Deoxyribonucleic Acid Homology Among *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus azotofixans*, and Other Nitrogen-Fixing *Bacillus* Strains

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Deoxyribonucleic acid homology experiments were performed among representative strains of *Bacillus polymyxa*, *Bacillus macerans*, and *Bacillus azotofixans* and other nitrogen-fixing *Bacillus* strains identified as *B. polymyxa*-like. All *B. azotofixans* strains showed less than 20% homology when *B. polymyxa* or *B. macerans* was used as a probe. The range of homology among *B. azotofixans* strains (strain P3L-5^T [T = type strain] was used as the probe) varied from 54 to 100%, indicating that these strains comprise a relatively homogeneous species. Strain Hino appears to be a variant of *B. azotofixans* because it exhibited only 28 to 44% homology with this species. Strains P3L-2, CR5D-16, and CR5D-23 showed less than 20% homology with *B. polymyxa*, *B. macerans*, and *B. azotofixans*, indicating that these strains do not belong to these species. Additional data will be necessary to fully clarify the taxonomy of these nitrogen-fixing *Bacillus* strains.

Since 1958, when Hino and Wilson (8) isolated a nitrogen-fixing *Bacillus* strain showing an atypical pattern of classification for *Bacillus polymyxa*, many other strains have been isolated from different soils and plants (10, 18) and have been tentatively identified as variants of this species.

To date, *B. polymyxa*, *Bacillus macerans*, and *Bacillus circulans* are accepted as the only nitrogen fixers among the genus *Bacillus*, although Seldin et al. (19) proposed the inclusion of *Bacillus azotofixans* as a new nitrogen-fixing species.

In this work, we performed deoxyribonucleic acid (DNA) homology experiments with representative strains of *B. polymyxa* and *B. macerans* and the strains reported to be *B. azotofixans* and *B. polymyxa*-like (10, 18) in an attempt to clarify the taxonomy of the nitrogen-fixing bacilli.

MATERIALS AND METHODS

Bacterial strains. All of the strains used in this work are listed in Table 1.

Propagation of cultures. Portions (2 ml) from 24-h cultures grown in liquid medium [glucose, 10 g; MgSO₄ · 7H₂O, 0.2 g; NaCl, 0.1 g; yeast extract, 1 g; (NH₄)₂HPO₄, 1 g; K₂HPO₄, 4 g; KH₂PO₄, 2.8 g; distilled water, 1,000 ml; pH 6.5] were inoculated into 200 ml of the same medium and incubated at 32°C for 18 h.

Isolation of chromosomal DNA. Cells from 200-ml cultures were concentrated in 5 ml of lysis buffer [25% sucrose, 0.05 M tris(hydroxymethyl)aminomethane (Tris), 0.001 M ethylenediaminetetraacetate, pH 8.0] and treated with 1 mg of lysozyme per ml for 30 min at 37°C. *B. polymyxa* strains were treated for 2.5 h at the same temperature. Sodium dodecyl sulfate was added to a final concentration of 1%, and the preparations were incubated for 30 min at 65°C. In order to purify the DNA, 1 mg of pronase (catalog no. P5147; Sigma Chemical Co.) per ml was added, and the preparations were incubated at 50°C for 2 h. The DNA was then precipitated with 2 volumes of cold ethanol, spooled with a glass stirring rod, dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (13), and shaken with an

equal volume of phenol neutralized with TES buffer (50 mM NaCl, 30 mM Tris, 5 mM ethylenediaminetetraacetate) at pH 7.5 for 15 min. The phenol treatment was repeated several times until very little interface was observed. The aqueous phase containing the DNA was spooled after ethanol precipitation and suspended in 0.1× SSC. Then, the preparations were treated with pancreatic ribonuclease (50 µg/ml) and ribonuclease T₁ (1 U/ml) for 30 min at 37°C. After two treatments with phenol the DNA was precipitated, spooled, and dissolved in 1 ml of DNA buffer (4 mM NaCl, 10 mM Tris, 0.1 mM ethylenediaminetetraacetate, pH 7.5). All DNA preparations were kept at 4°C with a few drops of chloroform.

DNAs from *Streptococcus* sp., *Staphylococcus aureus*, *Bacillus subtilis* IS75, and *Bacillus licheniformis* ATCC 8480 were kindly provided by investigators of the Public Health Research Institute of the City of New York, Inc. These DNA preparations were used as controls in some hybridization experiments.

Quantification of DNA. The amount of DNA in each preparation was quantified by using diphenylamine, as described by Johnson (9). The values obtained were compared with standards consisting of known amounts of pure calf thymus DNA (Sigma).

Preparation of DNA probes. Chromosomal DNAs of *B. polymyxa* 24.16^T (T = type strain), *B. macerans* NCTC 6355^T, *B. azotofixans* P3L-5^T, and strain Hino were used as probes in the hybridization experiments. These DNAs were nick translated as described by Davis et al. (5). The DNA probes were radiolabeled with both [³²P]deoxyadenosine triphosphate and [³²P]deoxythymidine triphosphate (Amersham Corp.) to a level of approximately 7 × 10⁷ cpm/µg of DNA.

Hybridization experiments. To a sample containing 0.5 µg of DNA in 170 µl of 100 mM Tris-hydrochloride (pH 7.4) 30 µl of 2 M NaOH and 100 µl of 20× SSC were added. This mixture was heated at 80°C for 10 min, cooled to room temperature, and then neutralized with 40 µl of 2 M Tris-hydrochloride. After this denaturation procedure, the DNA preparations were loaded onto nitrocellulose membranes by using a slot blot system (catalog no. SRC 072/0; Schleicher &

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TABLE 1. *Bacillus* strains used in this work

Strain(s)	Species	Comments
BE-1, BE-4, BE-5, P3E-2, P3E-20, P3E-30, P5L-4, TE-10, F-100, F-102, F-1532, F-203, F-215, P3L-3, P3L-5 ^T	<i>B. azotofixans</i>	Described by Seldin et al. ^a
Hino	<i>B. azotofixans</i>	Received from S. Hino, Hiroshima University, Hiroshima, Japan; initially identified as a <i>B. polymyxa</i> variant
P3L-2		Isolated by L. Seldin; initially identified as a variant of <i>B. macerans</i>
CR5D-16, CR5D-23		Received from J. L. Neal, Jr., Virginia Polytechnic Institute and State University, Blacksburg; initially identified as a <i>B. polymyxa</i> variant
LMD 24.16 ^T	<i>B. polymyxa</i>	Received from J. van der Toorn, Laboratory for Microbiology, Delft, Holland
NCTC 6355 ^T	<i>B. macerans</i>	Received from L. Rabinovitch, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
L	<i>B. polymyxa</i>	Received from M. Loutit, University of Otago, Otago, New Zealand
NCTC 10343	<i>B. polymyxa</i>	Received from L. Rabinovitch, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

^a See reference 19.

Schuell Inc.). Two or more additional dilutions of each DNA sample were also loaded. The filters were then placed between two sheets of Whatman 3MM filter paper and baked for 2 h at 80°C under a vacuum (12). The wet filters were slipped into a heat-sealable plastic bag, and about 25 ml of prehybridization solution (1) was added to the bag. Blots were prehybridized overnight at 65°C. Hybridizations were carried out for 24 h at 65°C in hybridization solution (1) to which about 7×10^6 cpm of denatured probe had been added for each filter (6.5 by 23 cm). The blots were then washed twice for 15 min in a solution containing $2 \times$ SSC and 0.1% sodium dodecyl sulfate and twice for 15 min in a solution containing $0.1 \times$ SSC and 0.1% sodium dodecyl sulfate. The washings were carried out at room temperature. Then the filters were dried at room temperature on a sheet of Whatman 3MM filter paper and wrapped in Saran Wrap. The filters were exposed to X-ray film by using a Du Pont Cronex Lightning Plus intensifying screen for 24 to 72 h at -70°C.

Quantification of DNA homology. After exposure to X-ray film, the individual "slots" were cut from the nitrocellulose membranes and placed into scintillation vials. Then 4 ml of Liquiscint (National Diagnostics) was added to each vial, and the radioactivity was measured with a scintillation counter. The percentage of homology was calculated by using the following equation: percent homology = (counts per minute with heterologous DNA/counts per minute with homologous DNA) $\times$ 100. Homologous DNA was the DNA

used as the probe in the hybridization experiments. When diluted DNA was used, the counts were calculated by using the average counts of the various dilutions. Whenever more than 25% homology was observed, the hybridization experiments were repeated three times.

RESULTS AND DISCUSSION

Several attempts have been made to construct classification schemes by using numerical taxonomic methods (15), API tests (11), or computer-assisted techniques (21) in order to accommodate strain variation in the genus *Bacillus*. These studies were performed by using morphological, physiological, and biochemical characteristics, but because of the diversity of this genus, it seems essential that these studies be supplemented by other data to prevent misidentification.

The nitrogen-fixing bacilli are a good example of this difficulty in identification. Since 1958, strain Hino (8) has

FIG. 1. Hybridization experiments. (A) *B. macerans* NCTC 6355^T used as the probe. (B) *B. polymyxa* LMD 24.16^T used as the probe. (C) *B. azotofixans* P3L-5^T used as the probe. The sources of unlabeled DNA were strains BE-1 (lane 1), BE-4 (lane 2), BE-5 (lane 3), P5L-4 (lane 4), P3E-2 (lane 5), P3E-20 (lane 6), P3E-30 (lane 7), TE-10 (lane 8), F-100 (lane 9), F-1532 (lane 10), F-215 (lane 11), F-102 (lane 12), F-203 (lane 13), P3L-3 (lane 14), Hino (lane 15), P3L-5 (lane 16), LMD 24.16^T (lane 17), L (lane 18), NCTC 10343 (lane 19), P3L-2 (lane 20), NCTC 6355^T (lane 22), CR5D-23 (lane 23), and CR5D-16 (lane 24). Lane 21 contained unlabeled *Streptococcus* DNA.

been classified as *B. polymyxa*, although its guanine-plus-cytosine content is similar to that of *B. macerans* (14). In the same way, Larson and Neal (10) tentatively identified several strains isolated from wheat roots as *B. polymyxa* variants. The review of Rhodes-Roberts (16) clearly demonstrated the need for an accurate study of the taxonomy of nitrogen-fixing *Bacillus* strains. As proposed by Priest (15), we performed DNA homology experiments with *B. polymyxa*, *B. macerans*, and the nitrogen-fixing *Bacillus* strains previously identified as *B. azotofixans* (19) or *B. polymyxa* and *B. macerans* variants (10, 19).

All of the *B. azotofixans* strains showed weak homology to *B. macerans* NCTC 6355^T (Fig. 1). The levels of homology are shown in Table 2, and all were less than 21%. In the same way, all strains belonging to *B. azotofixans* showed no more than 20% hybridization to *B. polymyxa* LMD 24.16^T (Table 2). Strains CR5D-16 and CR5D-23 also had low levels of homology to *B. polymyxa*, as did *B. macerans* NCTC 6355 and strain P3L-2. Figure 1 shows the differences in homology among the strains. Lane 21 contained a negative control, showing that there is no homology between *Streptococcus* DNA and *B. polymyxa*, *B. macerans*, or *B. azotofixans* DNA.

All of the *B. azotofixans* strains displayed 54 to 100% DNA relatedness (Fig. 1 and Table 2), indicating that they comprise a relatively homogeneous species (15). The DNA dilutions were compatible with the decreases in the counts on the nitrocellulose filters. Figure 2 shows these data, as

FIG. 2. Hybridization experiment in which strain P3L-5^T was used as a probe. The slots below each number correspond to a 1:2 dilution of the unlabeled DNA. The sources of unlabeled DNA were strain BE-1 (lane 1), strain BE-4 (lane 2), strain BE-5 (lane 3), strain P5L-4 (lane 4), strain P3E-2 (lane 5), strain P3E-20 (lane 6), strain P3E-30 (lane 7), strain TE-10 (lane 8), strain F-100 (lane 9), strain F-1532 (lane 10), strain F-102 (lane 11), strain F-203 (lane 12), strain P3L-3 (lane 13), strain Hino (lane 14), strain P3L-5^T (lane 15), *B. subtilis* IS75 (lane 16), *B. licheniformis* ATCC 8480 (lane 17), and *S. aureus* (lane 18).

TABLE 2. Levels of homology among strains

Source of unlabeled DNA	% Homology with labeled DNA from strain: ^a			
	<i>B. polymyxa</i> LMD 24.16 ^T	<i>B. macerans</i> NCTC 6355 ^T	<i>B. azotofixans</i> P3L-5 ^T	Hino
<i>B. azotofixans</i>				
BE-1	17	17	69	44
BE-4	8	3	57	32
BE-5	15	12	75	31
P5L-4	15	5	78	28
P3E-2	16	4	100	36
P3E-20	17	13	88	40
P3E-30	20	10	82	43
TE-10	16	21	69	34
F-100	18	9	75	43
F-102	12	19	60	28
F-1532	15	9	76	43
F-203	17	6	74	39
F-215	14	6	54	
P3L-3	11	15	97	42
P3L-5 ^T	11	6	100	39
Hino	12	20	38	100
<i>Bacillus</i> sp.				
P3L-2	8	6	11	12
CR5D-16	9	10	16	18
CR5D-23	13	8	18	19
<i>B. polymyxa</i> LMD 24.16 ^T	100	19	11	
<i>B. macerans</i> NCTC 6355 ^T	13	100	20	
<i>B. polymyxa</i>				
L	60	13	14	
NCTC 10343	100	9	11	

^a The hybridization experiments were repeated three times whenever more than 25% homology was observed. Values were obtained by determining the ratio of counts of heterologous DNA to counts of homologous DNA and multiplying by 100.

well as the results of hybridization between strain P3L-5^T DNA and *B. subtilis* and *B. licheniformis* DNAs. Strain Hino showed an intermediate level of homology to *B. azotofixans* P3L-5^T DNA (38%). For this reason strain Hino was used as a probe in hybridization experiments with all of the *B. azotofixans* strains. Figure 3 shows that strain Hino hybridized with all of the *B. azotofixans* strains, although the levels of homology varied from 28 to 44% (Table 2). These levels are in accordance with the results obtained when strain P3L-5^T was used as the probe. Based on the taxonomic criteria proposed by Gordon et al. (7), strain Hino is quite similar to *B. azotofixans*. Therefore, we were not able to separate it from *B. azotofixans*, although the levels of DNA homology were somewhat lower (Table 2). Many authors (2-4, 20) believe that levels of homology ranging from 20 to 60% represent "low, moderate or closely related but separate" species, but in the genus *Bacillus* there is controversy. Considering the diversity of this genus, Seki et al. (17) proposed that 50% DNA homology is sufficient to define a species. It appears that strain Hino is not as closely related to *B. azotofixans* strains as these strains are to one another, but we believe that it is too drastic a step to place strain Hino in a different species. Considering the taxonomic data (19), we propose to introduce strain Hino as a variant of *B. azotofixans*. Strains CR5D-16 and CR5D-23 were previously classified as *B. polymyxa* variants (10). However, less than 20% hybridization was detected between DNA samples from these strains and DNA samples from either *B. polymyxa*, *B. macerans*, or *B. azotofixans*, indicating that these strains probably belong to another species. The major taxonomic problem with strains CR5D-16 and CR5D-23 is the existence of few representative strains belonging to this group, and

FIG. 3. Hybridization experiment in which strain Hino DNA was used as a probe. The sources of unlabeled DNA were strain BE-1 (lane 1), strain BE-4 (lane 2), strain BE-5 (lane 3), strain P5L-4 (lane 4), strain P3E-2 (lane 5), strain P3E-20 (lane 6), strain P3E-30 (lane 7), strain TE-10 (lane 8), strain F-100 (lane 9), strain F-1532 (lane 10), strain F-102 (lane 11), strain F-203 (lane 12), strain P3L-3 (lane 13), strain P3L-5^T (lane 14), strain P3L-2 (lane 15), strain CR5D-16 (lane 16), strain CR5D-23 (lane 17), *S. aureus* (lane 18), strain Hino (lane 19), strain Hino DNA diluted 1:2 (lane 20), strain Hino DNA diluted 1:4 (lane 21), and strain Hino DNA diluted 1:8 (lane 22).

conditions for defining a new species, as described by Gordon (6), have yet to be fulfilled.

ACKNOWLEDGMENTS

We thank all members of the laboratory of D.D. and especially Yvette Weinrauch for their inestimable help. Thanks are also due to all colleagues who were kind enough to provide *Bacillus* strains.

Finally, we are grateful to Johanna Döbereiner, who provided financial support to L.S. through the National Academy of Science (BOSTID-NAS).

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