernatant (●) were determined

clostridia (Smith and Hobbs 1974). Our isolate proved to be a moderate halophile, tolerating a relatively narrow range of salt concentrations: between 1 and 2 M NaCl, with an optimum of 1.7 M NaCl (at 45° C); at lower temperatures less salt was required for growth, and optimum growth was found at 1.4–1.5 M NaCl at 30° C. A similar temperature dependence of salinity optimum is commonly found among the halophilic bacteria (Mullakhanbhai and Larsen 1975). Many microorganisms isolated from the Dead Sea display a great tolerance towards, and also a requirement for high divalent cation concentrations, e.g. the aerobic red halophilic bacterium *Halobacterium volcanii* has been reported to require divalent cation concentrations above 0.1 M for optimal growth, and to be able to grow at half its maximal growth rate in the presence of $MgCl_2$ concentrations as high as 1.4 M (Mullakhanbhai and Larsen 1975). Our halophilic *Clostridium* required much lower divalent cation concentrations, but proved also much less magnesium tolerant. This possibly precludes the organism from growing, unless at extremely slow rates, in its habitat, the Dead Sea, where magnesium concentrations as high as 1.8 M are found.

A few endospore-forming bacteria containing gas vacuoles have been described: they are the sulfate-reducing *Desulfotomaculum acetoxidans*, isolated from manure and from marine and freshwater sediments (Widdel and Pfennig 1977, 1981), and a number of fermentative anaerobic sporeformers isolated from soil by a group of Russian scientists (Krasil'nikov and Duda 1968, Krasil'nikov et al. 1971a, b). They described a number of clostridia producing vesicular caps on their endospores, which consist of gas vacuoles. These gas vacuoles develop concomitantly with the endospore, like in our isolate. Some of Krasil'nikov's strains resemble morphologically our Dead Sea isolate, but our strain differs from them in its obligate halophilic character, its habitat, and its mode of nutrition: Krasil'nikov's strains are all carbohydrate-fermenting clostridia, while strain MD-2 utilized carbohydrates only very inefficiently (though they may sometimes be growth-stimulating). For some of the strains isolated by the Russian group new species names have been proposed, such as *C. favososporum*, *C. corinoforum*, *C. syringum*, *C. caliptrosporum*, *C. conoidales* and *C. fluorescens* (Krasil'nikov et al. 1971b), but unfortunately no type strains seem to be available for study.

Comparison of the pressure resistance curve of the gas vacuoles attached to the endospores of *Clostridium* strain MD-2 with data on other gas-vacuolated organisms (Fig. 2) shows that the clostridial vacuoles resisted about twice as high pressures as *Halobacterium* gas vacuoles do, but they were less resistant than *Amoebobacter* and *Anabaena* vacuoles. When comparing the data it must be stressed that we determined the number of spores in which all vacuoles were collapsed, and not the percentage of vacuoles collapsed; last assay, involving determination of the turbidity of cell suspensions after the application of varying external pressures, proved impossible in our case because of clumping and precipitation of the spores, and because of the relatively low contribution of the gas vacuoles to the overall turbidity of the spore suspensions. Thus the pressure at which half of the gas vacuoles collapsed may have been somewhat lower than the 225 kN/m^2 measured.

Gas vacuoles attached to clostridial spores have been suggested to promote dispersal of the spores (Duda and Makar'eva 1978): the spores, which are oxygen-resistant, may rise to the water surface, and be dispersed by water currents, till reaching an anaerobic environment suitable for germination. Such a dispersal strategy may be effective, but is necessarily limited to cells occurring in sediments of low depth only. At the depth from which the organism was isolated by us, 60 m, no intact gas vacuoles can be expected to exist as the pressure of the water column by far exceeded the critical pressure of the gas vacuoles. Only in Dead Sea sediments overlayered by no more than 18 m water can development of gas vacuoles in the halophilic *Clostridium* be expected.

Two additional unexpected observations are worth mentioning: though resembling the clostridia in all other properties, strain MD-2 reacted Gram-negative in all stages of growth, and also when using a modification of the Gram strain developed for the staining of halophilic microorganisms (Dussault 1955). Thin sections viewed in the electron microscope also showed a Gram-negative type of cell wall (Fig. 3 B). The occasional observation of cells with developing endospores at both poles is parallelled by a similar observation with *C. oceanicum* in which cells with two endospores seem to be common, while electron microscopical sections do not show any trace of a crosswall between the spores (Smith 1970).

The data presented suggest our isolate is sufficiently different from both the currently and the previously recognized species in the genus *Clostridium* to warrant its designation as a new species, and we propose the name *Clostridium lortetii*, named for M. L. Lortet, the French bacteriologist who first isolated clostridia from the Dead Sea, more than eighty years ago. Strain MD-2, the type strain, has been deposited in the American Type Culture Collection as ATCC 35059.

The following summarized species description is given: *Clostridium lortetii*. lortet'i.i. M. L. gen.n. *lortetii*, named for M. L. Lortet, French bacteriologist.

Rods, 2.5–10 × 0.5–0.6 μm, with rounded ends. Motile by means of peritrichous flagella. Sporulating cells terminally swollen. Spores round, terminal, with attached gas vacuoles. Gram-negative.

Surface colonies are circular, 0.5–1 mm in diameter, translucent, gray, with glossy surface. Sometimes spreading, with irregular margin.

Halophilic, grows optimally at NaCl concentrations between 1 and 2 M. Good growth in medium containing glutamic acid, yeast extract, nutrient broth and casamino acids. A number of carbohydrates (glucose, fructose, maltose, sucrose, starch) stimulate growth. Gelatin not hydrolyzed.

Fermentation products include acetate, propionate, n-butyrate, isobutyrate, isovalerate and hydrogen. Sulfide formed from cysteine. Optimum temperature for growth is 37–45° C. Has been found in sediment from the Dead Sea. The G + C content of the DNA is 31.5 mol% (by buoyant density). Reference strain: Strain MD-2 ATCC 35059.

Acknowledgements. We thank M. Shilo (The Hebrew University of Jerusalem) for stimulating discussions, A. E. Walsby (University of Bristol) who first identified the cell inclusions of strain MD-2 as gas vacuoles, Ahmad Montazer-Zouhoor (Department of Genetics and Development, University of Illinois) and the Center for Electron Microscopy at the University of Illinois for help with the electron microscopy, The Dead Sea Works Ltd. for logistic support enabling the obtaining of sediment samples, and R. S. Wolfe for use of laboratory facilities enabling completion of the work.

This study was supported in part by a grant from the Israeli Ministry of Energy and Infrastructure and USPH grant No. AI 12277.

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Received February 20, 1983/Accepted June 23, 1983