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Novel Anaerobic Ultramicrobacteria Belonging to the *Verrucomicrobiales* Lineage of Bacterial Descent Isolated by Dilution Culture from Anoxic Rice Paddy Soil

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The use of dilution culture techniques to cultivate saccharolytic bacteria present in the anoxic soil of flooded rice microcosms allowed the isolation of three new strains of bacteria, typified by their small cell sizes, with culturable numbers estimated at between 1.2×10^5 and 7.3×10^5 cells per g of dry soil. The average cell volumes of all three strains were 0.03 to 0.04 μm^3, and therefore they can be termed ultramicrobacteria or "dwarf cells." The small cell size is a stable characteristic, even when the organisms grow at high substrate concentrations, and thus is not a starvation response. All three strains have genomic DNA with a mol% G+C ratio of about 63, are gram negative, and are motile by means of a single flagellum. The three new isolates utilized only sugars and some sugar polymers as substrates for growth. The metabolism is strictly fermentative, but the new strains are oxygen tolerant. Sugars are metabolized to acetate, propionate, and succinate. Hydrogen production was not significant. In the presence of 0.2 atm of oxygen, the fermentation end products or ratios did not change. The phylogenetic analysis on the basis of 16S ribosomal DNA (rDNA) sequence comparisons indicates that the new isolates belong to a branch of the *Verrucomicrobiales* lineage and are closely related to a cloned 16S rDNA sequence (PAD7) recovered from rice paddy field soil from Japan. The isolation of these three strains belonging to the order *Verrucomicrobiales* from a model rice paddy system, in which rice was grown in soil from an Italian rice field, provides some information on the possible physiology and phenotype of the organism represented by the cloned 16S rDNA sequence PAD7. The new isolates also extend our knowledge on the phenotypic and phylogenetic depths of members of the order *Verrucomicrobiales*, to date acquired mainly from cloned 16S rDNA sequences from soils and other habitats.

Soils contain a high diversity of microorganisms (30, 41, 52), responsible for the cycling of organic and inorganic compounds within the soil habitat and interacting with plants and with other soil microorganisms. Microscopy and flow cytometry have revealed that many of the bacterial cells in soil are very small, with diameters of less than 0.3 μm and cell volumes of less than 0.1 μm^3 (2, 4, 13, 37). Although many bacterial species have been isolated from soils, it is not known if these isolates are "typical" soil bacteria. It has been suggested that often only the easily cultured fraction of the soil microflora has been isolated and that the more numerous, difficult-to-isolate bacteria have been more or less ignored (10, 52). This view is being strengthened by using modern molecular ecological methods to investigate the microbial diversity of such habitats and to detect the presence of as-yet-uncultivated microorganisms (30, 48, 55). Studies on the phylogenetic diversity of soil microorganisms have shown that many phylogenetic groups are present, often from lineages for which no or only a few cultivated representatives are known (30, 55). With relatively few exceptions, speculations on the role of an organism in its habitat are very difficult without laboratory culture studies. Thus, even with the rapid advance of molecular ecological methods allowing the investigation of a habitat at the organismic level without cultivation, the need to isolate representative strains of microorganisms typical for a habitat remains.

Rice paddy soil is a complex system in which processes leading to methane emission have been quantified (14) and their importance to world climate has been recognized (36). Since these soils are flooded, they are largely anoxic and typically (but not exclusively) methanogenic. The fluxes of carbon and electrons through such a system are similar to those in other anaerobic habitats, such as sediments and anaerobic waste treatment systems (14). As part of an ongoing study into the diversity of microorganisms in a model soil system, in this case the flooded soil in which rice plants are grown under laboratory conditions, novel bacteria were isolated by liquid dilution culture techniques and characterized, and they were found to belong to the *Verrucomicrobium* lineage of bacterial descent. This major lineage of bacterial evolution is presently represented by only one known species isolated from an aquatic habitat and a number of partial 16S ribosomal DNA (rDNA) sequences obtained from environmental DNA extracted from various soils (29, 30, 33, 54, 57). The new isolates from anoxic soil, interestingly, displayed a stable, small cell size, with cell volumes of less than 0.1 μm^3. Here we report on the isolation and characteristics of these new isolates.

MATERIALS AND METHODS

Medium preparation. Two different anoxic media were used in this study. These differed in the composition of the mineral salts solution which formed the basis of each medium. The mineral salts solution of the dilute medium (DM) contained 0.25 g of NaCl, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of KCl, 0.1 g of NH_4Cl, 0.05 g of KH_2PO_4, and 0.1 g of $CaCl_2 \cdot 2H_2O$ per liter of H_2O, while that of the

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freshwater medium (FM) contained 1.0 g of NaCl, 0.4 g of $MgCl_2 \cdot 6H_2O$, 0.5 g of KCl, 0.25 g of NH_4Cl, 0.2 g of KH_2PO_4, and 0.15 g of $CaCl_2 \cdot 2H_2O$ per liter of H_2O. Further preparation was the same for both media. The appropriate mineral salts solution was autoclaved at 121°C for 40 min and allowed to cool under a headspace of N_2 plus CO_2 (80:20 [vol/vol]) with a slight overpressure (ca. 6 kPa). Once cool, 30 ml of 1 M $NaHCO_3$, 3 ml of 0.5 M Na_2S, 5 ml of vitamin solution 2, and 1 ml each of a trace element solution (58), selenite/tungstate solution (53), and vitamin solution 1 were added per liter from sterile stock solutions. For some media, 5 ml of a freshly prepared, filter-sterilized (0.2-μm pore size) 0.4 M L-cysteine solution was added instead of Na_2S or no reducing agent was added at all. The pH was adjusted to 7.2 (unless noted otherwise) with sterile 1 M HCl or 0.5 M Na_2CO_3, and the medium was dispensed aseptically into sterile culture vessels. Screw-capped bottles were filled, leaving a small gas bubble, or serum bottles were partially filled (with the headspace gassed with N_2 plus CO_2 [80:20 {vol/vol}]) and closed with butyl rubber stoppers. The $NaHCO_3$ and Na_2S solutions were autoclaved in vessels closed with black rubber stoppers, under headspaces of CO_2 and of N_2, respectively. The trace element and selenite or tungstate solutions were autoclaved under air. Vitamin solution 1 was sterilized by filtration and contained (per liter of H_2O) 40 mg of 4-aminobenzoate, 10 mg of (+)-biotin, 100 mg of nicotinic acid, 50 mg of hemicalcium D-(+)-pantothenate, 150 mg of pyridoxamine hydrochloride, 100 mg of thiamine chloride hydrochloride, and 50 mg of cyanocobalamin. Vitamin solution 2 was sterilized by filtration and contained (per liter of H_2O) 10 mg of DL-6,8-thioctic acid, 10 mg of riboflavin, and 4 mg of folic acid.

Soil extract was prepared as described by Cote and Gherna (16).

Unless noted otherwise, L-isomers of organic and amino acids and D-isomers of sugars were used. Substrate and other supplement stock solutions of 100 mM to 2 M were sterilized by autoclaving, except for heat-labile compounds, which were sterilized by filtration (0.2-μm pore size). Amorphous cellulose, sterilized by autoclaving, was prepared as described by Aksenova et al. (1), except that 1 M L-cysteine was used instead of 25 mM disodium EDTA. Pectin was dissolved and sterilized by autoclaving for 10 min as described by Schink et al. (44). An aqueous slurry of flowers of sulfur was autoclaved at 110°C for 30 min and added to the medium at approximately 100 mmol/liter as required. Amorphous ferric hydroxide was prepared as described by Lovley and Phillips (31) and added from an autoclaved stock solution at 30 mmol Fe(III) per liter to FM with 2 mM L-cysteine as the reducing agent instead of sulfide and with 2 mM Na_2SO_4 as an additional sulfur source. A control experiment showed that all the strains could grow in this medium. To determine the effects of oxygen on the fermentation balances, anoxic FM without sulfide or L-cysteine, but supplemented with 2 mM Na_2SO_4, was dispensed into sterile 125-ml serum bottles, which were sealed with butyl rubber stoppers under a headspace of N_2 plus CO_2 (80:20 [vol/vol]) and then supplemented with appropriate volumes of 100% oxygen to obtain the desired final partial pressure. Nutrient agar (Difco Laboratories, Detroit, Mich.), supplemented with 4 mM filter-sterilized glucose, was used to test growth on plates.

The agar-deep method for isolating pure cultures was described by Pfennig (38). Purity was checked microscopically, by growth tests on various growth substrates in FM, and by testing growth on nutrient agar plates supplemented with 4 mM glucose. Complex purity test medium consisted of FM supplemented with (per liter) 0.5 g of yeast extract, 2 mmol of glucose, 5 mmol of fumarate, 5 mmol of pyruvate, and 2 mmol of acetate.

All incubations, other than those in the counting experiments, were at 30°C in the dark unless otherwise noted.

Isolation of bacteria. Rice (*Oryza sativa*, var. Roma, type japonica) was grown in the laboratory as described by Frenzel et al. (20) in flooded soil obtained from wetland rice fields of the Italian Rice Research Institute in Vercelli, Italy. Three-tube most-probable-number counts were made from a soil core from such a laboratory rice culture in which the rice plants were 90 days old. The core was taken by pressing a plastic tube into the soil to a depth of about 15 cm. Only the lower 10-cm part of the core was used. The core (23.65 g of dry soil) was suspended in 100 ml of DM under N_2 plus CO_2 (80:20 [vol/vol]), mixed well by stirring with a magnetic stirring bar at about 150 rpm for 5 min, stored at 4°C, and used within 6 h. This mixture was diluted in 10-fold steps, adding 1 ml of the previous dilution to 9 ml of uninoculated DM supplemented with the appropriate growth substrate, in glass tubes. These were sealed with black rubber stoppers, and the headspace was gassed with N_2 plus CO_2 (80:20 [vol/vol]). Sodium dithionite was added at 100 μM as a supplementary reducing agent at this stage. The inoculated tubes were incubated at 25°C, in the dark, for 3 months. Tubes were assessed as positive if bacterial cells were present (microscopically) and the substrate had been degraded. The most-probable-number index was calculated from the dry weight of the soil (by drying to constant weight at 105°C), the dilution factor, and tables for three parallel dilution series based on a statistical treatment of such counting methods (7).

Other bacterial strains. *Acetobacterium woodii* WB1 (DSM 1030) and *Pelobacter acetylenicus* WoAcy1 (DSM 3246) were from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and were cultivated in FM with 20 mM ethylene glycol and 20 mM acetoin as growth substrates, respectively.

Characterization methods. The methods for gram staining (32) and lysis in 3% (wt/vol) KOH (22) used *A. woodii* and *P. acetylenicus* as gram-positive and gram-negative controls, respectively. The tests for catalase and urease activities and for indole production from L-tryptophan were described by Janssen and Harfoot (24). Esculin hydrolysis was tested by observing the disappearance of the characteristic fluorescence at 366 nm in a culture containing 0.1 g of esculin per liter, after neutralization with NaOH after growth. Gelatin hydrolysis was tested by adding 120 g of gelatin per liter of the normal growth medium; after the culture had grown, the failure to form a stable gel at 4°C was scored as positive. Antibiotics were added from filter-sterilized (0.2-μm pore size) aqueous stock solutions. Genomic DNA was isolated, and its mol% G+C content was determined by high-pressure liquid chromatography as described by Janssen et al. (25).

Electron microscopy. Bacteria were fixed prior to negative staining by adding glutaraldehyde to a final concentration of 1% (vol/vol). The fixed cells were adsorbed to carbon-coated Formvar on 400-mesh copper grids and stained with 2% (wt/vol) uranyl acetate (pH 4.0) or 2% (wt/vol) sodium phosphotungstate (pH 7.0). The preparations were examined immediately in a Philips 301G electron microscope operated at 80 kV.

The cell volumes (V) were calculated, assuming the cells to be nearly spherical or ellipsoid in shape, from the cell length (l) and half the cell diameter (r) according to the following formula: $V = 4/3 \times \pi \times l/2 \times r^2$.

Analytical methods. The organic end products of fermentation and glucose utilization were determined by high-pressure liquid chromatography (28). Hydrogen production was measured by sampling the gas phase of cultures grown in serum bottles, using Dyntatech gas-tight gas syringes (Precision Sampling Corporation, Baton Rouge, La.), and injection into a stainless steel column (70 cm long, 5.3-mm internal diameter) containing Molecular Sieve 5Å, 80/100 mesh (Supelco, Bellefonte, Pa.), held at 70°C. The carrier gas was H_2-free synthetic air (N_2:O_2, 89.5:20.5, vol/vol; Messer-Griesheim, Krefeld, Germany) which was first passed through a column containing Molecular Sieve 5Å, 80/100 mesh, and then through a column containing hopcalite (2-mm grain size; Dräger, Lübeck, Germany). The detector was a RGD2 HgO-to-Hg conversion detector (Trace Analytical, Stanford, Calif.) operated at 280°C.

Sulfide production was tested by using a copper precipitation assay (15). Ammonia was determined colorimetrically (11). Nitrate utilization and nitrite production were determined by ion chromatography (3). The optical densities of bacterial cultures were measured at 440 nm in 10-mm quartz cuvettes, correcting for uninoculated medium. Dry mass growth yields were calculated from culture densities by using a gravimetrically determined conversion factor obtained from washed (25 mM ammonium acetate) cell pellets harvested from two 1-liter cultures by centrifugation and dried to a constant mass at 105°C.

16S rDNA sequence determination and analysis. The extraction of genomic DNA, PCR-mediated amplification of the 16S ribosomal DNA (rDNA), and sequencing of the purified PCR products was as described by Rainey et al. (40). Sequence reaction mixtures were electrophoresed by using a model 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif.)

Phylogenetic analysis. The 16S rDNA sequences obtained in this study were manually aligned with reference sequences obtained from the Ribosomal Database Project by using the ae2 editor (33). Due to the fact that various reference 16S rDNA sequences obtained from the databases are incomplete, three data sets were produced. Nucleotide positions are numbered according to the system of Brosius et al. (8). The first data set, comprising the sequences generated in this study and the reference sequences from representatives of the main lines of descent, contained information on 1,238 unambiguously aligned nucleotides between positions 32 and 1477 present in all sequences. By using only the sequences generated in this study and the sequence of *Verrucomicrobium spinosum*, a second data set containing information on 1,477 unambiguously aligned nucleotides between positions 32 and 1524 was produced. A third data set, made up of the sequences generated in this study, a number of reference sequences, and the sequences of three cloned 16S rDNAs (PAD7, PAD50, and FIE19), comprised 265 unambiguously aligned nucleotides between positions 1115 and 1391. Evolutionary distances were calculated by the method of Jukes and Cantor (26). Phylogenetic dendrograms were reconstructed by using treeing algorithms contained in the PHYLIP package (19). Bootstrap analyses of the neighbor-joining data (1,000 resamplings) were used to evaluate the tree topologies recovered (18).

Nucleotide sequence accession numbers. The 16S rDNA sequences obtained in this study are deposited with EMBL under the accession numbers X99390 (strain VeGlc2), X99391 (strain VeCb1), and X99392 (strain VeSm13). The strain designations (when available) and accession numbers of the sequences used as representatives of various main lines of bacterial descent are as follows: *Agrobacterium tumefaciens* DSM 30150 (M11223), *Arthrobacter globiformis* DSM 20124^T (M23411), *Bacillus subtilis* NCDO 1769^T (X60646), *Bacteroides fragilis* ATCC 25285^T (M61006), *Burkholderia cepacia* ATCC 25416^T (M22518), *Chlamydia psittaci* ATCC VR125 (M13769), *Clostridium butyricum* ATCC 19398^T (M59085), *Escherichia coli* (J01695), *Gemmata obscuriglobus* UQM 2246^T (X56305), *Isosphaera pallida* IS1B (X64372), *Pirellula staleyi* ATCC 27377^T (M34126), *Planctomyces limnophilus* IFAM 1008^T (X62911), *Spirochaeta aurantia* ATCC 25082^T (M57740), *Streptomyces ambofaciens* ATCC 23877^T (M27245), and *Verrucomicrobium spinosum* DSM 4136^T (X90515). The cloned 16S rDNA sequences used in the analysis (PAD7, PAD50, and FIE19) were obtained from the Ribosomal Database Project (33).

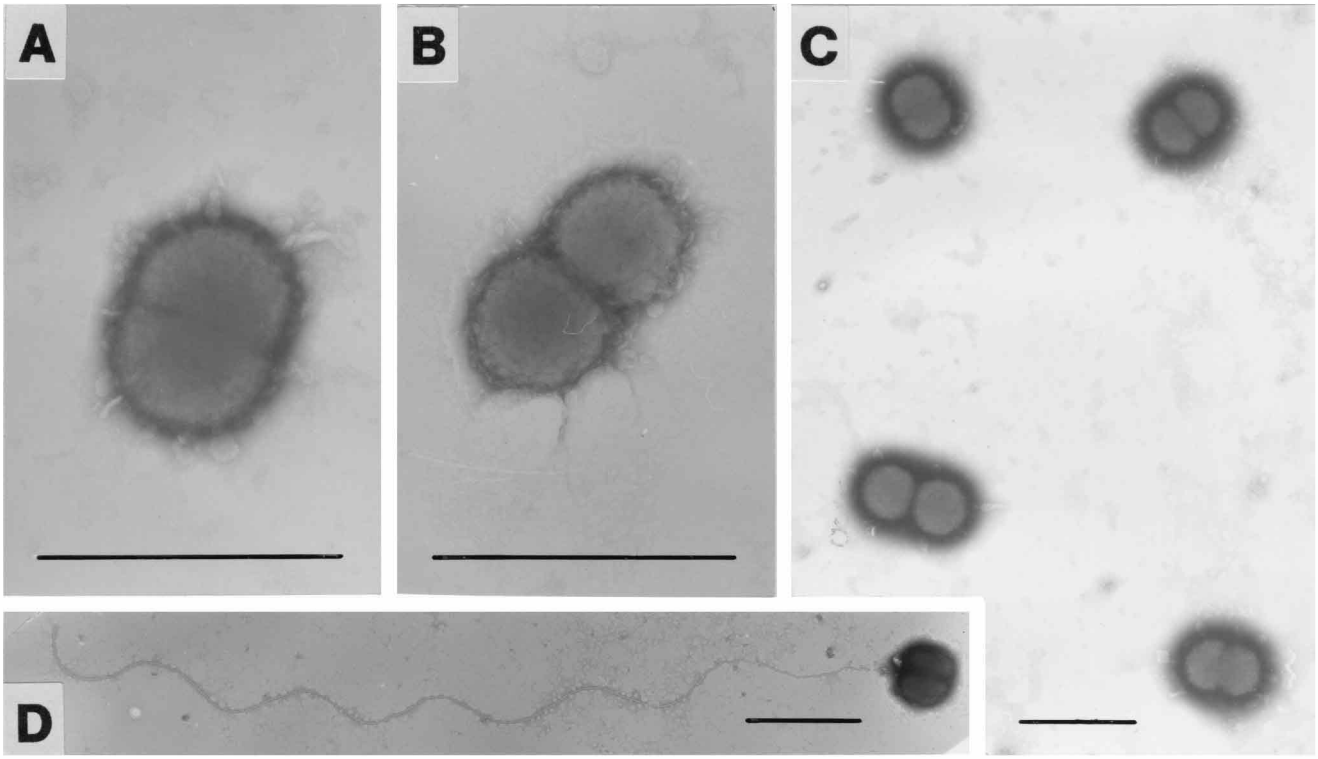

FIG. 1. Morphology of the newly isolated strains as shown by transmission electron microscopy of negatively stained whole cells. (A) Cell of strain VeGlc2 undergoing division. (B) Paired cells of strain VeSm13. (C) Cells of strain VeGlc2, showing cells at various stages of division. (D) Paired cells of strain VeCb1, showing the flagellum. Scale bars in all panels = 1 μm.

RESULTS

Isolation of new strains. Serial liquid dilution of rice paddy soil in anoxic DM with 2 mM cellobiose, 4 mM glucose, or a mixture of seven sugars (1 mM each of glucose, cellobiose, fructose, xylose, arabinose, maltose, and galactose) as the growth substrates yielded cultivatable cell counts of between 5.9×10^5 and 7.3×10^6 cells per g of dry soil from laboratory rice cultures. The initial, less-dilute tubes of the dilution series were dominated by large rod-shaped bacteria, often with endospores. The terminal, positive tubes of the dilution series were dominated by smaller rod-shaped bacteria. In the subterminal, positive tubes of the dilution series, the cultures which developed after growth were dominated by very small coccal to oval-shaped cells. Based on the development of these morphotypes, the counts of these organisms were tentatively estimated at 1.2×10^5 to 7.3×10^5 cells per g of dry soil. The subterminal positive cultures of the liquid dilutions, containing these morphotypes, were used to inoculate agar-deep dilution series, with the same growth substrates as used for the initial counting and with FM. White lens-shaped colonies developed, which were used to inoculate further agar-deep dilution series. This procedure was repeated to isolate three strains of these coccal to oval-shaped organisms in pure culture. The purity of these cultures was verified by using complex purity test medium, by microscopy, and by analyzing the fermentation end products on various fermentable growth substrates, as well as by incubating the cultures on glucose-nutrient agar under air. Strains VeCb1, VeGlc2, and VeSm13 originated from subterminal positive tubes of liquid dilution series with, respectively, 2 mM cellobiose, 4 mM glucose, and the mixture of seven sugars. The three strains were maintained by subculture at approximately monthly intervals by using FM supplemented with 4 mM glucose for strains VeGlc2 and VeSm13 and with 2 mM cellobiose for strain VeCb1. These growth substrates were used for all subsequent experiments, unless stated otherwise, with FM as the growth medium.

Morphological and cytological characteristics. The cells of strains VeCb1, VeGlc2, and VeSm13 were ellipsoid to nearly spherical in shape (Fig. 1). The mean size of the cells was about 0.5 μm long and 0.35 μm in diameter, as determined by examination of negatively stained cells with an electron microscope (Table 1). The cell volumes were calculated from these dimensions and averaged between 0.03 and 0.04 μm^3. The small cell size was confirmed by phase-contrast microscopic observations of living cells, using a calibrated eyepiece micrometer. Division appeared to be by binary fission, producing two daughter cells of equal size. Cells were usually observed in pairs (Fig. 1C), of which usually only one cell had a flagellum. The flagellum was subpolarly inserted; had a diameter of 12 to 14 nm, a wavelength of about 1.3 μm, and an amplitude of about 150 nm; and was up to 8.5 μm long (Fig. 1D). Motility was observed in living fresh preparations examined by phase-

TABLE 1. Mean cell dimensions of strains VeCb1, VeGlc2, and VeSm13, as measured from negatively stained cells observed by electron microscopy

Strain (*n*)	Measurement (SD)		
	Length (μm)	Width (μm)	Volume (μm^3)
VeCb1 (16)	0.49 (0.08)	0.33 (0.07)	0.030 (0.017)
VeGlc2 (40)	0.50 (0.11)	0.37 (0.05)	0.039 (0.018)
VeSm13 (9)	0.47 (0.12)	0.37 (0.09)	0.040 (0.026)

TABLE 2. Compounds supporting the growth of strains VeCb1, VeGlc2, and VeSm13 under anaerobic conditions[a]

Substrates supporting growth[b]	Growth[c] of:		
	VeCb1	VeGlc2	VeSm13
Glucose (4 mmol/liter)	+	+	+
Galactose (4 mmol/liter)	+	+	+
Xylose (4 mmol/liter)	+	+	+
Arabinose (4 mmol/liter)	+	+	+
L-Rhamnose (4 mmol/liter)	−	−	+
Cellobiose (2 mmol/liter)	+	+	−
Lactose (2 mmol/liter)	+	+	+
Maltose (2 mmol/liter)	+	+	−
Sucrose (2 mmol/liter)	+	+	−
Melibiose (2 mmol/liter)	+	+	−
Xylan (1 g/liter)	+	+	−
Starch (1 g/liter)	+	+	−
Pectin (1 g/liter)	+	+	+

[a] Unless noted otherwise, D-isomers of sugars and L-isomers of organic and amino acids were tested.

[b] Substrates not able to support growth of any strain (in millimoles per liter, unless noted otherwise): fructose (4), mannose (4), ribose (4), cellulose (1g/liter); fumarate (20), malate (20), succinate (20), lactate (20), citrate (10), D,L-3-hydroxybutyrate (20), crotonate (20), tartrate (20), pyruvate (20); ethylene glycol (20), 1,2-propanediol (20), glycerol (20) ± acetate (2), acetoin (20), mannitol (20); alanine (20), glutamate (20), glycine (20), threonine (20), lysine (20), aspartate (20), valine (20), leucine (20).

[c] Symbols: +, growth ($\Delta OD_{440} > 0.2$; succinate, acetate, and propionate produced); −, no growth ($\Delta OD_{440} < 0.05$; succinate, acetate, and propionate not produced). (OD_{440}, optical density at 440 nm.)

TABLE 3. Fermentation end products and balances of glucose fermentation by strain VeGlc2[a]

Substrate or product (mmol/liter)	Carbon balance (mol of C/100 mol of substrate)	Available H (mol/100 mol of substrate)
Glucose (3.67)	600.0	2,400
Acetate (1.68)	91.6	366
Propionate (2.25)	183.9	858
Succinate (0.97)	105.7	370
CO_2 (0.71)[b]	19.3	
Cells (1.58)[c]	172.2	732
Total products	572.7	2,326

[a] Calculations were made as described by Gottschalk (21), using $C_4H_7O_3$ as the empirical formula for cell material (39). The carbon balance (substrate carbon recovered as products) was 95%, and the available H balance was 97%.

[b] CO_2 production and utilization could not be determined because it was used as the buffer in the growth medium; instead, net CO_2 production was calculated by assuming the formation of 1 mol of CO_2 per mol of acetate and the utilization of 1 mol of CO_2 per mol of succinate.

[c] Calculated from a mean (of two experiments) dry mass yield of 162.3 mg/liter.

contrast microscopy. The cells were not embedded in a polysaccharide matrix or capsule. The cells stained gram negative and lysed in the KOH test. No spores were ever observed, even in medium supplemented with 10% (vol/vol) soil extract plus increased concentrations of $CaCl_2 \cdot 2H_2O$ (0.3 g/liter), $MnCl_2 \cdot 4H_2O$ (50 mg/liter), and thiamine chloride hydrochloride (30 mg/liter). The genomic DNAs of strains VeCb1, VeGlc2, and VeSm13 had mol% G+C ratios of 63.3 (standard deviation [SD] = 1.7, $n = 3$), 63.4 (SD = 0.1, $n = 4$), and 63.5 (SD = 0.2, $n = 10$), respectively.

Metabolism. The spectra of growth substrates able to be utilized by strains VeGlc2 and VeCb1 were identical. Sugars and some sugar polymers supported growth (Table 2). Cellulose was not utilized. Strain VeSm13 used a more limited range of sugars. Organic and amino acids and alcohols did not support growth. All the growth-supporting substrates were fermented to acetate, propionate, and succinate by all three strains. The maximum H_2 levels measured in the headspace of cultures growing on glucose was 0.52 Pa, corresponding to less than 10^{-4} mol of H_2 per mol of glucose fermented. Fermentation balances for growth on glucose by strain VeGlc2 (Table 3) showed that close to 2 mol of succinate plus propionate (reduced end products) were produced per mol of acetate, as would be expected if succinate formation is the pathway to reoxidize reduced electron carriers. The specific growth yield of strain VeGlc2 on glucose was 44.2 g of dry cell mass per mol of glucose.

Strains VeCb1, VeGlc2, and VeSm13 did not reduce elemental sulfur, sulfate, thiosulfate, nitrate, or amorphous ferric hydroxide. When grown under 0.2 atm of O_2, the ratio of end products of glucose fermentation by strains VeCb1 and VeGlc2 was not changed, but growth was slower.

Miscellaneous characteristics. Strains VeCb1, VeGlc2, and VeSm13 did not produce sulfide from L-cysteine or indole from L-tryptophan. Urea and esculin were hydrolyzed, but gelatin was not. Catalase activity was not detected. Strains VeCb1 and VeGlc2 grew under air on nutrient agar plates supplemented with 4 mM glucose, forming very small (<1-mm-diameter) colonies which were difficult to break up. Strain VeSm13 also grew on such plates and formed slightly mucoid, raised colonies. All three strains could grow under anoxic conditions without a reducing agent (with 2 mM Na_2SO_4 added as a sulfur source) and under strictly anaerobic conditions with either 1.5 mM sulfide or 2 mM L-cysteine as the reducing agent.

Strain VeGlc2 grew in medium supplemented with 15 g of NaCl per liter but not with 20 g/liter. Growth of this strain was possible at 10°C and fastest at 30 to 35°C but not possible at 40°C. Strain VeGlc2 was able to grow at pH values of 6.6 to 8.0. Values outside this range were not tested. When FM (1 g of NaCl per liter [pH 7.2]) with 4 mM glucose as the growth substrate was used at 30°C, strain VeGlc2 grew with a doubling time of 5.9 h. Strain VeGlc2 was resistant to bacitracin and benzylpenicillin at concentrations of 1,000 μg/ml but more sensitive to other cell wall antibiotics and other antibiotics (Table 4).

Phylogenetic analyses. Almost complete 16S rDNA sequence data (>95% of the *Escherichia coli* sequence [8]) were obtained for strains VeCb1, VeGlc2, and VeSm13. The phylogenetic dendrogram shown in Fig. 2 was reconstructed from evolutionary distances by the neighbor-joining method with the 16S rDNA sequences generated in this study and reference

TABLE 4. Growth of strain VeGlc2 at different antibiotic concentrations

Antibiotic	Target	Growth[a] at concn (μg/ml)			
		1	10	100	1,000
Bacitracin	Cell wall	+[a]	+	+	+
Benzylpenicillin	Cell wall	+	+	+	+
Ampicillin	Cell wall	+	+	−	−
Vancomycin	Cell wall	+	+	−	−
D-Cycloserine	Cell wall	+	+	−	−
Kanamycin	Protein synthesis	+	+	−	−
Streptomycin	Protein synthesis	+	+	−	−
Chloramphenicol	Protein synthesis	+	−	−	−
Rifampin	RNA polymerase	+	−	−	−

[a] +, growth; −, no growth.

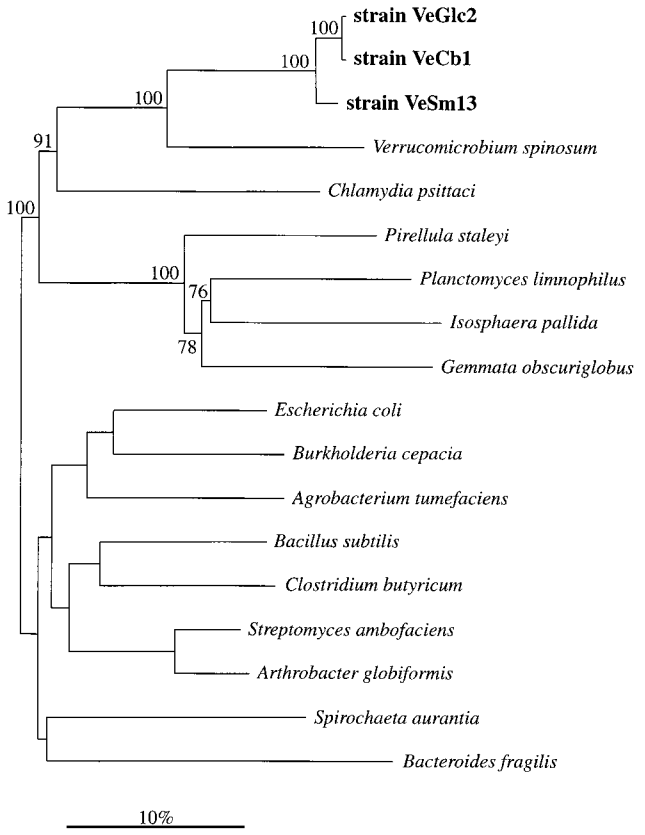

FIG. 2. Phylogenetic dendrogram reconstructed from evolutionary distances (26) by the neighbor-joining method (43), indicating the position of strains VeCb1, VeGlc2, and VeSm13 within the radiation of representatives of the main lineages of the domain *Bacteria*. Scale bar = 10 inferred nucleotide substitutions per 100 nucleotides. Numbers given at the nodes represent bootstrap values.

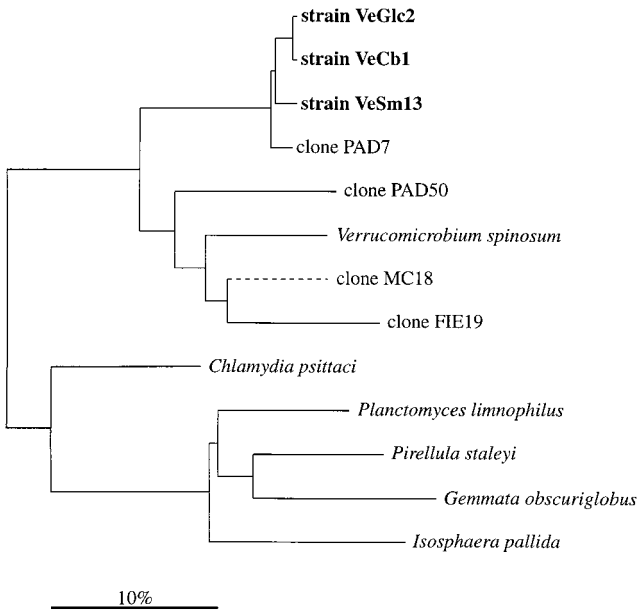

FIG. 3. Phylogenetic dendrogram reconstructed from evolutionary distances (26) by the neighbor-joining method (43), indicating the relationships between strains VeCb1, VeGlc2, and VeSm13; related taxa; and cloned 16S rDNA sequences from soils. Scale bar = 10 inferred nucleotide substitutions per 100 nucleotides. The position of environmental clone MC18 was added on the basis of its position as shown in the analysis presented by Ward-Rainey et al. (57).

sequences from representatives of various main lines of bacterial descent, containing information on 1,238 unambiguously aligned nucleotide positions present in all sequences between positions 32 and 1477. Phylogenetic analysis based on the comparison of 16S rDNA sequence data showed that strains VeCb1, VeGlc2, and VeSm13 are phylogenetically most closely related to *V. spinosum* (Fig. 2). This relationship is supported by the results of bootstrap analyses, in which the cluster comprising strains VeCb1, VeGlc2, and VeSm13 and *V. spinosum* was recovered in all cases (Fig. 2). The 16S rDNA sequence similarity values between the new isolates and the reference strains, based on the data set used for the reconstruction of the phylogeny shown in Fig. 2, are in the range of 69.1 to 81.7%.

By using only the sequences generated in this study and the sequence of *V. spinosum*, a more complete data set was produced, containing information on 1,477 unambiguously aligned nucleotide positions present in these sequences between positions 32 and 1524. 16S rDNA sequence similarities of strains VeCb1, VeGlc2, and VeSm13 to their closest relative, *V. spinosum*, calculated by using this second data set, were in the range of 79.9 to 81.0%. Strains VeCb1 and VeGlc2 showed 99.7% 16S rDNA sequence similarity to each other and 95.9 to 96.0% similarity to strain VeSm13.

The phylogenetic dendrogram shown in Fig. 3 resulted from the analysis of a third, more limited, data set made up of the sequences generated in this study, a number of reference sequences, and the sequences of three cloned 16S rRNA genes recovered from soils (PAD7 and PAD50 [33] and FIE19 [33, 54]), which comprised 265 unambiguously aligned nucleotides between positions 1115 and 1391. A fourth cloned sequence (MC18 [30]) was added to the graphic on the basis of its position as shown in the analysis presented by Ward-Rainey et al. (57). These 16S rRNA genes, isolated from soils, were found to cluster within the group comprising the new isolates and *V. spinosum*. The 16S rDNA sequence similarities between these cloned 16S rDNA sequences and the cultured members of this cluster over the 265 nucleotide positions able to be compared were in the range of 81.9 to 97.4%. The highest 16S rDNA sequence similarity between a cloned sequence and a cultured relative was found in the case of the cloned sequence PAD7 and strains VeCb1, VeGlc2, and VeSm13, at 97.4%.

DISCUSSION

Isolation of soil ultramicrobacteria. The use of dilution culture techniques to obtain bacterial isolates which are significant in or at least typical of a habitat has recently been employed to isolate marine ultramicrobacteria (9, 47). Enrichment cultures normally select for fast-growing organisms, while dilution cultures select for organisms present in high numbers in the sample, given they are able to grow in the media used. The use of dilution cultures to cultivate saccharolytic bacteria present in the anoxic soil of flooded rice microcosms allowed the isolation of the three new isolates of bacteria, typified by their small cell size. The lower tubes of the dilution series, which act as "normal" enrichment cultures, were dominated by larger rod-shaped bacteria, often with endospores. The theory of dilution cultures (9) allows the conclusion to be drawn that the organisms in the more-dilute tubes were present in higher numbers at the time of sampling and may thus represent more-typical inhabitants of the habitat under study. However, as the total number of microorganisms in the anoxic rice paddy soil is

likely to be more than 10^8 cells per g of dry soil (12), the three new isolates, with culturable numbers estimated at between 1.2×10^5 and 7.3×10^5 cells per g of dry soil, should be termed typical (rather than numerically dominant) representatives of the paddy soil microflora until more data are available.

The cells of the three newly isolated strains are small. The average cell volumes of all 3 strains were 0.03 to 0.04 μm^3. Bacteria of this size can be termed ultramicrobacteria (51), using the definition of cells with volumes of less than 0.1 μm^3 or diameters of less than 0.3 μm (47). The small cell sizes of the three new isolates appear to be stable characteristics of these strains, in the same way as the small cell size (0.05 to 0.06 μm^3) of a newly isolated marine ultramicrobacterium is a stable characteristic (47). This is in contrast to the miniaturization of cells which occurs under starvation conditions (27, 35). The presence of small cells seems typical of soils (2, 4, 13, 37), and these very small cell forms, sometimes termed dwarf cells (5), appear to have intact genomes (6). It has been asked if these are starvation forms of normal bacteria or if they form a separate population (5). Similar questions have long been asked of aquatic and marine ultramicrobacteria (35, 49), which appeared to be metabolically active (17, 34, 50; see also references cited by Schut et al. [47]) but apparently not cultivatable (23, 42, 59). The recent isolation of such a marine ultramicrobacterium (47) suggests that at least some of these forms are a truly separate population in which small size is a stable characteristic. The three new strains from anoxic rice soil similarly show a stable small size, even when growing at high (4 mM) substrate concentrations, and thus this is not a starvation response. This suggests that at least part of the soil population which has been observed to consist of such small cells (2, 4, 13) is indeed a separate population.

Characteristics of new isolates. The new isolates resemble each other physiologically and morphologically. Strain VeGlc2 displayed resistance to some antibiotics effective against bacterial cell wall synthesis. This resistance to some β-lactam antibiotics suggests that further examination of the cell envelope structure and chemistry is warranted.

The three new isolates utilized only sugars and some sugar polymers as substrates for growth, suggesting a role in the breakdown of plant polymers in the rice paddy soil. The strains are not cellulolytic and thus probably grow on the sugars released during the degradation of plant matter during cell lysis or by degrading plant polymers such as xylan, starch, and pectin. These growth substrates were fermented to acetate, propionate, and succinate, suggesting that the succinate-propionate pathway (21) is used to reoxidize reduced electron carriers. The metabolism and metabolic pathways of one of the isolates, strain VeGlc2, are currently being investigated in greater detail. The metabolism was strictly fermentative, but the new strains were oxygen tolerant. Oxygen tolerance may be a selective advantage in the paddy soil, which is anoxic during the growing season but is drained, and thus can become aerobic, once the crop is harvested. Similar changes in the conditions of flooding and drying, and thus in the length of the anoxic periods, are also encountered in many "aerobic" soils.

Phylogenetic relationships. The phylogenetic analysis on the basis of 16S rDNA sequence comparisons shows the new isolates to be most closely related to the aquatic bacterium *V. spinosum*, which until now was the only cultured member of the order *Verrucomicrobiales* (57). The results of the sequence comparisons and bootstrap analyses indicate that the new isolates represent an additional branch within the *Verrucomicrobiales* lineage. The phylogenetic depth as measured by 16S rDNA sequence similarities is now as low as 81.7% within the *Verrucomicrobiales* lineage, a depth comparable to that within the *Planctomycetales* lineage, the lowest value for which is 76.7% (56).

A novel lineage of bacterial descent, represented by four cloned 16S rDNA sequences, was detected by Liesack and Stackebrandt (30) in a forest soil and later found to be related to *V. spinosum* on the basis of comparative analysis of the sequences (57). In addition, cloned sequences of the 16S rDNAs of other members of this bacterial lineage have been recovered from the bacterioplankton of the Pacific Ocean and from a number of soils, including rice paddy field soil (29, 33, 54). Although inclusion of some of these cloned 16S rDNA sequences from various habitats greatly reduces the amount of sequence data available for comparative analysis, the recovery of the cloned 16S rDNA sequences within the *Verrucomicrobiales* lineage can be considered to be significant, since the reference organisms of the *Planctomycetales* form a cluster (Fig. 3) that was also found when a larger sequence data set was analyzed (Fig. 2) (57). This analysis (Fig. 3) shows that one of the cloned 16S rDNA sequences obtained from rice paddy soil (clone PAD7) is closely related to our three new strains. Other cloned 16S rDNA sequences from soils (clones PAD50, MC18, and FIE19) apparently form a lineage of descent with *V. spinosum*. From a taxonomic point of view, the 16S rDNA sequence data and the results of its analysis could be used to support the creation of a new genus for strains VeCb1, VeGlc2, and VeSm13. Similarly the results could support the inclusion of the as-yet-uncultured organism represented by the cloned 16S rDNA sequence PAD7 from rice paddy soil (33) in the same genus, after its isolation and physiological and chemotaxonomic characterization.

V. spinosum was isolated from the surface waters of a eutrophic lake (45, 46). A number of cloned 16S rDNA sequences from soils (29, 30, 33, 54, 57) have been shown to belong to the lineage representing the order *Verrucomicrobiales*, although to date, the strains from which these sequences originate have not been isolated. Our isolation of three strains belonging to the order *Verrucomicrobiales* from a model rice paddy system in which rice was grown in soil from an Italian rice field confirms the presence of these bacteria in soils and also provides some information on the possible physiology and phenotype of the organism only known from a cloned 16S rDNA sequence (clone PAD7).

V. spinosum is, in contrast to the new strains, not an ultramicrobacterium, with cells of about 0.8 to 1.0 μm in diameter and 1.0 to 3.8 μm long (45). Cells of *V. spinosum* display numerous prosthecae and fimbriae, structures to data not observed in the new isolates. *V. spinosum* is able to grow fermentatively on a range of sugars and can also grow under aerobic conditions. Thus, *V. spinosum* and the three new strains may have some metabolic similarities. Whether the other organisms detected on the basis of their 16S rDNA sequences have similar physiologies cannot be ascertained until knowledge of the range of physiological and genetic diversity of the *Verrucomicrobium* lineage of bacterial descent has been expanded.

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