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Bacillus thermoamylovorans sp. nov., a Moderately Thermophilic and Amylolytic Bacterium

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A moderately thermophilic, facultatively anaerobic, amylolytic bacterium was isolated from palm wine, a tropical alcoholic beverage that was sampled in Senegal. The cells were gram positive, catalase positive, non-spore forming, rod shaped, and slightly motile with peritrichous flagella. The strain which we examined did not possess cytochrome and produced L-(+)-lactate, acetate, ethanol, and formate but not hydrogen during carbohydrate fermentation. Growth occurred at pH values ranging from 5.4 to 8.5, and optimum growth occurred at around pH 7.0. The optimum temperature for growth was around 50°C, and the upper temperature limit for growth was 58°C. The guanine-plus-cytosine content of the DNA was 38.8 ± 0.2 mol%. A sequence analysis of the 16S rRNA gene revealed that the new organism is closely related phylogenetically to members of genus *Bacillus*. Despite the lack of spores, we propose that on the basis of phylogenetic characteristics, the new isolate should be classified as a new *Bacillus* species, *Bacillus thermoamylovorans*. The type strain is strain DKP (= Collection of Institut Pasteur CNCM I-1378).

Palm wine is the collective name of alcoholic beverages that result from spontaneous fermentation of the sap of any species of tree belonging to the large family *Palmae*. This traditional beverage is prepared in the tropical areas of Africa, Asia, and South America where palm trees are common (27). The sap is collected from a newly made slit at the base of an immature male inflorescence on a tree. Leaking sap is collected in a calabash, through a channel made from a plaited palm leaf (48). This method allows daily collection of about 10 liters of sap per tree (16). The sap contains about 10 to 15% (wt/vol) sucrose (12, 45), amino acids (51), and vitamins (2, 6, 38, 49).

High densities of bacteria (10^7 to 4×10^8 cells ml^{-1}) and yeasts (2×10^5 to 10^8 cells ml^{-1}) (45) give palm wine a milky flocculent appearance (29). The bacteria and yeasts isolated from palm wine belong almost exclusively to the genera *Lactobacillus* and *Saccharomyces*, respectively (30, 41, 45, 46, 51), which are both adapted to the high osmotic pressures and acidity that occur in fermented beverages (beer, grape wine, and cider) (21). Microbiological studies of palm wine have shown that the stage of fermentation markedly affects the diversity of the microflora.

During the early stages of production, when the sap has a neutral pH (pH 7.0 to 7.2) and contains a high concentration of sucrose, numerous species belonging mostly to the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Saccharomyces*, *Schizosaccharomyces*, *Candida*, and *Pichia* have been isolated from palm wine (30, 41, 51); a few *Clostridium* or *Bacillus* spp. and gram-negative *Serratia* and *Klebsiella* spp. have also been isolated (30, 45). This diverse microbiota ferments the sucrose within about 30 h (12, 38), and this fermentation leads to acidification of the medium through the production of organic acids, mostly lactic acid. The decrease in pH results in (i) the selection of acidophilic species and (ii) the disappearance of gram-negative bacteria, as well as pathogenic bacteria, that are not adapted to low pH values (45).

After about 4 days of fermentation, *Lactobacillus* species tend to disappear, while members of the genera *Streptococcus* and *Micrococcus* become dominant along with yeasts (30, 41, 45). Later, the accumulation of ethanol and the low pH of the medium favor the growth of acetic acid-producing bacteria, which oxidize ethanol to acetic acid. Despite the selectivity of the ecosystem, some *Clostridium* and *Bacillus* spp. survive in this environment, probably because of their ability to sporulate. As the mesophilic bacteria involved in palm wine production have already been studied (6, 30, 41, 45, 46, 51) and as thermophilic lactobacilli belonging to the subgenus *Thermobacterium* have been isolated previously from grape wine by Barre (5), we focused on the thermophilic anaerobic flora in palm wine. During this study we isolated two thermophilic species, *Clostridium thermopalmarium* (23) and a thermophilic amylolytic bacterium whose characteristics are described and discussed in this paper.

MATERIALS AND METHODS

Origin of the isolate. Strain DKP^T (T = type strain) was isolated from a sample of palm wine collected in Rufisque (20 km east of Dakar), Senegal.

Culture methods and media. Hungate's anaerobic techniques (17, 25) were used in the experiments described below. Strain DKP^T was cultured on a basal medium containing (per liter) 5.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 5.0 g of Biotrypcase (bioMérieux, Craponne, France), 1.0 g of KH_2PO_4, 0.4 g of $MgCl_2 \cdot 6H_2O$, 1.0 g of NH_4Cl, 5.0 mg of $FeSO_4 \cdot 7H_2O$, 25 ml of a mineral solution (4), 1 ml of a trace element solution (4), and 1 ml of Tween 80. The pH of this medium was adjusted to 7.5 with 10 M KOH, and the medium was boiled and cooled under a stream of O_2-free N_2 at room temperature. It was then distributed into 60-ml serum bottles (20 ml of medium per bottle) and Hungate tubes (4.5 ml of medium per tube) (28). After the medium was autoclaved at 110°C for 45 min, energy sources were injected into the serum bottles and Hungate tubes from separately sterilized stock solutions to give a final concentration of 0.3% (wt/vol).

A vitamin-free, chemically defined medium was used to determine vitamin requirements. This medium contained (per liter) 1.0 g of KH_2PO_4, 0.4 g of $MgCl_2 \cdot 6\ H_2O$, 0.2 g of NH_4Cl, 5.0 mg of $FeSO_4 \cdot 7H_2O$, 25 ml of a mineral solution (4), 1 ml of a trace element solution (4), 1 ml of Tween 80, 0.26 mmol of DL-cysteine, 0.26 mmol of L-lysine, 0.26 mmol of L-tryptophan, 0.26 mmol of L-(+)-asparagine, 0.26 mmol of L-glutamine, 0.26 mmol of DL-tyrosine, 0.26 mmol of DL-phenylalanine, 0.26 mmol of DL-threonine, 0.26 mmol of DL-aspartic

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acid, 0.26 mmol of L-isoleucine, 0.26 mmol of DL-serine, 0.26 mmol of DL-valine, 0.26 mmol of L-histidine-HCL, 0.26 mmol of DL-leucine, 0.26 mmol of glycine, 0.26 mmol of DL-alanine, 0.26 mmol of DL-proline, 0.26 mmol of L-arginine, 0.26 mmol of DL-methionine, and 0.26 mmol of L-glutamic acid (all amino acids from Sigma Chemical Co., St. Louis, Mo.). The pH of the medium was adjusted to 7.5 with 10 M KOH. The medium was then distributed under a stream of O_2-free N_2 into Hungate tubes (4.5 ml of medium per tube) and autoclaved at 110°C for 40 min. Glucose, vitamins, and purine and pyrimidine bases were injected into the Hungate tubes from filter-sterilized solutions.

Unless indicated otherwise, all experiments were conducted at the optimum temperature for growth (50°C) and pH 7.5.

Enrichment and isolation procedure. Enrichment cultures were obtained by adding 10 ml of palm wine, 1 ml of 10% $NaHCO_3$, and 0.2 ml of 2% $Na_2S \cdot 9H_2O$ to a serum bottle containing 20 ml of basal medium supplemented with 0.3% glucose. After 24 h at 50°C, a 1-ml sample was injected into another serum bottle containing basal medium. This process was repeated twice. Pure cultures were obtained by repeated application of the roll tube method of Hungate (17). Single colonies were removed, preparations were diluted serially in Hungate tubes containing 4.5 ml of basal medium supplemented with 0.3% glucose, and the tubes were incubated for 24 h at 50°C. Strain DKP^T was stored in liquid growth medium at room temperature and was used for further characterization.

Sporulation test. Two agar-based media were used to test for sporulation. Medium A contained (per liter) 5.0 g of Bacto Peptone (Difco), 3.0 g of meat extract (Difco), 5 mg of $MnSO_4$, and 16 g of agar (Difco).

Medium B, which was used for plates, was the same as the basal medium except that 1% glucose and 1.6% agar were added. The pH of medium A and the pH of medium B were adjusted to pH 7.2 with 10 M KOH. After autoclaving at 110°C for 45 min, the media were distributed into plates. The plates were inoculated with 0.3-ml portions of an overnight liquid culture and then incubated at 50°C for up to 5 days to determine the presence of spores.

The heat resistance of cells was determined in basal medium supplemented with 0.3% glucose. After 1, 2, and 20 days of incubation at 50°C, duplicate cultures were heated at 80°C for 5 and 10 min and subcultured into fresh medium (inoculum, 20% [vol/vol]), and the resulting preparations were incubated 48 h at 50°C. In addition, the viability of cells after 1, 2, and 20 days of incubation was checked by subculturing before heating.

Analytical techniques. Bacterial growth was monitored by measuring the increase in turbidity at 600 nm in anaerobic Hungate tubes inserted directly into a Shimadzu model UV 160A spectrophotometer. The effect of oxygen on the growth and metabolism of strain DKP^T was determined in flasks which contained basal medium supplemented with 1% glucose and were agitated. Hungate tubes containing basal medium supplemented with 0.3% glucose with air or O_2-free N_2 in the headspace were also used. Flasks and Hungate tubes were inoculated (inoculum, 10%, [vol/vol]) with overnight cultures and incubated for 48 h at 50°C.

The fermentation pattern was determined in duplicate by using basal medium containing the carbohydrate being tested at a final concentration of 0.3% (wt/vol) and 0.017% bromothymol blue. Stock carbohydrates were sterilized separately by filtration.

The vitamin requirements were determined by using the vitamin-free chemically defined medium supplemented with glucose at a final concentration of 0.3% (wt/vol). A vitamin solution, an adenine-guanine-uracil solution, and a xanthine solution were prepared as described by Rogosa et al. (37) and were sterilized separately by filtration. The final concentrations of the vitamins and purine and pyrimidine bases tested were as follows: nicotinic acid, thiamine-HCL, calcium D-pantothenate, and riboflavin, 992 μg liter^{-1}; *p*-aminobenzoic acid, 551 μg liter^{-1}; pyridoxine, 1,984 μg liter^{-1}; vitamin B_{12}, 1 μg liter^{-1}; DL-biotin, 10 μg liter^{-1}; folic acid, 1 μg liter^{-1}; and adenine, guanine, uracil, and xanthine, 5.157 mg liter^{-1}. Actively growing cultures obtained after three successive transfers in basal medium were used for inoculation. Inocula were obtained from suspensions of organisms that had been washed twice in saline. For each growth factor, the test was performed in triplicate.

The temperature range for growth was determined by using thermostatically controlled water baths. Catalase activity was tested with a 3% (vol/vol) hydrogen peroxide solution by using a pellet resulting from centrifugation of 1.5 ml of an overnight culture. Aerobiosis was achieved by shaking cultures growing in basal medium containing 1% glucose. Indole production and ammonium production were assayed with Kovács and Nessler's reagents (Sigma), respectively. Nitrate reduction was determined with Griess's reagent (Sigma). H_2S was measured photometrically as colloidal CuS after reaction with a mixture containing 50 mM HCl and 5 mM $CuSO_4$ (9).

Glucose and fermentation product contents were determined by high-performance liquid chromatography (HPLC), using a model Analprep 93 pump (Touzart et Matignon, Vitry sur Seine, France) and a type ORH 801 column (Interaction Chemicals, Inc., Mountain View, Calif.) equipped with a differential refractometer detector (Knauer, Berlin, Germany). A 20-μl portion of a cell-free supernatant was injected into the column, which was maintained at 35°C. A 25 mM H_2SO_4 solution was used as the solvent at a flow rate of 0.7 ml · min^{-1}. Hydrogen was quantified by using a Girdel model Serie 30 gas chromatograph equipped with a thermal conductivity detector and a stainless steel column (1 m by 3.2 mm) packed with Carbosphere SS (60/80 mesh); the column temperature was 150°C, the carrier gas was He (10^5 Pa), the injector and detector temperature was 210°C, and the power of the filament was 90 mA. CO_2 and O_2 contents were determined with a Chrompack model CP 9000 gas chromatograph equipped with a thermal conductivity detector and two stainless steel columns (1.5 m by 2.2 mm). One column was packed with molecular sieve 5A (60/80 mesh), and the other column was packed with silica gel (GC grade); the column temperature was 60°C; the carrier gas was He (10^5 Pa), the injector temperature was 70°C, the detector temperature was 150°C, and the power of the filament was 90 mA. L-(+)-Lactic dehydrogenase and D-(−)-lactic dehydrogenase (Boeringer Mannheim, Mannheim, Germany) were used to assess the stereoisomerism of the lactic acid produced by the fermentation of glucose.

For cytochrome analysis, 3 g of wet cells suspended in 10 ml of 20 mM Tris hydrochloride buffer (pH 7.6) was sonicated eight times at 0.5 cycle · s^{-1} for 2 min. The suspension was centrifuged at 30,000 × *g* for 20 min at 5°C to remove the cell debris. The resulting cell extract was separated into a supernatant fraction and a particulate fraction by centrifugation at 140,000 × *g* for 2 h. The resulting dark gelatinous pellet was resuspended in the same buffer; this represented the particulate fraction. Both the soluble fraction and the particulate fraction were examined for cytochromes by determining their air-oxidized and dithionite-reduced spectra (300 to 600 nm), as well as their redox difference spectra (dithionite-reduced spectrum minus air-oxidized spectrum), with a Shimadzu model UV 300 spectrophotometer.

Morphological characteristics. Morphological properties were determined by phase-contrast microscopy, using slides coated with a thin layer (0.5 mm) of purified agar (Difco). For electron microscopy preparations were negatively stained with 4% (wt/vol) uranyl acetate in distilled water. For transmission electron microscopy cells were fixed for 1 h in 0.07 M sodium cacodylate buffer (pH 7.3) containing 1.2% (wt/vol) glutaraldehyde and 0.05% ruthenium red. After the samples were washed in cacodylate buffer containing ruthenium red, they were postfixed with 1% OsO_4 in cacodylate buffer. The samples were embedded in Epon, and ultrathin sections were stained with 2% uranyl acetate in 50% ethanol and then with lead citrate. Micrographs were taken with a JEOL model 1200 CX electron microscope.

DNA base composition. The guanine-plus-cytosine (G+C) content of the DNA was determined by workers at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. After disruption with a French pressure cell, the DNA was isolated and purified by chromatography on hydroxyapatite. The G+C content was determined by HPLC as described by Meshbah et al. (26); nonmethylated lambda DNA (Sigma) was used as the internal standard.

SDS-PAGE. Approximately 90 mg of cells was harvested from three bottles that contained basal medium supplemented with 0.3% of glucose and had been incubated at 50°C for 48 h. Whole-cell protein extracts were prepared and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Pot et al. (33). Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of strains by using the Pearson product moment correlation coefficient, and an unweighted pair group using mathematical average cluster analysis were performed by the techniques described by Pot et al. (33), using the GELCOMPAR software package (version 2.0) (52). The protein profile of strain DKP^T was compared with a database consisting of normalized protein fingerprints derived from reference strains belonging to almost all previously described species of lactic acid bacteria (32).

16S rRNA sequence studies. Purification of genomic DNA, amplification, and purification of the 16S rRNA gene from isolate DKP^T were performed by using a technique described previously (24, 35). The purified PCR product was sequenced directly. Sequencing was performed with an ABI automated DNA sequencer by using a Prism dideoxy terminator cycle sequencing kit as recommended by the manufacturer (Applied Biosystems, Ltd.). The primers used for sequencing have been described previously (35). The 16S ribosomal DNA sequence obtained from the sequencing data was aligned, by using sequence editor ae2, with the sequences of various members of the bacterial phylum whose 16S rRNA sequences were obtained from the Ribosomal RNA Database Project and from GenBank (22). Positions of sequence and alignment uncertainty were omitted from the analysis, and the pairwise evolutionary distances for 1,128 nucleotides were computed from levels of similarity by using the Olsen correction parameter (31) of Jukes and Cantor (18). Dendrograms were constructed from evolutionary distances by using the program of De Soete (10). A transversion analysis was performed by using the program DNAPARS implemented in the PHYLIP package (13). Tree topology was reexamined by using 100 bootstrapped data sets; the SEEQBOOT, DNADIST, FITCH, and CONSENSE programs available on TREECON (50) were used for this purpose.

Nucleotide sequence accession numbers. The strain DKP^T 16S rRNA sequence which we determined has been deposited in the GenBank database under accession number L27478. The EMBL accession numbers of the 16S rRNA sequences of *Sporosarcina urea* and *Saccharococcus thermophilus* are X62173 and X70430, respectively.

RESULTS

Colony morphology. After 48 h of growth on basal medium, strain DKP^T colonies were small, white, lens shaped with smooth edges, and 2 to 3 mm in diameter.

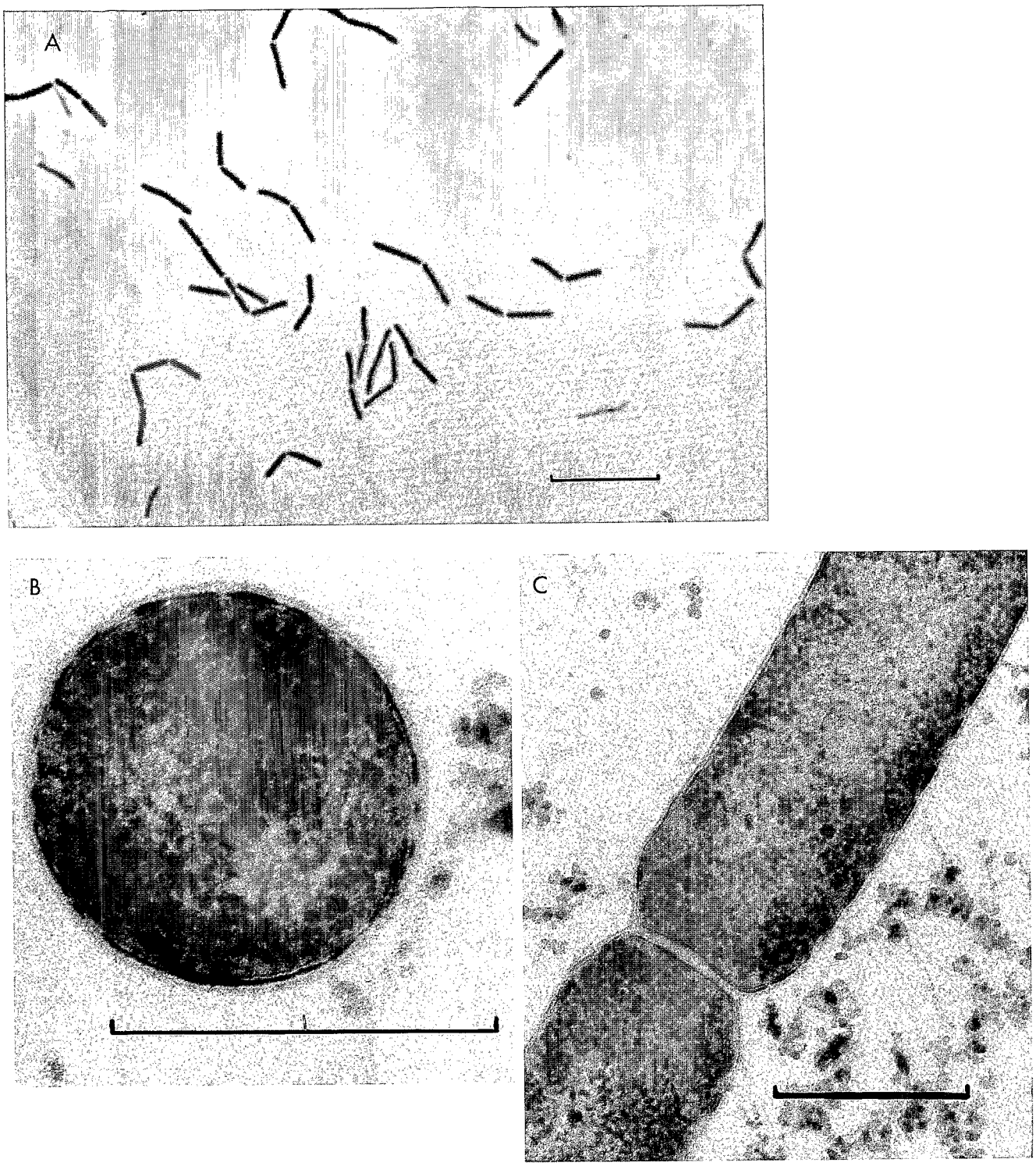

FIG. 1. (A) Phase-contrast photomicrograph of strain DKP^T. Bar = 10 μm. (B) Ultrathin transverse section of isolate DKP^T, showing the cell wall structure. Bar = 0.5 μm. (C) Ultrathin longitudinal section of isolate DKP^T, showing the septum. Bar = 0.5 μm.

Cell morphology. Strain DKP^T cells were straight rods that occurred either singly or in short chains of two to four cells (Fig. 1A). Although the cells possessed peritrichous flagella, they exhibited only very slight motility as determined by phase-contrast microscopy. The cells were 0.45 to 0.5 μm wide and 3.0 to 4.0 μm long. The Gram stain reaction was positive, but cells in older cultures lost the ability to retain the Gram stain. Thin sections revealed a typical gram-positive cell envelope profile (Fig. 1B and C). Strain DKP^T did not grow on solid medium A, which was used to induce sporulation of bacilli.

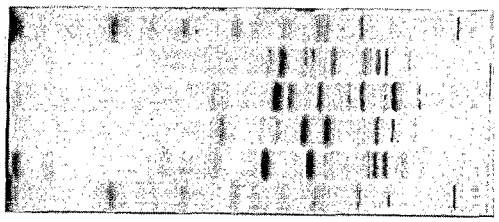

FIG. 2. Profiles obtained by electrophoretic analysis of whole-cell proteins of the following organisms: strain DKP^T, *Lactobacillus paracasei*, *Lactobacillus acidophilus*, and *Lactobacillus brevis*.

Strain DKP^T and *Bacillus coagulans*, which was used as a standard, exhibited good growth on solid medium B. After 48 h, *B. coagulans* sporulated, but strain DKP^T did not. In addition 24- and 48-h and 20-day-old cultures that had been incubated at 50°C exhibited no heat resistance when they were heated at 80°C for 5 or 10 min, indicating that no spores were present.

DNA base composition. The average G+C content of strain DKP^T DNA, based on three determinations, was 38.8 ± 0.2 mol%.

Growth conditions and metabolic properties. In medium containing glucose as the energy source, strain DKP^T required yeast extract or Biotrypcase for growth. No growth was observed when gelatin or casein replaced yeast extract. However, a small amount of growth was observed in the vitamin-free chemically defined medium when glucose was used as an energy source. Colonies grew on plates incubated in air. Strain DKP^T also grew in liquid medium that had been agitated and in anaerobic medium with O_2 in the headspace; in the latter medium the O_2 was completely consumed and CO_2 was produced. Strain DKP^T also grew under strict anaerobic conditions in a prereduced medium. This indicated that strain DKP^T is a facultative anaerobe. During anaerobic growth on glucose, strain DKP^T produced lactate, formate, acetate, and ethanol as the only end products. No H_2 was produced by strain DKP^T even when it was cocultured with an hydrogenotrophic methanogen. At the end of the time course the fermentation balance measured was: 1.0 glucose → 0.89 lactate + 1.0 formate + 0.56 acetate + 0.57 ethanol.

Our data accounted for a level of carbon recovery of 99%. The molar ratio of acetate, ethanol, and formate was 1:1:2. The lactate produced was 96% L-(+)-lactate. Under aerobic conditions, strain DKP^T produced lactate, acetate, and CO_2 but not formate during glucose metabolism, and acetate production increased compared with acetate production under anaerobic conditions. Under aerobic conditions, the end products of glucose metabolism were: 1.0 glucose → 0.6 lactate + 1.21 acetate + 0.06 ethanol + 1.21 CO_2.

However, strain DKP^T lacked cytochromes but was positive for catalase activity. Nitrate reduction and sulfate reduction were negative, and indole and H_2S were not produced. In medium containing arginine as an energy source, NH_3 was produced but no growth occurred.

Under anaerobic conditions, growth was optimal at approximately 50°C; 58°C was the highest temperature at which growth occurred. The organism grew at pH values ranging from 5.4 to 8.5; the optimum pH was 7.0. In basal medium containing glucose as the energy source at pH 7.0 and 50°C, the maximum doubling time was 40 min.

Fermentation of sugars. The following sugars (final concentration, 0.3% [wt/vol]) were fermented within 24 h: L-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, L-rhamnose, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, trehalose, starch, glycogen, gentiobiose, and gluconate. D-Xylose, D-galactose, *N*-acetyl-D-glucosamine, lactose, sucrose, melezitose, and D-turanose were fermented slowly (within 48 to 96 h). Glycerol, erythritol, D-arabinose, L-xylose, ribitol, L-sorbose, galactitol, inositol, D-mannitol, D-glucitol, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose, inulin, raffinose, xylitol, D-lyxose, D-tagatose, D- and L-fucose, D-arabitol, and L-arabitol were not fermented.

Vitamin requirements. Thiamine, DL-biotin, and purine and pyrimidine bases (adenine, guanine, uracil, xanthine) stimulated the growth of strain DKP^T but were not essential. Vitamin B_{12}, pyridoxine, nicotinic acid, *p*-aminobenzoic acid, calcium D-pantothenate, folic acid, and riboflavin had no significant effect on growth.

SDS-PAGE. The electrophoretic patterns of the soluble cellular proteins, as determined by the PAGE method (Fig. 2), showed that strain DKP^T is not similar to any previously described *Lactobacillus* or *Carnobacterium* species and that it does not belong to the genus *Enterococcus*, *Lactococcus*, or *Vagococcus*. Strain DKP^T was also compared with the most relevant species of the genera *Leuconostoc*, *Pediococcus*, and *Tetragenococcus* and with a number of representative strains belonging to the genera *Streptococcus* and *Bacillus*, including *Bacillus stearothermophilus*, *Bacillus thermoglucosida*, and *Bacillus thermoleovora* strains and two *Bacillus kaustophilus* strains. Low correlation values were obtained, indicating that strain DKP^T does not belong to any of the species mentioned above (data not shown).

16S rRNA sequence. Using 10 primers, we determined an almost complete sequence consisting of 1,542 bases for the 16S ribosomal DNA gene of isolate DKP^T. Sequence positions 8 to 1542 (*Escherichia coli* numbering as described by Winker and Woese [53]) had 70 of the 70 signature nucleotides and/or nucleotide pairs which indicate that an organism is a member of the domain *Bacteria* (53). The results of a sequence alignment, followed by a phylogenetic analysis of the rRNA gene sequence in which representatives of the families of the domain *Bacteria* were used, indicated that strain DKP^T belongs to the subphylum containing gram-positive bacteria with DNA G+C contents of less than 55 mol%, which includes members of the genera *Bacillus* (groups 1 to 5 as defined by Ash et al. [3]), *Sporolactobacillus*, *Sporosarcina*, and *Saccharococcus*. Further analysis indicated that the position of isolate DKP^T is equidistant from members of *Bacillus* groups 1, 2, and 5, with levels of similarity ranging from 93.9% for members of group 1 (*Bacillus cereus*, *Bacillus subtilis*, *Bacillus circulans*, *Bacillus pantothenticus*) and group 5 (*B. kaustophilus*, *B. stearothermophilus*, *Bacillus thermoglucosidasius*, and *Saccharococcus thermophilus*) to 93.4% for members of group 2 (*Bacillus smithii*, *Bacillus globisporus*, and *Sporosarcina urea*). Members of group 3 (*Bacillus polymyxa*) and *Bacillus amylolyticus*) and group 4 (*Bacillus aneurinolyticus*) were more distantly related (level of similarity, 89.1%). Figure 3 is a dendrogram gener-

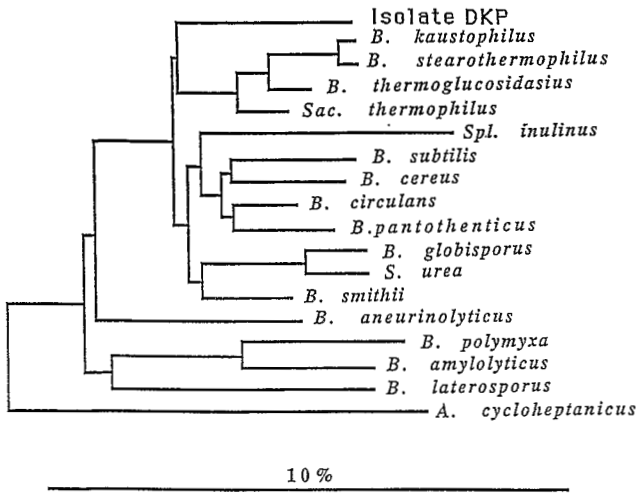

FIG. 3. Dendrogram showing the position of isolate DKP^T among representatives of the genus *Bacillus* and related bacteria. The dendrogram was derived from the similarity matrix shown in Table 1. Abbreviations: *B.*, *Bacillus*; *Sac.*, *Saccharococcus*; Spl., *Sporolactobacillus*; *A.*, *Alicyclobacillus*; *S.*, *Sporosarcina*.

ated by the method of De Soete (10) from the evolutionary distance matrix (Table 1) and shows these relationships. The G+C content of the 16S rRNA of isolate DKP^T was 55.5 mol%; this value is similar to the values determined for members of groups 1 to 5 but slightly lower than the values determined for members of group 5 (average G+C content, 58 mol%). A transversion analysis, implemented in the PHYLIP package, yielded results similar to those shown in Fig. 3, indicating that the results were not biased by the G+C content of the 16S ribosomal DNA gene used in the analysis.

The following evidence supports the hypothesis that strain DKP^T occupies an intermediate position in the cluster containing members of *Bacillus* groups 1, 2, and 5. (i) Only two of the nine signature nucleotides that are found in members of the thermophilic bacilli belonging to group 5 as defined by Rainey et al. (34) were found in strain DKP^T, indicating a lack of close affiliation. And (ii) certain nucleotides, nucleotide pairs, and nucleotide stretches were found in strain DKP^T exclusively or occurred rarely in the 62 members of the genus *Bacillus* and related genera (e.g., the genus *Sporosarcina*) whose 16S rRNA sequences are available from the Ribosomal Database Project; these nucleotides, nucleotide pairs, and nucleotide stretches include C at position 189, GAUAA at positions 193.3 to 193.7, GAUGGC at positions 198 to 203, GCCAUCACUU at positions 214 to 224, U at position 264, U and G at positions 458 and 474, UCUUGCGGCUCAACCG CAA at positions 610 to 628), G and C at positions 835 and 851), U and A at positions 999 and 1041), G and C at positions 118 and 1155, and UCUAG at positions 1120 to 1124 (*E. coli* numbering as described by Winker and Woese [53]). A bootstrap analysis of the data performed by using programs in the TREECON package and the PHYLIP package revealed low levels of relatedness with members of all three groups; the levels of relatedness with group 1, 2, and 5 strains were 54, 22, and 36%, respectively.

DISCUSSION

Strain DKP^T, which was isolated from a palm wine sample harvested near Dakar, Senegal, is a moderately thermophilic facultative anaerobe. Its type of metabolism, its low G+C content (38.8 mol%), and its typical gram-positive cell wall (Fig. 1B) suggest that strain DKP^T could belong to the genus *Bacillus* or the genus *Lactobacillus* (15, 44).

The morphological and physiological characteristics and nutritional requirements of strain DKP^T were consistent with the description of the genus *Lactobacillus* (19, 42). Like lactobacilli, strain DKP^T is non-spore forming, saccharolytic, and chemoorganotrophic. It requires Biotrypcase or yeast extract for good growth. It produces lactate, acetate, ethanol, and formate but not hydrogen during sugar fermentation. Furthermore, NH_3 is produced during arginine fermentation, as described previously for heterofermentative lactobacilli (1, 19). The ratio of the molar yields of acetate, ethanol, and formate is 1:1:2; this fermentation ratio is typical of the phosphoroclastic split of pyruvate into formate and acetyl phosphate, where acetyl phosphate produces acetate and ethanol. Unpublished data have shown that the lactate concentration varies considerably according to the culture conditions. In an acidic medium, the yield of lactate increases concomitantly with decreases in the yields of acetate, ethanol, and formate. Under glucose-limiting conditions in a continuous culture, a decrease in lactate production and an increase in the production of acetate, ethanol, and formate occur. These results are consistent with those reported previously for lactic bacteria (7, 11, 36, 47). Furthermore, like members of the genus *Lactobacillus*, strain DKP^T lacks cytochrome. Nevertheless, it has been demonstrated that under aerobic conditions, lactobacilli reoxidize $NADH_2$, with oxygen serving as the final electron acceptor, since they possess flavin-containing oxidases and peroxidases (19). This process probably results in an increase in acetate production by strain DKP^T in aerobiosis, as described previously for *Lactobacillus plantarum* (40).

Despite the phenotypic similarities between strain DKP^T and the genus *Lactobacillus*, there are important differences between these taxa. (i) Strain DKP^T possesses peritrichous flagella, which is unusual in lactobacilli (19, 42). (ii) Strain DKP^T is catalase positive. (iii) Strain DKP^T exhibits optimal growth in neutrophilic media, whereas lactobacilli grow best in slightly acidic media (pH 5 or less), with optimal growth occurring at pH 5.5 to 6.2 and growth often reduced at neutral or slightly alkaline pH values (19). (iv) Strain DKP^T exhibits an optimum temperature for growth of 50°C and the upper temperature limit for growth is 58°C; most lactobacilli grow best at mesophilic temperatures and have an upper temperature limit for growth of around 40°C (the so-called "thermophilic" lactobacilli may have an upper temperature limit of 55°C, but no strain capable of growth at temperatures above 55°C is known [19]). In addition, a comparison of the electrophoretic pattern of the soluble cellular proteins of strain DKP^T with representative patterns of lactic acid bacteria (Fig. 2; data not shown) also indicated that strain DKP^T does not belong to any previously described species of lactic bacteria.

The genus *Sporolactobacillus*, which contains only one species, *Sporolactobacillus inulinus* (20), is phylogenetically closely related to the genera *Bacillus* and *Lactobacillus*. However, *Sporolactobacillus inulinus* differs from strain DKP^T by its mesophilic and homolactic characteristics. Moreover, this species produces D-(−)-lactate and does not ferment pentoses. The genus *Sporolactobacillus* differs from strain DKP^T and the genus *Lactobacillus* by spore production and from the genus *Bacillus* by its lack of catalase and cytochrome. The taxonomic position of *Sporolactobacillus inulinus* has been discussed on the basis of the results of a 16S rRNA sequence analysis (14, 43, 44), and despite the lack of heme proteins such as catalase and cytochrome, this organism is considered a member of the family *Bacillaceae*.

TABLE 1. 16S rRNA similarity matrix[a]

Taxon	% Similarity to:																			
	Alicyclobacillus cycloheptanicus	Isolate DPK^T	*Saccharococcus thermophilus* ATCC 43125	*Bacillus stearothermophilus* ATCC 12980	*Bacillus thermoglucosidasius* ATCC 43742	*Bacillus kaustophilus* NCIB 8547	*Bacillus pantothenticus* ATCC 14576	*Bacillus cereus* ATCC 14579	*Bacillus circulans* ATCC 4513	*Bacillus subtilis*	*Sporolactobacillus inulinus* ATCC 15538	*Bacillus aneurinolyticus* NCIB 10056	*Bacillus laterosporus* ATCC 64	*Bacillus amylolyticus* NCIB 8144	*Bacillus polymyxa* ATCC 842	*Bacillus smithii* DSM 4216	*Sporosarcina urea ATCC 614*	*Bacillus globisporus* DSM 4
Alicyclobacillus cycloheptanicus	100																	
Isolate DKP^T	86.4	100																
Saccharococcus thermophilus ATCC 43125	87.3	94.0	100															
Bacillus stearothermophilus ATCC 12980	87.4	93.3	96.7	100														
Bacillus thermoglucosidasius ATCC 43742	87.4	94.8	97.5	97.3	100													
Bacillus kaustophilus NCIB 8547	87.7	93.4	96.7	99.3	97.3	100												
Bacillus pantothenticus ATCC 14576	86.5	94.0	95.2	93.4	94.7	93.4	100											
Bacillus cereus ATCC 14579	86.4	93.4	94.9	93.6	94.5	93.6	95.3	100										
Bacillus circulans ATCC 4513	86.2	94.4	95.8	93.8	95.0	93.8	96.9	96.2	100									
Bacillus subtilis	86.1	93.6	94.8	92.7	94.1	92.7	95.0	95.4	96.3	100								
Sporolactobacillus inulinus ATCC 15538	85.0	91.6	92.7	91.1	91.9	91.1	92.7	92.4	93.3	92.8	100							
Bacillus aneurinolytius NCIB 10056	87.1	91.6	92.4	91.9	92.1	91.9	91.7	91.1	92.4	91.4	89.3	100						
Bacillus laterosporus ATCC 64	85.5	90.1	90.2	90.2	89.9	90.2	90.6	90.8	90.7	89.6	88.1	91.4	100					
Bacillus amylolyticus NCIB 8144	86.5	89.4	90.8	89.5	90.2	89.8	90.6	90.1	91.1	90.1	88.5	90.8	90.6	100				
Bacillus polymyxa ATCC 842	86.3	88.8	90.3	88.8	89.3	89.3	89.7	88.8	90.3	89.0	89.1	90.2	89.8	94.4	100			
Bacillus smithii DSM 4216	87.0	93.9	95.9	94.4	95.4	94.2	95.4	95.3	96.6	95.1	92.6	92.4	90.9	91.6	90.3	100		
Sporosarcina urea ATCC 614	86.0	93.4	94.7	92.7	94.1	92.7	94.0	93.6	95.4	93.4	91.5	91.5	89.7	89.4	89.6	94.9	100	
Bacillus globisporus DSM 4	86.7	92.9	94.3	92.3	93.4	92.5	93.5	93.3	95.1	93.3	91.6	91.8	90.1	89.5	90.4	95.2	97.6	100

[a] Values were determined by using Olsen's modification of the method of Jukes and Cantor (see Materials and Methods). Most of the sequences used in this analysis were obtained from the Ribosomal Data Project, version 3.0 (22). The *Sporosarcina urea* and *Saccharococcus thermophilus* sequences were obtained from the EMBL. A total of 1,128 unambiguous nucleotides were used in the analysis.

An analysis of the 16S rRNA gene of strain DKP^T revealed that this organism is closely related phylogenetically to members of the genus *Bacillus* (Fig. 3) as defined by Ash et al. (3); the position of this isolate is equidistantly from the position of members of groups 1, 2, and 5. However, a detailed analysis of the signature nucleotides found in members of these three groups did not reveal the precise position of isolate DKP^T in the cluster containing members of groups 1, 2, and 5, indicating that strain DKP^T is not closely affiliated with any of these three groups. Phenotypically, members of the genus *Bacillus* and strain DKP^T are similar since *Bacillus* species may be facultative anaerobes and usually produce catalase. *Bacillus* species use oxygen as the final electron acceptor; oxygen can be replaced by alternative electron acceptors in some species (8). However, strain DKP^T differs from the previously described *Bacillus* species since it is non-spore forming and does not contain heme proteins such as cytochromes. The possibility that strain DKP^T produces specific spores which do not exhibit all of the characteristics of *Clostridium* spores, as described in *Thermoanaerobacter finnii* (39), was ruled out since strain DKP^T did not exhibit any heat resistance at 80°C.

According to Stackebrandt and Woese (44), the genera *Bacillus*, *Lactobacillus*, and *Streptococcus* and a few other genera seem to form a unit, microaerophilic to aerobic in phenotype, which is relatively coherent phylogenetically and is a subbranch of the clostridia. Since the primitive atmosphere of the earth was anaerobic, it has been suggested that among the genera *Clostridium*, *Bacillus*, and *Lactobacillus*, the genus *Clostridium* is the most ancient. This hypothesis is consistent with the fact that almost all species belonging to this genus are strictly anaerobic and is supported by the results of 16S rRNA studies (44). Accordingly, it has been hypothesized that the genus *Bacillus* is the most recent genus, because almost all *Bacillus* species are facultatively anaerobic. The genus *Lactobacillus* probably appeared between the genera *Clostridium* and *Bacillus*. Lactobacilli and, more generally, lactic bacteria possess metabolism which is intermediate between anaerobiosis and aerobiosis. We postulate that strain DKP^T, because of its phylogenetic and phenotypic characteristics, could be a primitive member of the genus *Bacillus*.

We propose that strain DKP^T should be placed in the genus *Bacillus* as a new species, *Bacillus thermoamylovorans*.

Description of *Bacillus thermoamylovorans* sp. nov. *Bacillus thermoamylovorans* (ther.mo.a.my.lo.vo'rans. Gr. adj. *thermos*, hot; Gr. n. *amylum*, starch; L. v. *vorare*, to devour; M. L. adj. *thermoamylovorans*, utilizing starch at high temperatures). Gram-positive, straight, rod-shaped eubacteria; cells are 0.45 to 0.5 μm wide and 3 to 4 μm long, and occur singly or in short chains. Slightly motile by means of peritrichous flagella.

Spores are not detected under a range of conditions; cells are killed by heating at 80°C for 5 min.

Facultative anaerobe. Catalase positive. No cytochrome is produced. NO_3^- and SO_4^{2-} are not reduced. Indole and H_2S are not produced.

Heterolactic fermentation of hexoses occurs; the end products are lactate, acetate, ethanol, and formate but not H_2.

Chemoorganotrophic. Biotrypcase or yeast extract is required for luxuriant growth. Vitamins and nucleic acid derivatives stimulate growth but are not essential.

Moderately thermophilic. The optimum temperature for growth is about 50°C; the upper temperature limit for growth is 58°C.

Neutrophilic. The optimal pH for growth ranges from 6.5 to 7.5; the pH range for growth is 5.4 to 8.5.

The G+C content of the DNA is 38.8 ± 0.2 mol%.

The results of a 16S rRNA sequence analysis indicate the position of this organism is equidistant from the positions of members of groups 1, 2, and 5 of the genus *Bacillus*.

Isolated from palm wine, an African alcoholic beverage, in Rufisque (near Dakar), Senegal.

The type strain is DKP (= Collection of Institut Pasteur CNCM I-1378).

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