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Bacillus methanolicus sp. nov., a New Species of Thermotolerant, Methanol-Utilizing, Endospore-Forming Bacteria

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The generic position of 14 strains of gram-positive bacteria able to use methanol as a growth substrate was determined. All are obligately aerobic, thermotolerant organisms that are able to grow at temperatures of 35 to 60°C. Nine of the strains produce oval spores at a subterminal-to-central position in slightly swollen rod-shaped cells. DNA-DNA hybridization studies, 5S rRNA sequence analysis, and physiological characteristics revealed that all 14 strains cluster as a well-defined group and form a distinct new genospecies. Analysis of the 16S and 5S rRNA sequences indicated that this new species is distinct from *Bacillus brevis* but closely related to *B. firmus* and *B. azotoformans*. The name proposed for this new species is *B. methanolicus*. The type strain, PB1, has been deposited in the National Collection of Industrial and Marine Bacteria as NCIMB 13113.

Bacteria able to grow on methanol at elevated temperatures are of technological interest for single-cell protein production, solvent degradation in aerobic thermophilic biotreatment processes, and fermentative production of amino acids (1, 25, 42). Following an earlier report on growth of a mixed culture with spore-forming bacteria on methanol (45), a claim appeared in the patent literature (31) concerning *Bacillus* strains that grow on methanol with optimum growth temperatures of ca. 55°C and maximum growth temperatures of ca. 65°C. In recent years, several other groups have successfully employed continuous culture techniques for isolation of pure cultures of *Bacillus* strains that grow rapidly on methanol at 55°C (5, 15, 24, 29, 42). Metabolism of methanol, ethanol, and glucose (7, 9, 23, 47) and environmental control of metabolic fluxes (2–4, 14, 15, 30) have been studied in detail in these organisms, grown in batch and continuous cultures under a variety of nutrient limitations and steady-state as well as transient-state conditions. The methylotrophic *Bacillus* strains display a strong resistance to high methanol concentrations, and the molar growth yields on methanol at the optimum growth temperatures in methanol-limited chemostats are among the highest reported for methylotrophic bacteria (6, 24). Enzyme analysis revealed that all isolates employ a novel NAD-dependent methanol dehydrogenase for methanol oxidation and the ribulose monophosphate pathway for formaldehyde assimilation (7–9).

On the basis of a number of phenotypic tests, Al-Awadhi et al. (5) concluded that seven of their isolates were *Bacillus brevis* strains. A further isolate was a sheathed, filamentous, gram-positive, endospore-forming, obligately aerobic bacterium that could not be allocated to any previously described genus. In the present investigation, 14 organisms, including most of Al-Awadhi's isolates, were further characterized, and a classification of these strains based on various properties, including DNA-DNA hybridization, is proposed.

MATERIALS AND METHODS

Test strains and cultivation conditions. The thermotolerant methanol-utilizing *Bacillus* strains PB1 (NCIMB 13113), C1 (NCIMB 13114), AR2, TS1, TS2, and TS4, isolated by Dijkhuizen et al. (24); 4(55) (NCIMB 12523), S1 (NCIMB 12524), WM5.2 (NCIMB 12525), TFB (NCIMB 12526), WM5.1 (NCIMB 12527), S2 (NCIMB 12528), and KA (NCIMB 12529), isolated by Al-Awadhi et al. (5); and 40M, isolated by Govorukhina and Trotsenko (29), were the subject of chemotaxonomic, molecular systematic, and phenotypic tests. The mesophilic trimethylamine-utilizing *Bacillus* strain S2A1 (19) was included in the 5S rRNA sequence analysis. The nonmethylotrophs *B. firmus* DSM 12 and *B. brevis* DSM 30 were used as reference strains in some of the physiological tests. All strains were stored without supplements as frozen stocks at −80°C and routinely grown in Tryptone soya broth (TSB; Oxoid CM131; 30 g/liter) adjusted to pH 7.5. For plates, TSB was solidified with 1.5% (wt/vol) agar (TSBA). Growth of the methylotrophic *Bacillus* strains in batch cultures and carbon-, oxygen- or nitrogen-limited continuous cultures has been described previously (7, 9, 14). DNA, 16S rRNA, 5S rRNA, cell wall fractions, fatty acids, and lipids were isolated from cells grown in batch cultures in trimethylamine (25 mM; strain S2A1 only) or methanol (100 mM; all other strains) mineral medium supplied with vitamins (24).

Morphology. Colony morphology was examined in isolated colonies grown on TSBA for 2 days at 55°C. Cellular

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   1 UUUUUGGAGA GUUUGAUCCU GGCUCAGGAC GAACGCUGGC GGCGUGCCUA AUACAUgcaa
  61 gucgaacgGA CUGAUGGGAG CUUGCUCCCU GAAGUCAGCG GCGGACGGGU GAGUAACACG
 121 UGGGCAACCU GCCUGUAAGA CCGGGAUAAC UUCGGGAAAC CGGAGCUAAU ACCGGAUAAU
 181 CCUCUUUCCC GCAUGGGAAA GAGCUGAAAG AUGGCUUCGG CUAUCACUUA CAGAUGGGCC
 241 CGCGGCGCAU UAGCUAGUUG GUGAGGUAAC GGCUCACCAA GGCGACGAUG CGUAGCCGAC
 301 CUGAGAGGGU GAUCGGCCAC ACUGGGACUG AGACACGGCC CAGACUCCUA CGGGAGGCAG
 361 CAGUAGGGAA UCUUCCGCAA UGGACGAAAG UCUGACGGAG CAACGCCGCG UGAGCGAAGA
 421 AGGCCUUCGG GUCGUAAAGC UCUGUUGUCA GGGAAGAACA AGUACCGUUC GAAUAGGGCC
 481 GUACUUUGAC GGUACCUAAC CAGAAAGCCA CGGCUAACUA CGUGCCAGCA GCCGCGGUAA
 541 UACGUAGGUG GCAAGCGUUG UCCGGAAUUA UUGGGCGUAA AGCGCGCGCA GGCGGUUCCU
 601 UAAGUCUGAU GUGAAAGCCC ACGGCUCAAC CGUGGAGGGU CAUUGGAAAC UGGGGAACUU
 661 GAGUGCAGAA GAGGAGAGCG GAAUUCCACG UGUAGCGGUG AAAUGCGUAG AGAUGUGGAG
 721 GAACACCAGU GGCGAAGGCG GCUCUCUGGU CUGUAACUGA CGCUGAGGCG CGAAAGCGUG
 781 GGGAGCAAAC AGGAUUAGAU ACCCUGGUAG UCCACGCCGU AAACGAUGAG UGCUAAGUGU
 841 UAGAGGGUUU CCGCCCUUUA GUGCUGCAGC UAACGCAUUA AGCACUCCGC CUGGGGAGUA
 901 CGGCCGCAAG GCUGAAACUC AAAGGAAUUG ACGGGGGCCC GCACAAGCGG UGGAGCAUGU
 961 GGUUUAAUUC GAAGCAACGC GAAGAACCUU ACCAGGUCUU GACAUCCUCU GACAAUCCUG
1021 GAGACAGGAC GUUCUUCGGG GGACAGAGUG ACAGGUGGUG CAUGGUUGUC GUCAGCUCGU
1081 GUCGUGAGAU GUUGGGUUAA GUCCCGCAAC GAGCGCAACC CUUGACCUUA GUUGCCAGCA
1141 UUCAGUUGGG CACUCUAAGG UGACUGCCGG UGACAAACCG GAGGAAGGUG GGGAUGACGU
1201 CAAAUCAUCA UGCCCCUUAU GACCUGGGCU ACACACGUGC UACAAUGGAU GGUACAAAGG
1261 GCUGCGAGAC CGCGAGGUUG AGCCAAUCCC AAAAAACCAU UCUCAGUUCG GAUUGCAGGC
1321 UGCAACUCGC CUGCAUGAAG CCGGAAUCGC UAGUAAUCGC GGAUCAGCAU GCCGCGGUGA
1381 AUACGUUCCC GGGCCUUGUA CACACCGCCC GUCACACCAC GAGAGUUUGU AACACCCGAA
1441 GUCGGUGAGG UAACCGUAAG GAGCCAGCCG CCUAAGGUGG GACAGAUGAU UGGGGUGAAG
1501 UCGUAACAAG GUAGCCGUAU CGGAAGGUGC GGCUGGAUCA CCUCCUUUCU
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FIG. 1. 16S rRNA sequence of *B. methanolicus* sp. strain C1. Lowercase letters indicate nucleotides whose assignment is uncertain. Underlined boldface regions are target sites of sequencing or amplification primers.

morphology was examined in gram-stained smears of these cultures. Cell dimensions were measured by a calibrated eyepiece graticule. Cell diameters were recorded as being either less than 1 μm (code = 0) or greater than 1 μm (code = 2). Cell lengths were recorded as either less than 1 μm (code = 0), between 4 and 6 μm (code = 1), or greater than 6 μm (code = 2). Spore morphology was examined in cultures grown on mannitol mineral medium plates (1% [wt/vol] mannitol, 1.5% [wt/vol] agar) for 5 days. The mineral medium used was that described by Dijkhuizen et al. (24).

Physiological tests. Tests with the thermotolerant methylotrophic *Bacillus* strains were performed at 55°C. Tests with reference strains *B. firmus* and *B. brevis* were performed at 37°C. All tests were inoculated with overnight peptone water broth cultures derived from overnight plate cultures. Acid production from sugars and sugar alcohols and hippurate hydrolysis were detected by using the media and methods of Gordon et al. (28). Degradation of casein (1% [wt/vol], skim milk) was determined in TSBA; clearing of the medium surrounding the growth was scored as positive. Starch (1% [wt/vol], soluble) hydrolysis was detected in the same basal medium by flooding the plates with an iodine solution (28). Nitrate and nitrite reduction, growth in 0.02% NaN_3, oxidase reaction, and the presence of amylase were examined by the methods of Cowan (20). Tolerance to saline was determined by inoculating bottles containing 10 ml of TSB supplemented with 2 or 5% (wt/vol) NaCl. Tolerance to acid pH was tested by adjusting TSB to pH 6.0 or 6.5. The abilities of strains to use mannitol, cellobiose, pyruvate, acetate, succinate, methanol, ethanol, and isopropanol were determined by using mineral medium supplied with a vitamin mixture (24). The substrates tested were added to a final concentration of 1% (wt/vol), and the pH was adjusted to 7.5. All physiological tests were performed in triplicate, and

TABLE 1. 16S rRNA sequence similarity values for *B. methanolicus* C1 and other bacilli[a]

Organism	% Similarity to:															
	B. methanolicus	*B. azotoformans*	*B. firmus*[1]	*B. lentus*	*B. coagulans*[2]	*B. megaterium*[3]	*B. subtilis*[4]	*B. cereus*[5]	*B. globisporus*[6]	*B. stearothermophilus*[7]	*B. aneurinolyticus*	*B. laterosporus*	*B. brevis*	*B. polymyxa*[8]	*B. cycloheptanicus*	*E. coli*
B. methanolicus		96.3	96.1	95.7	95.4	95.4	95.4	94.1	92.8	93.7	91.5	90.1	90.2	89.0	85.9	78.8
B. azotoformans	96.1		96.1	95.7	93.9	95.4	94.8	94.3	93.1	92.5	92.5	90.8	90.9	89.6	85.6	79.6
B. firmus[1]	95.9	96.0		96.3	94.9	95.8	94.9	94.5	93.5	92.0	91.8	89.9	88.9	90.2	85.4	78.5
B. lentus	95.5	95.4	96.2		95.3	95.5	94.3	94.7	93.4	92.3	91.7	89.3	88.7	89.5	84.9	78.8
B. coagulans[2]	95.1	93.5	94.7	95.0		94.0	94.2	93.8	92.3	92.2	91.4	89.3	89.4	89.2	85.0	78.3
B. megaterium[3]	95.1	95.1	95.5	95.0	93.4		94.2	94.7	93.0	91.6	91.1	90.3	89.0	89.4	85.2	79.0
B. subtilis[4]	94.9	94.8	94.5	93.9	93.7	93.8		94.3	91.8	92.0	90.9	89.1	88.8	89.1	85.0	78.0
B. cereus[5]	93.7	94.1	93.8	94.5	93.3	94.3	94.0		92.1	91.9	90.4	89.8	88.3	88.9	85.0	78.3
B. globisporus[6]	92.5	92.8	93.1	93.1	92.0	92.6	91.3	91.8		91.3	90.8	88.3	88.1	88.7	84.5	78.8
B. stearothermophilus[7]	93.3	92.2	91.7	91.8	91.8	91.3	91.6	91.3	89.9		90.6	88.1	88.4	88.1	85.8	78.5
B. aneurinolyticus	91.1	92.3	91.5	91.3	90.9	90.7	90.5	90.0	89.3	90.2		90.0	89.8	89.2	85.2	78.5
B. laterosporus	90.1	90.6	89.8	89.1	89.1	90.1	89.0	89.5	87.8	87.9	89.6		94.1	88.5	83.6	78.1
B. brevis	90.1	90.6	88.5	88.3	89.1	88.8	88.6	88.2	87.8	88.3	89.6	94.0		87.2	82.7	77.8
B. polymyxa[8]	88.9	89.2	90.0	89.3	89.0	88.9	88.6	88.2	88.3	87.7	88.9	88.4	87.1		85.6	77.8
B. cycloheptanicus	85.9	85.4	84.9	84.4	84.5	84.8	84.7	84.6	84.1	85.8	84.9	83.6	83.2	85.4		77.1
E. coli	78.3	79.5	78.4	78.7	78.2	78.9	77.9	78.1	78.8	78.1	78.3	78.3	77.4	77.4	76.6	

[a] Mean values are given for the groups of related bacilli defined in Fig. 2. The values on the lower left are overall similarity values; the values on the upper right are based on a set of data reduced to those positions which are invariant in at least 40% of the entire set of sequences.

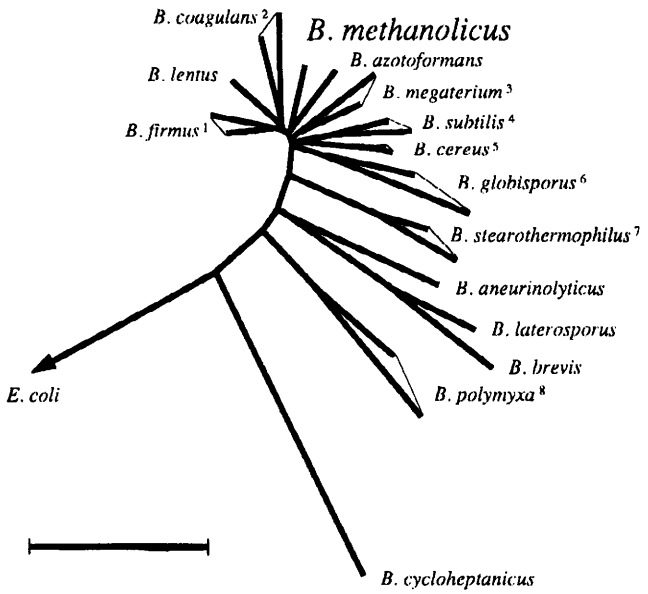

FIG. 2. Distance matrix tree showing the relationships between methanol-utilizing *B. methanolicus* C1 and other bacilli. The phylogenetic distances were calculated by including those alignment positions which are invariant in at least 40% of the entire set of 16S rRNA sequences. Groups of more closely related bacilli whose relationships are supported by distance and parsimony analyses are indicated by triangles. These groups are as follows: 1, *B. firmus*, *B. benzoevorans*, and *B. circulans*; 2, *B. coagulans*, *B. acidoterrestris*, *B. badius*, and *B. smithii*; 3, *B. megaterium*, *B. fastidiosus*, *B. maroccanus*, *B. psychosaccharolyticus*, and *B. simplex*; 4, *B. subtilis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. lautus*, *B. lentimorbus*, *B. licheniformis*, *B. popillae*, and *B. pumilus*; 5, *B. cereus*, *B. anthracis*, *B. medusa*, *B. mycoides*, and *B. thuringiensis*; 6, *B. globisporus*, *B. fusiformis*, *B. insolitus*, *B. pasteurii*, *B. psychrophilus*, and *B. sphaericus*; 7, *B. stearothermophilus*, *B. kaustophilus*, and *B. thermoglucosidasius*; 8, *B. polymyxa*, *B. amylolyticus*, *B. alvei*, *B. azotofixans*, *B. gordonae*, *B. larvae*, *B. macquariensis*, *B. macerans*, *B. pabuli*, and *B. pulvifaciens*. Bar, 0.05 K_{nuc}.

results were recorded after 1, 3, and 5 days. Poly-β-hydroxybutyric acid accumulation was determined as described by Braunegg et al. (12) and Suzuki et al. (46).

Antibiotic sensitivity. Cultures were incubated overnight in peptone water broth. Disposable bioassay plates were prepared with 200 ml of Oxoid Sensitest agar (CM409) with 3% (wt/vol) agar. The plates were seeded with a 3-ml culture volume and allowed to dry. Oxoid antibiotic sensitivity discs were then placed on these plates, which were incubated overnight. The zone width from the edge of the disc to the edge of the bacterial growth was then measured.

Computer analysis. Physiological and morphological data were analyzed by using the simple matching, Jaccard, and pattern difference coefficients (44). Clustering was achieved by using the unweighted pair group method with arithmetic averages algorithm (44).

Determination of G+C contents of DNAs and DNA homology studies. Purification of DNA was performed as described by Meyer and Schleifer (37). The melting point of the purified DNA was determined by using the method of Marmur and Doty (36), and the G+C content was calculated by using the method of De Ley et al. (22). DNA from *Escherichia coli* B (Sigma Chemical Co., St. Louis, Mo.), with a G+C content of 51.7 mol%, was used as the reference. DNA-DNA hybridization studies were performed by using the DNA filter method described by Kilpper et al. and Kilpper-Bälz et al. (33, 34).

16S rRNA analysis. Determination of the 16S rRNA primary structure was done by direct sequencing of the RNA and sequencing of cloned in vitro-amplified rDNA. RNA was isolated from strain C1 as described by Embley et al. (26). Sequencing of 16S rRNA was performed by using reverse transcriptase as described by Lane et al. (35). Sequence ambiguities were resolved by using terminal transferase (21). A DNA fragment containing the greater part of a 16S rRNA gene (homologous to positions 54 to 1542 of *E. coli* 16S rRNA), the intrageneric spacer, and a small 5′-terminal part of a 23S rRNA gene (homologous to positions 1 to 130 of *E. coli* 23S rRNA) was amplified in vitro by applying the polymerase chain reaction technique (41) in combination with site-specific primers (5′-CATGCAAGTCGARCG-3′ [16S rRNA specific] and 5′-GGGTTYCCCCATTCGG-3′ [23S rRNA specific]). The amplified rDNA fragment was cloned as pC1-611-118 in the vector pBluescript (Stratagene, La Jolla, Calif.). DNA sequencing was done as described by Chen and Seeburg (17). Oligonucleotide primers were synthesized by standard methods by using a Biosearch Cyclone DNA synthesizer. The sequences of these primers are complementary to highly conserved regions of 16S rRNA. The new sequence was added to an alignment of about 500 almost complete 16S rRNA primary structures of (eu)bacteria. Alignment of sequences was performed with respect to conserved primary structures, as well as secondary structures. Phylogenetic distance values (K_{nuc}; 32) were calculated, including those positions which had been determined for both of the particular sequence pairs. Phylogenetic trees were reconstructed by applying the distance, parsimony, and bootstrapped parsimony methods by using the programs NEIGHBOR, FITCH, DNAPARS, and DNABOOT as implemented in Felsenstein's PHYLIP program package (27).

5S rRNA analysis. Isolation, sequencing, and phylogenetic analysis of 5S ribosomal RNA were performed by published procedures (16, 18).

```
Bacillus
strain

PB1                  : CCUGGUGGCG*AUGGCGAGAAGGUCACACCCGUUCCCAUACCGAACACGGAAGUUAAGCG
C1                   : ----------*----------------------------------------------------
AR2                  : ----------*----------------------------------------------------
TS1                  : YY--------*----------------------------------------G-----------
TS4                  : YY--------*----------------------------------------G-----------
S1                   : ----------*----------------------------------------------------
S2                   : YY--------*----------------------------G-----------G-----------
WM5.1                : YY--------*-------------------------------R--------------------
4(55)                : ----------*-----------A----------------------------------------
KA                   : YY--------*U----------------------------G--------------------
40M                  : YY--------*----------------------------G----------------------
S2A1                 : YYY-----AUU*A-----AG----U-------------C--------A-CC-AG--A--
B. firmus            : U-Y-------*--A-----------------------------------------------
B. megaterium        : U--R------*--A----AG-----------------------------------------
B. subtilus          : U------AU-*-------AG----------------G-----------------------
B. stearothermophilus : ---A----U-*--A---GAGG--AA-------------C-------------------C
B. brevis            : U------AU-*------GAGG--A-------------------------CC-------C

PB1                  : UCUCAGCGCCGAUGGUAGUUGGGGCAUU*GCCCCUGCGAGAGUAGGACGCUGCCAGGC
C1                   : ----------------------------*-----------------------U--------
AR2                  : ----------------------------*----------------------------------
TS1                  : ----------------------------*-----------Y----------------------
TS4                  : ----------------------------*-------------------------U--------
S1                   : ----------------------------*----------------------------------
S2                   : ----------------------------*----------------------------------
WM5.1                : ----------------------------*----------------------------------
4(55)                : ----------------------------*----------------------------------
KA                   : ----------------------------*----------------------------------
40M                  : ----------------------------*-----------------------U----------
S2A1                 : CU--------------------GUC-*C------U-----C-------C--UC--G
B. firmus            : ----------R----------GC-G*U------U-----------------R---
B. megaterium        : CU-U------R----------A-U--*-U------K-----------U----Y-----
B. subtilus          : CU----------------C----GU--*C------U-----------AUC-------
B. stearothermophilus : CUC-----------------CAGC--------A--------U------U----
B. brevis            : CUC-------A------C---CUC-GCAG-GAG-C-G-----------UC-------
```

FIG. 3. Alignment of 5S rRNA sequences of methylotrophic bacteria (determined in this study) and *B. firmus* ATCC 14575, *B. megaterium* KM, *B. subtilis* 168, *B. stearothermophilus* 799, and *B. brevis* ATCC 8185 (49). Only nucleotides that differ from the *Bacillus* strain PB1 sequence are shown. Asterisks indicate alignment gaps. Y, C or U; R, A or G; K, G or U.

TABLE 2. Physiological characteristics of thermotolerant methylotrophic *Bacillus* strains,[a] *B. firmus*, and *B. brevis*[b]

Test	Result obtained with *Bacillus* sp. strain:												*B. firmus* result	*B. brevis* result
	C1	PB1	AR2	TS1	TS2	TS4	S1	S2	TFB	WM5.1	WM5.2	KA		
Fermentation of:														
Fructose	0	0	2	0	2	2	0	0	0	0	0	0	—	—
Glycerol	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Glycogen	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Inulin	0	0	0	0	0	0	0	0	0	0	0	0	2	2
Lactose	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Maltose	2	2	2	0	2	2	2	2	0	2	2	2	—	—
Mannitol	2	2	2	2	2	2	2	2	2	2	2	2	0	1
Raffinose	2	2	2	0	0	0	0	2	—	2	2	2	0	0
Ribose	2	2	0	2	0	0	0	2	0	2	2	0	—	—
Salicin	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Sorbitol	0	2	0	0	0	0	2	—	2	0	2	—	0	2
Starch	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Sucrose	0	0	0	0	0	0	0	0	0	0	0	0	2	2
Trehalose	2	0	2	0	2	2	0	0	2	0	0	0	0	0
Growth on:														
Methanol	2	2	2	2	2	2	2	2	2	2	2	2	0	0
Acetate	—	0	0	2	0	0	—	0	0	0	0	0	—	—
Ethanol	2	0	2	2	2	2	0	—	0	0	—	0	—	—
Pyruvate	2	0	2	—	2	2	0	2	1	0	0	0	—	—
Isopropanol	0	0	1	0	2	0	0	0	0	0	0	—	—	—
Growth in:														
2% NaCl	2	0	2	2	2	2	0	2	0	2	2	2	—	—
0.02% NaN_3	0	0	1	2	2	1	0	0	2	2	0	1	2	0
Growth at pH 6.0	1	0	0	0	0	0	0	0	0	0	0	0	2	0
Hydrolysis of:														
Casein	0	0	0	0	0	0	0	0	0	0	0	0	1	2
Starch	1	0	1	0	1	1	2	1	2	0	0	0	2	2
Colony morphology														
Transparent	2	0	2	2	2	2	2	0	2	0	0	0	—	—
Rough	2	2	2	2	2	2	2	0	2	2	2	0	—	—
Raised[c]	0	2	0	0	0	0	0	0	0	2	2	0	—	—
Crenated[d]	2	2	2	2	2	2	2	0	2	2	2	0	—	—
Cellular morphology														
Diameter	0	2	0	0	0	0	2	2	2	—	2	0	2	2
Length	1	1	2	1	2	1	1	1	0	—	1	2	0	2
Filaments	0	2	2	0	2	2	0	0	0	—	0	2	0	2
Chains[e]	0	2	0	0	0	0	0	0	0	—	2	2	0	0

[a] All strains produced acid from glucose but not from *i*-erythritol, inositol, xylose, galactose, or adonitol. All strains grew at pH 6.5 and displayed oxidase activity. All methanol-utilizing bacilli showed catalase activity. None of the strains hydrolyzed hippurate or were able to reduce nitrate or nitrite. No growth was observed in the presence of 5% NaCl. The methylotrophic bacilli grew on mannitol but failed to grow on succinate or cellobiose (*B. brevis* and *B. firmus* were not tested). All colonies contained nonmotile, rod-shaped cells that stained positive in the Gram test. The methylotrophic bacilli grew at temperatures between 35 and 60°C.

[b] Scores: 2, positive; 1, weakly positive; 0, negative; —, test not done or results unsatisfactory. Scores for cell lengths are described in Materials and Methods.

[c] Score 0, colony is flat.

[d] Score 0, colony is convex.

[e] Score 0, single cells.

Other analytical methods. Preparation of cell wall fractions and determination of the diamino acid were carried out as described by Schleifer and Kandler (43). Fatty acid, lipid, and hopanoid extraction and analysis were carried out as described by Bringer et al. (13).

Nucleotide sequence accession numbers. The 16S rRNA sequence has been deposited in the EMBL data library under accession number X64465. The 5S rRNA sequences have been deposited in the EMBL data library under accession numbers Z11816 to Z11827.

RESULTS AND DISCUSSION

16S rRNA sequence. The 16S rRNA primary structure of strain C1 is shown in Fig. 1. The sequence was compared with all published homologous sequences of bacilli (10, 11,

TABLE 3. Antibiotic sensitivity of thermotolerant methylotropic *Bacillus* strains, *B. firmus*, and *B. brevis*

Antibiotic[a]	Sensitivity[b] of strain:												*B. firmus* sensitivity	*B. brevis* sensitivity
	C1	PB1	AR2	TS1	TS2	TS4	S1	S2	TFB	WM5.1	WM5.2	KA		
S3 500	2	2	0	0	0	0	1	2	2	2	2	2	0	0
OB5	2	1	1	1	1	1	1	1	1	2	2	2	1	1
E10	—	2	1	2	2	2	1	2	2	2	2	2	1	1
VA5	1	2	2	—	1	1	1	1	1	—	1	1	1	1
NV5	1	1	1	1	—	1	—	1	1	2	1	1	1	1
N10	2	1	1	1	1	—	1	1	1	2	1	1	1	1
PB300	1	2	1	1	1	1	1	1	1	2	2	1	1	1
FD10	2	2	1	—	1	1	2	1	1	2	1	1	1	1
AMP10	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C30	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CN10	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K5	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S10	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TE10	1	1	1	1	1	1	1	1	1	1	1	1	1	1
W5	1	1	—	0	—	1	1	1	1	1	1	1	1	1

[a] S3 500, compound sulfonamide (500 μg); OB5, cloxacillin (5 μg); E10, erythromycin (10 μg); VA5, vancomycin (5 μg); NV5, novobiocin (5 μg); N10, neomycin (10 μg); PB300, polymyxin B (300 μg); FD10, fusidic acid (10 μg); AMP10, ampicillin (10 μg); C30, chloramphenicol (30 μg); CN10, gentamicin (10 μg); K5, kanamycin (5 μg); S10, streptomycin (10 μg); TE10, tetracycline (10 μg); W5, trimethoprim (5 μg).

[b] 2, highly sensitive; 1, sensitive; 0, not sensitive; —, test not done or unsatisfactory.

40, 48). A matrix of sequence similarity values is shown in Table 1. Mean values are given for groups of more closely related bacilli. The phylogenetic tree shown in Fig. 2 is based on a matrix of phylogenetic distances which had been calculated by including those alignment positions which are invariant in at least 40% of the entire set of sequences. The combined 16S rRNA data show that *Bacillus* strain C1 is more closely related to *B. azotoformans* and *B. firmus* than to *B. brevis*, as originally thought (5).

DNA-DNA hybridization studies. DNA-DNA hybridization studies showed that the thermotolerant methanol-utilizing *Bacillus* strains are closely related and form a distinct taxon. The DNA similarity values obtained at optimal hybridization conditions (25°C below the T_m of the homologous hybridization) were 60% and higher.

5S rRNA sequences. 5S rRNA sequences of the methylotrophic bacteria determined in this investigation are shown in Fig. 3. These sequences were compared with published data on nonmethylotrophic mesophilic and thermophilic *Bacillus* species (49). The 5S rRNA data confirm that the thermotolerant methanol-utilizing *Bacillus* strains cluster as a well-defined group, separate from mesophilic trimethylamine-utilizing *Bacillus* strain S2A1 and nonmethylotrophic thermophilic organisms such as *B. stearothermophilus*. The 5S rRNA data also provide further evidence that the thermotolerant methylotrophic bacilli are closely related to *B. firmus* and *B. megaterium* but not to *B. brevis*.

Morphology and physiological characteristics. All of the methanol-utilizing strains studied were obligately aerobic and able to grow in various media at temperatures of 35 to 60°C. Their physiological characteristics are listed in Tables 2 and 3. All strains formed circular colonies on TSBA after 2 days of incubation, in most cases with rough surfaces and crenated, undulating edges. Colonies of strains S2 and KA were different with respect to most of the properties tested (Table 2) (5). All colonies contained nonmotile, rod-shaped cells that stained gram positive. Strain KA forms filamentous cells during all growth stages. Some of the other isolates also formed filamentous cells but only towards the end of growth, e.g., in the centers of TSBA colonies. In methanol-limited continuous cultures of strains C1 and AR2, most of the cells were present as short chains of rod-shaped cells. Reduced growth rates caused formation of strongly helical filaments (data not shown) in both of these cultures. Similar cellular structures were detected in colonies of all of the methylotrophic isolates grown on mannitol mineral agar for 2 days. Under these conditions, however, they constituted only a minority of the population. With the exception of strains C1, TS1, TS2, WM5.2, and TFB, cells of all of the strains sporulated on TSBA and/or on mannitol mineral agar. Sporulating cells were swollen and possessed oval spores at a subterminal-to-central position. The G+C content of the DNAs of strains C1, PB1, AR2, TS1, TS4, S2, 4(55), WM5.1, and KA was determined as 48 to 50%. The above data indicate that all endospore-forming isolates may be assigned to the genus *Bacillus* (39).

Previously, we reported (24) that isolation of these methanol-utilizing strains in pure cultures was difficult when methanol agar plates were used. This may be due to accumulation of toxic formaldehyde from methanol. With various alternative techniques, six methanol-utilizing strains were isolated (strains PB1, C1, AR2, TS1, TS2, and TS4). All six initially produced subterminal oval endospores, but some strains lost this ability upon subcultivation, resulting in isolation of endospore-deficient mutants. All of these methylotrophic *Bacillus* strains contained *meso*-diaminopimelic acid as a cell wall diamino acid. This murein type is also present in the cell walls of *B. subtilis*, *B. pumilus*, *B. megaterium*, *B. cereus*, *B. firmus*, *B. fastidiosus*, and *B. brevis* (43). Lipid analysis indicated the presence of squalene and phosphatidylethanolamine in strain C1. Hopanoids (38) were not detected. The fatty acid profile of strain C1 consists mainly of 13-methyltetradecanoic acid (*iso*-C_{15}; 27% of total fatty acid composition), 12-methyltetradecanoic acid (*anteiso*-C_{15}; 16%), 14-methylpentadecanoic acid (*iso*-C_{16}; 12%), 13-methylpentadecanoic acid (*anteiso*-C_{16}; 13%), 15-methylhexadecanoic acid (*iso*-C_{17}; 4%), and 14-methylhexadecanoic acid (*anteiso*-C_{17}; 14%). Cells of strains M40 and C1 grown on methanol in nitrogen-limited continuous cultures contained a storage polymer that was identified as poly-β-hydroxybutyric acid. Sudan black staining of cells of strains PB1 and AR2 grown on methanol in oxygen-limited

continuous cultures revealed the presence of intracellular lipophilic material, most likely poly-β-hydroxybutyric acid. These findings suggest that the ability to synthesize poly-β-hydroxybutyric acid is a feature common to the entire group of methylotrophic *Bacillus* strains.

Determination of the average linkage (simple matching coefficient/unweighted pair group clustering) of the methanol-utilizing *Bacillus* strains on the basis of 68 characteristics (Table 2 and 3) revealed that all of the isolates are linked at >80% similarity, which indicates that the strains are closely related.

The thermotolerant methylotrophic test strains can readily be separated from mesophilic *B. firmus* on the basis of growth temperature; growth at pH 6.0; methanol utilization; acid production from glycerol, inulin, mannitol, and sucrose; and hydrolysis of casein (Table 2). *B. brevis* differs with respect to methanol utilization; acid production from inulin, salicin, and sucrose; and hydrolysis of casein (Table 2).

Molecular systematic, chemotaxonomic, and phenotypic data confirm that all of the 14 strains examined in this study are closely related and belong to the genus *Bacillus*. Until the internal heterogeneity of this group of organisms is studied in more detail, we propose that all 14 isolates be considered as belonging to a new species, for which we propose the name *B. methanolicus*.

Description of *B. methanolicus* Arfman, Dijkhuizen, Kirchhof, Ludwig, Schleifer, Bulygina, Chumakov, Govorukhina, Trotsenko, White, and Sharp sp. nov. (me.tha'n-oli.cus. M. L. n. *methanolicum*, methanol; M. L. masc. adj. *methanolicus*, relating to methanol). Cells are rod shaped and stain gram positive. Older cultures may contain filamentous cells. No motility has been observed. Sporulating cells are swollen and possess oval spores at a subterminal-to-central position, although the ability to sporulate has been lost in some cultures. Growth is obligately aerobic and occurs at temperatures between 35 and 60°C, with optimum growth at around 55°C. The G+C content of the DNA is 48 to 50 mol%. Phenotypic characteristics are shown in Tables 2 and 3. Organisms can be isolated from soil samples, aerobic (thermophilic) waste water treatment systems, and volcanic hot springs. Type strain: *B. methanolicus* PB1 NCIMB 13113.

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