Bacillus azotofixans sp. nov., a Nitrogen-Fixing Species from Brazilian Soils and Grass Roots

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A new species, *Bacillus azotofixans*, is described. This taxon is based upon 16 soil and root-associated strains that exhibit acetylene-reducing ability and nitrogen-fixing *Bacillus* sp. strain Hino. *B. azotofixans* is phenotypically similar to *Bacillus polymyxa* and *Bacillus macerans*. However, 13 tests (nitrate reduction; production of acid and gas from xylose, arabinose, lactose, ribose, and glycerol; resistance to lysozyme; liquefaction of gelatin; starch hydrolysis; decomposition of casein and pectin; production of dihydroxyacetone; susceptibility to *B. polymyxa* phages) differentiate it from *B. polymyxa*, and 12 characteristics (spore position; Voges-Proskauer test; nitrate reduction; production of acid and gas from xylose, arabinose, lactose, ribose, and glycerol; growth at 45°C; hydrolysis of starch; decomposition of pectin; formation of crystalline dextrans) differentiate it from *B. macerans*. The guanine-plus-cytosine contents of five strains ranged from 47.9 to 52.5 mol%. All strains reduced acetylene much more efficiently than *B. polymyxa* or *B. macerans*. In four strains, nitrogen fixation was confirmed by micro-Kjeldahl analysis of acetylene-reducing cultures. Acetylene reduction was not inhibited by nitrate and was not dependent on yeast extract or thiamin plus biotin. The proposed type strain of *B. azotofixans* is strain P3L-5 (= ATCC 35681).

Classically, all *Bacillus* strains capable of fixing molecular nitrogen were found to belong to *Bacillus polymyxa* (7, 9) or *Bacillus macerans* (23). In later work, other (nonsymbiotic) nitrogen-fixing *Bacillus* isolates were tentatively identified as strains of *Bacillus circulans* (10). To date, no strains of other *Bacillus* species have been accepted with certainty as nitrogen fixers, although recently it was claimed that several nitrogen-fixing bacilli resembled *Bacillus cereus* or *Bacillus licheniformis* (1). Most of the recently isolated nitrogen-fixing bacilli have been identified clearly as *B. polymyxa* (16-20) or *B. macerans* strains (17) or as *B. polymyxa*-like (24) organisms. The original work of Hino and Wilson (9) on nitrogen fixation by a *Bacillus* strain was done with an isolate that has been classified as an atypical *B. polymyxa* strain (4, 14, 19). However, the deoxyribonucleic acid (DNA) of this strain revealed a guanine-plus-cytosine (G + C) content beyond the range reported for *B. polymyxa*, which is typical of *B. macerans* (14).

In a previous report (20), we described 18 acetylene-reducing *B. polymyxa* strains from soil and 6 other strains with this property that were tentatively identified as "variants" of *B. polymyxa*. Since then, 10 other acetylene-reducing bacilli have been isolated in our laboratory from washed or surface-sterilized roots of different grasses; these strains had properties that were remarkably similar to those of the six aforementioned isolates. In this paper, we describe the characteristics of all of the strains belonging to this group of nitrogen-fixing bacilli.

MATERIALS AND METHODS

Bacterial strains. The strains used and their sources are listed in Table 1. Of these, six were isolated from two different Brazilian soils and have been described elsewhere (20). A total of 10 new strains were isolated by a modification of the method described by Baldani and Döbereiner (2) from washings of either unsterilized or surface-sterilized macerated roots of different grasses. The suspensions were pasteurized (10 min, 80°C) to select for *Bacillus* species. Appropriate dilutions were plated onto nitrogen-free thiamine-biotin

agar (20) and incubated anaerobically. Further enrichment and purification, including two aerobic single-colony reisolation steps, were performed as described by Seldin et al. (20).

Maintenance and propagation of cultures. All strains were stored aerobically at room temperature on GB agar slants (20) supplemented with 1% CaCO₃. Cultures were transferred to fresh slants at 6-month intervals. Long-term preservation of strains was in sterile soil by the method of Gordon et al. (6). Inoculations of media were made from 24- to 48-h-old cultures in GB broth tubes (20) at 32°C. Unless otherwise stated, the preferred incubation temperature was 32°C.

Properties of strains. Most cultural and biochemical tests were performed by using the methods and media of Gordon et al. (6). For all tests that required complex media (temperature range of growth, growth inhibition by NaCl and by pH 5.7, hydrolysis of starch, decomposition of pectin and tyrosine, resistance to lysozyme, and liquefaction of gelatin), appropriately adjusted GB solid or liquid media were employed. The basal medium of Gordon et al. (6) was used to test for acid and gas production from carbohydrates. The sugars used were generally in the D-(+) form, except for L-(-)-arabinose and D-(-)-ribose. Cytochrome oxidase was determined by the standard paper strip Kovacs oxidase test. Cellular morphology, form and position of spores, and swelling of the sporangia were observed by phase-contrast microscopy of wet mounts and on crystal violet-stained smears. Photographs of sporulated cultures were made by using a Zeiss phase-contrast microscope. Capsules were observed on slides prepared by the Duguid method (3). Polyphosphate inclusions were detected in preparations stained with 1% toluidine blue (3). Motility was observed in fresh wet mounts of young (24-h) bacterial cultures in GB broth. Sensitivity to three different bacteriophages specific for *B. polymyxa* was tested by the method of Seldin et al. (21).

In all tests, *B. polymyxa* LMD 24.16^T (T = type strain), *B. polymyxa* L, and *B. macerans* NCTC 6355^T, as well as nitrogen-fixing *Bacillus* sp. strain Hino, were included as controls. *B. polymyxa* strains LMD 24.16^T and L, *B. macerans* strain NCTC 6355^T, and strain Hino were in all aspects similar to their previously published descriptions (4, 6, 9).

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TABLE 1. *Bacillus* strains used in this study

Strain(s)	Source and other information
F-100, F-102, F-203, F-215, F-1532	From Fundão garden soil, Rio de Janeiro, Brazil; isolation and tentative identification described by Seldin et al. ^a
I-9	From Itaguaí soil, Rural University of Rio de Janeiro, Rio de Janeiro State, Brazil; described by Seldin et al. ^a
BE-1, BE-4, BE-5	From surface-sterilized roots of <i>Bachiaria ruziziensis</i> var. Kennedy growing at Rural University of Rio de Janeiro.
P3E-2, P3E-20, P3E-30	From surface-sterilized wheat roots from Paraná State, Brazil
P3L-3, P3L-5 ^T , P5L-4	From wheat roots from Paraná State, Brazil
TE-10	From surface-sterilized wheat roots from Brasília, Federal District, Brazil
Hino	Received from S. Hino, Hiroshima University, Hiroshima, Japan; nitrogen fixer described in 1958 and tentatively classified as <i>B. polymyxa</i> ^b
<i>B. polymyxa</i> LMD 24.16 ^T (=ATCC 842 ^T)	Received from J. van der Toorn, Laboratory for Microbiology, Delft, Holland
<i>B. polymyxa</i> L	Received from M. W. Loutit, University of Otago, New Zealand; nitrogen-fixing strain isolated from soil
<i>B. polymyxa</i> NRRL B-371(=ATCC 8526)	Received from P. Jurtshuk, Jr., University of Houston, Houston, Tex.
<i>B. macerans</i> NCTC 6355 ^T (=ATCC 8244 ^T)	Received from L. Rabinovitch, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil
<i>B. macerans</i> P3L-2	Source same as strain P3L-3; nitrogen-fixing isolate identified as <i>B. macerans</i> in our laboratory

^a See reference 20.^b See reference 9.

Extraction of DNA and T_m analysis. DNA was extracted by the method of Marmur (12), with the following minor modifications. Cells from 800-ml overnight cultures at 32°C were concentrated in 40 ml of 25% sucrose containing 0.05 M tris(hydroxymethyl)aminomethane and 0.001 M ethylenediaminetetraacetate at pH 8.0 and treated with 1 mg of lysozyme (Calbiochem-Behring) per ml for 3 h at 37°C. Subsequently, the ethylenediaminetetraacetate concentration was increased to 0.01 M, and the preparation was treated with 1% sodium dodecyl sulfate for 30 min at 65°C. After several deproteinization steps, the DNA was precipitated and dissolved in 0.1× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). These partially purified DNA preparations were made 1× SSC by adding 10× SSC and then treated with 50 µg of ribonuclease (5× crystallized; Sigma Chemical Co.) per ml for 30 min at 37°C and subsequently with 500 µg of protease (type p-5005; Sigma) for 2 h at 37°C. After additional deproteinization steps, the DNA was precipitated and finally dissolved in 0.1× SSC; melting temperature (T_m) analysis was performed when the ratio of optical density at 260 nm to optical density at 280 nm was 1.8 or higher.

The G + C content of the DNA was determined by thermal denaturation, using the method of Marmur and Doty (13), at 260 nm with a Beckman Acta III spectrophotometer in double-beam mode and a Haake circulating water bath, which was regulated manually. The sample cuvette temperature was monitored with a glass-embedded thermistor in the cuvette. The T_m determined in 0.1× SSC was converted to the expected T_m in 1× SSC by adding 15.4°C, as described by Mandel and Marmur (11). The G + C content was calculated by the formula of Marmur and Doty (13). T_m analysis was performed on DNA from five new strains. These strains (strains F-100, I-9, BE-4, P3L-5, and TE-10) were selected to include one strain for each different origin from which the 16 isolates studied were obtained. The DNAs of *B. polymyxa* LMD 24.16^T, *B. macerans* NCTC 6355^T, and nitrogen-fixing *Bacillus* sp. strain Hino were also analyzed as references. All experiments were performed at least three times, and mean values and standard deviations were then calculated. Protein-free ribonuclease-treated *Escherichia coli* DNA (type 262109; Calbiochem-Behring) was used as a control and had a T_m of 74.8°C, corresponding to a G + C content of 51.0 mol%.

Nitrogen fixation. Acetylene reduction was tested by measuring the ethylene production of cultures in 18-ml vials as described previously (20). The medium employed was a modification of the semisolid glucose-malate medium of Patriquin et al. (15), in which malate, CaCl₂, and KOH were omitted, the agar and glucose concentrations were doubled, and 1% CaCO₃ was added to stabilize the pH. The vials were incubated for 24 h at 32°C, after which acetylene was introduced. Readings were made after 1 h at 32°C.

Nitrogen fixation was confirmed by micro-Kjeldahl analysis of 5-day-old cultures in the semisolid medium. These cultures had ceased to reduce acetylene, and the glucose of the medium had been completely depleted. Vials inoculated from heat-killed cultures were used as controls. Six replicate cultures were tested for each strain.

The influence of nitrate on acetylene reduction was determined by testing for acetylene reduction, using the modified Patriquin medium supplemented with up to 0.5% NaNO₃.

RESULTS AND DISCUSSION

Previous reports (9, 14, 19, 20) clearly show the difficulty encountered in identifying to species level nitrogen-fixing *Bacillus* isolates that differ from well-accepted *Bacillus* species. Thus, nitrogen-fixing *Bacillus* sp. strain Hino was very tentatively classified as *B. polymyxa* (9), but the authors warned that it was different from this species in many respects, including G + C content. However, a major taxonomic problem was the existence of only one representative strain, and Ouelette et al. (14) suggested that a final conclusion regarding the taxonomic position of this strain should await the isolation and description of additional similar strains. No other strains with characteristics similar to those of strain Hino have been described, except for the 16 strains isolated in our laboratory.

These strains were isolated from at least five different localities, indicating a geographical and ecological spread (Table 1). The six strains obtained roughly 3 years ago have maintained their properties up to now. Ten additional strains isolated very recently are identical to this group. Nitrogen-fixing *Bacillus* sp. strain Hino, which was isolated from Japanese soil, was described in 1958 and has been kept in the laboratory since then. This strain is similar to our isolates. This group of strains belongs to the genus *Bacillus*, since their cells are rod shaped, they grow and form spores

aerobically, and they are catalase positive. They are phenotypically related to *B. polymyxa* and *B. macerans*, but differ from these and other *Bacillus* species in many respects. Therefore, the conditions for defining a new species, as described by Gordon (5), seem to have been fulfilled. We propose that this species be named *Bacillus azotofixans*. A species description is given below.

Bacillus azotofixans n. sp. (a. zo. to. fix'ans. Gr. n. *azote* nitrogen; M.L. part. adj. *fixans* fixing, to hold in place; N.L. adj. *azotofixans* nitrogen fixing) cells are straight rods. After 24 to 48 h in GB broth, they occur singly and are weakly motile. The cells are 3 to 6 by 1 μm . In older GB broth cultures, long forms are produced. Strains sporulate well after 5 days on aerobic GB agar; in growth obtained from agar slants, the bulk of the organisms occur as spores. The spores are oval to ellipsoidal and predominantly central to subterminal and distend the sporangium to spindle shape

(Fig. 1). Capsules with discrete boundaries are produced. Aerobic cultures in medium supplemented with 0.1% $(\text{NH}_4)_2\text{HPO}_4$ contain inclusions of polyphosphate. Young (24-h) GB broth cultures are gram variable, containing both gram-positive and gram-negative cells. On GB agar under both aerobic and anaerobic conditions, colonies develop after 48 h at 32°C. Colonies are 1 to 2 mm in diameter, whitish, circular to slightly irregular, and convex, with entire to undulate margins. They are mucoid and opaque, with a butyrous texture, and do not adhere to the agar. Colonies gradually become flatter and duller, and after 2 weeks reveal three to five concentric rings when viewed by transmitted light. Strains produce moderate growth after 48 h of anaerobic incubation on nitrogen-free thiamine-biotin agar (20). Under aerobic conditions, little or no growth is produced in nutrient broth or on the agar of Gordon et al. (6). TY broth and agar (22) permit moderate growth. Abundant growth is

FIG. 1. Photomicrographs of wet mounts of sporulated cultures of *B. azotofixans*. (A) Strain F-100. (B) Strain BE-4. (C) Strain P3L-5^T. (D) Strain TE-10. Bar = 10 μm .

TABLE 2. DNA base compositions of five selected strains and three reference strains

Strains	G+C content (mol%) ^a
F-100.....	47.9 ± 0.2
I-9.....	50.1 ± 0.5
BE-4.....	52.5 ± 0.7
P3L-5 ^T	51.6 ± 1.5
TE-10.....	51.0 ± 1.5
Hino.....	51.2 ± 0.3
<i>B. polymyxa</i> LMD 24.16 ^T	46.1
<i>B. macerans</i> NCTC 6355 ^T	53.1 ± 0.5

^a Triplicate determinations in 0.1 × SSC were calculated by the method of Mandel and Marmur (11).

produced after 24 to 48 h in GB broth. Visible growth occurs in a pellet on the bottom. The minimum and maximum temperatures for growth are 15 to 20 and 37 to 40°C, respectively. No growth occurs at 10 or 42°C. Moderate growth is produced at pH 5.7. Strains grow in the presence of 1% NaCl. At 2 and 3% NaCl, variable growth occurs. Growth is inhibited by 5% NaCl. No growth occurs in the presence of 0.001% lysozyme. Facultatively anaerobic. Aerobic growth is catalase positive and oxidase negative. Voges-Proskauer positive. Voges-Proskauer broth acidified to pH 4.5 to 5.1. Acid and gas are produced from glucose, sucrose, galactose, maltose, raffinose, cellobiose, inulin, and mannitol. Acid and gas are not produced from xylose, arabinose, lactose, ribose, sorbose, and glycerol. Nitrate is not reduced. Citrate is not utilized as a carbon source. Casein and pectin are not decomposed. Gelatin is not liquefied. Tyrosine is not decomposed. Starch hydrolysis is negative, but slight clearing of the medium occurs beneath colonies. Dihydroxyacetone is not produced from glycerol. No crystalline dextrans are formed in rolled oat medium. Nitrogen is fixed under anaerobic conditions. Not susceptible to *B. polymyxa* typing phages EPy-1, SPy-1, and SPy-2. The base composition of the DNA ranges from 47.9 to 52.5 mol% G + C (average, 50.7 mol%) (Table 2).

The type strain is strain ATCC 35681 (= P3L-5). The description of the type strain is the same as that given above for the species; the base composition of the DNA is 51.6 ± 1.5 mol%.

Nitrogen fixation. For all newly isolated strains that grew on nitrogen-free isolation plates, the ability to fix molecular nitrogen was checked by assaying acetylene-reducing activity. As Table 3 shows, all isolates effectively reduced acetylene, and all values were in the range from 69.5 to 240.3 nmol of ethylene per ml per h. These values were consistently higher than those obtained for selected *B. polymyxa* and *B.*

TABLE 3. Acetylene reduction and micro-Kjeldahl analysis of the isolated *Bacillus* strains and selected controls

Strain	Acetylene reduction (nmol of ethylene per ml per h) ^a	Micro-Kjeldahl analysis (mg of N per g of glucose) ^b
F-100	194.5	3.7
F-102	87.5	
F-203	105.0	
F-215	155.7	
F-1532	191.7	
I-9	162.5	3.6
BE-1	154.6	
BE-4	119.6	3.3
BE-5	108.6	
P3E-2	153.5	
P3E-20	69.5	
P3E-30	110.5	
P3L-3	109.7	
P3L-5 ^T	240.3	3.8
P5L-4	124.8	
TE-10	111.5	6.0
<i>B. polymyxa</i> NRRL B-371	35.7	0.4
<i>B. polymyxa</i> L	32.5	0.6
<i>B. polymyxa</i> LMD 24.16 ^T	0	
<i>B. macerans</i> P3L-2	15.6	0.2
<i>B. macerans</i> NCTC 6355 ^T	0	

^a Determined in semisolid medium after incubation for 24 h at 32°C. Values are averages of triplicate vials.

^b Determined by courtesy of J. Döbereiner, Embrapa, Rio de Janeiro State, Brazil.

macerans strains (Table 3), suggesting a higher efficiency of the nitrogen fixation process. Table 3 also shows that neither type strain reduced acetylene under these conditions, suggesting the absence of a nitrogen-fixing system. A micro-Kjeldahl analysis performed on five selected isolates showed values in the range from 3.3 to 6.0 mg of N per g of glucose, whereas reference strains *B. polymyxa* NRRL B-371, *B. polymyxa* L, and *B. macerans* P3L-2 fixed only 0.2 to 0.6 mg of N per g of glucose (Table 3). This observation seems to confirm the higher efficiency of nitrogen fixation by the new isolates.

For four strains, nitrate (NaNO₃) at concentrations up to 0.5% did not influence the efficiency of acetylene reduction in semisolid medium, and at 0.5% nitrate the values indicating acetylene reduction were roughly equal to the control values (Table 4). This observation is in agreement with the

TABLE 4. Influence on acetylene reduction of 0.5% nitrate, 0.1% yeast extract, and thiamine plus biotin

Strain	Acetylene reduction (nmol of ethylene per ml per h)					
	0% Nitrate	0.5% Nitrate	0% Yeast extract	0.1% Yeast extract	No thiamine plus biotin	Thiamine (1 µg/ml) plus biotin (µg/ml)
F-100	149.4 ± 23.2 ^a	148.5 ± 45.7	96.5 ± 48.0	152.8 ± 29.2	96.5 ± 48.0	131.4 ± 4.6
BE-4	71.3 ± 12.0	79.7 ± 28.0	80.8 ± 9.5	88.9 ± 27.0	77.8 ± 4.6	79.1 ± 15.8
P3L-5 ^T	102.6 ± 22.5	77.3 ± 15.4	56.5 ± 21.4	102.6 ± 22.5	69.1 ± 2.2	77.2 ± 15.7
TE-10	75.1 ± 16.7	68.2 ± 21.1	48.7 ± 28.6	75.1 ± 16.7	48.7 ± 28.6	45.9 ± 30.4
<i>B. polymyxa</i> NRRL B-371	13.2 ± 1.0	0	0	13.2 ± 1.0	0	0
<i>B. macerans</i> P3L-2	13.6 ± 1.7	0	0	13.6 ± 1.7	0	0

^a Results are means ± standard deviations of three different experiments.

observed absence of a nitrate reductase in these strains. Acetylene reduction by *B. polymyxa* NRRL B-371 and *B. macerans* P3L-2 (both nitrate reducers) was completely inhibited by 0.5% nitrate.

Omission of 0.1% yeast extract from the semisolid modified Patriquin medium did not substantially lower the efficiency of acetylene reduction by the four selected strains (Table 4), although somewhat lower values were generally observed in the vials that lacked yeast extract. Acetylene reduction by reference strains *B. polymyxa* NRRL B-371 and *B. macerans* P3L-2 did not occur without yeast extract (Table 4). Addition of 1 µg of thiamine per ml and 1 µg of biotin per ml to the medium lacking yeast extract did not result in a substantial increase in acetylene-reducing efficiency (Table 4), suggesting that nitrogen fixation by our strains did not depend on these vitamins. Also, acetylene reduction by *B. polymyxa* NRRL B-371 and *B. macerans* P3L-2 was not stimulated by these vitamins.

As Table 5 shows, 12 biochemical characteristics other than nitrogen fixation separate *B. azotofixans* from *B. polymyxa*. These (negative) properties are production of dihydroxyacetone, nitrate reduction, production of acid and gas from xylose, arabinose, lactose, ribose, and glycerol, growth in 0.001% lysozyme, liquefaction of gelatin, hydrolysis of starch, and decomposition of casein and pectin. Furthermore, *B. azotofixans* is resistant to *B. polymyxa* phages. *B. azotofixans* differs from *B. macerans* by 12 properties. These include negative properties (production of crystalline dextrans; hydrolysis of starch; nitrate reduction; decomposition of pectin; growth at 45°C; production of acid and gas from xylose, arabinose, lactose, ribose, and glycerol) and a positive property (Voges-Proskauer test). Furthermore, the spore position of *B. azotofixans* is central to subterminal instead of subterminal to terminal. Also, the observed G + C

contents were higher than the value determined for *B. polymyxa* LMD 24.16^T DNA and were, with the possible exception of strain F-100, beyond the upper limit of the range reported for *B. polymyxa* DNA (8, 14). On the other hand, these values, although somewhat lower than the value determined for *B. macerans* NCTC 6355^T DNA (Table 2), partially coincided with the range reported for *B. macerans* (8, 14).

A set of characteristics essential for membership in *B. azotofixans* is contained in the following short description: facultatively anaerobic endospore-forming rods that have central to subterminal spores bulging the sporangia to a spindle shape and are capable of fixing nitrogen very efficiently; strongly fermentative, producing acid and gas from glucose and mannitol but not from xylose and arabinose; Voges-Proskauer positive; nitrate reductase negative; no production of dihydroxyacetone; no production of crystalline dextrans; average G + C content, 50.7 ± 1.6 mol% (six strains, including strain Hino).

The strains in this nitrogen-fixing species may be interesting to microbiologists, agronomists, and microbial ecologists alike, since they fix nitrogen efficiently, produce resistant forms, and are apparently widespread in soil, having been isolated in five different localities in Brazil and also in Japan. The fact that these strains were isolated both from nonrhizosphere soil and from grass roots suggests that they may occur either as free-living bacteria or as root-associated bacteria. Furthermore, they fix nitrogen efficiently in the presence of nitrate, and nitrogen fixation is not dependent on yeast extract or thiamin plus biotin.

ACKNOWLEDGMENTS

We are particularly indebted to Johanna Döbereiner for her help and stimulation. We also thank R. E. Gordon for confirming some of

TABLE 5. Differential characteristics of *B. azotofixans*, *B. polymyxa*, and *B. macerans*

Characteristics	<i>B. azotofixans</i>	<i>B. polymyxa</i> ^a	<i>B. macerans</i> ^b
Nitrogen fixation	+ ^c	v	v
G+C content range (mol%)	47.9 – 52.5	43.1 – 45.6 (44 – 48) ^d	49 – 51 (50 – 54) ^d
Spore position ^e	C-ST	C-T	ST-T
Voges-Proskauer test	+	+	–
Acid and gas produced from:			
Xylose	–	+	+
Arabinose	–	+	+
Lactose	–	+ ^f	+ ^f
Ribose	–	+ ^f	+ ^f
Glycerol	–	+ ^f	+ ^f
Growth at 45°C	–	–	+
Growth in 0.001% lysozyme	–	v	–
Nitrate reduction	–	+	+
Starch hydrolysis	w–	+	+
Decomposition of casein	–	+	–
Liquefaction of gelatin	–	+ ^f	–
Decomposition of pectin	–	+	+
Production of:			
Crystalline dextrans	–	–	+
Dihydroxyacetone	–	+	–
Susceptibility to phages	–	+ ^g	–
EPY-1, SPY-1, and SPY-2			

^a Unless indicated otherwise, the data are from references 4 and 6 and were confirmed in our laboratory with *B. polymyxa* LMD 24.16^T, *B. polymyxa* L, and *B. macerans* NCTC 6355^T.

^b Data from reference 14.

^c +, Positive; –, negative; v, variable; w–, very weak to negative.

^d Data from reference 14; data in parentheses from reference 8.

^e C-ST, Central to subterminal; C-T, central to terminal; ST-T, subterminal to terminal.

^f Data obtained in our laboratory.

^g Data from reference 21.

our results and for her helpful comments, H. Rocha for kindly providing the T_m facilities, and A. Drosowicz for the use of the gas chromatograph. Thanks are also due to all colleagues who provided strains.

This work was supported by grants from the National Research Council of Brazil (CNPq) and FINEP.

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