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Identification of *Bacillus subtilis* NRRL B-3275 as a Strain of *Bacillus pumilus*

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The physiological and biochemical properties of a species of *Bacillus* previously identified as *B. subtilis* NRRL B-3275 (B-3275) were compared with those of seven strains of *B. pumilus* and five strains of *B. subtilis*. The biotin requirement of B-3275, its inability to hydrolyze starch, and its failure to reduce nitrate indicate that the organism is more closely related to the *B. pumilus* strains than to those of *B. subtilis*. Hybridization of deoxyribonucleic acid (DNA) from B-3275 with that of the strains of *B. pumilus* showed a binding efficiency (compared with the homologous reaction) of 58 to 99%, depending on the strain. Hybridization with the DNA from any of the strains of *B. subtilis* did not exceed 24%. DNA from B-3275 was unable to transform two amino acid auxotrophic markers to prototrophy in a highly competent strain of *B. subtilis* 168. We conclude that B-3275 is a strain of *B. pumilus* which we designate as *B. pumilus* NRRL B-3275.

In recent years considerable attention has been devoted to aberrant forms of bacteria. In particular, the physiology of L-forms has been studied in many pathogenic strains. However, these studies have been hampered by the lack of good systems for genetic exchange among the parent bacterial strains or the derived L-forms. The isolation of a strain of *Bacillus subtilis* which gives rise to osmotically fragile, spherical bodies during growth in a high salt medium (1) provided a model to investigate transformation in L-forms of *B. subtilis*. Because we were unable to demonstrate transformation of *B. subtilis* 168 with deoxyribonucleic acid (DNA) isolated from *B. subtilis* NRRL B-3275 (B-3275), we initiated taxonomic studies to investigate more fully its relationship to *B. subtilis* 168. B-3275 and known strains of *B. pumilus* are susceptible to transduction by PBS1 (Lovett and Young, *in preparation*). Because preliminary genetic analysis suggests that the orientation of several auxotrophic markers in B-3275 is similar to that in *B. subtilis* 168, it is imperative that the identity of B-3275 be established. In the present communication, evidence is presented for reclassifying B-3275 as a strain of *B. pumilus*, referred to hereafter as *B. pumilus* NRRL B-3275.

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MATERIALS AND METHODS

Organisms. The *B. pumilus* and *B. subtilis* strains examined are listed in Table 1. B-3275 was obtained from H. R. Burmeister; *B. pumilus* BD 2002 was provided by D. Dubnau. *B. amyloliquefaciens* H and *B. licheniformis* FDO-12 (both used only for DNA studies) were obtained from N. Welker and C. Thorne, respectively.

Classification of B-3275. The procedures and media of Smith, Gordon, and Clark (9) were used, except that the temperature of incubation was 37 instead of 28 C.

Extraction of DNA for physical studies. Bacteria were grown to stationary phase with rotary shaking at 37 C in 800 ml of minimal medium (10) containing 0.05% acid-hydrolyzed casein and L-tryptophan (50 μg/ml). D-Biotin (0.05 μg/ml) was added to all strains of *B. pumilus*. Cells were harvested by centrifugation, washed once with TES buffer (3), *p*H 7.0, and suspended in 50 ml of the buffer. Lysozyme and pancreatic ribonuclease (Worthington Biochemical Corp.) were added at 200 and 75 μg/ml, respectively, and the suspension was held at 37 C for 1 hr. Sodium lauryl sulfate (recrystallized once from USP grade, Fisher Scientific Corp.) was added to 1% (w/v), and the suspension was placed at 65 C for 30 min (6). After homogenization in a Ten Broeck homogenizer, the lysate was gently shaken with an equal volume of phenol (Mallinckrodt, washed once with TES buffer) and centrifuged at 1,000 × *g* at 25 C for 30 min. The aqueous phase was extracted three more times with phenol and finally precipitated sequentially in ethyl alcohol and isopropanol (6). The final precipitate was suspended in 0.15 M NaCl and 0.015 M sodium

citrate (SSC) and dialyzed against 1,000 volumes of SSC for 48 hr at 4 C. Tritium-labeled DNA was prepared by supplementing the growth medium with 200 mg of deoxyadenosine and 2 mc (30 μg) of [*methyl*] -^{3}H-thymidine (New England Nuclear Corp.). The absorbancy ratio of a typical DNA preparation (260: 230:280 nm) was 1.0:0.46:0.55. The concentration of DNA was estimated by assuming that 1 optical density unit at 260 nm is equivalent to 40 μg of DNA.

Thermal denaturation. Determinations of melting temperatures (T_m) were performed by the procedure of Marmur and Doty (7). Base compositions were calculated by the equation: per cent guanine + cytosine (%GC) = (T_m − 69.3) 2.439 (7).

DNA hybridization. Hybrid formation and detection were carried out as described by Denhardt (2) with the following modifications. Membrane filters (Schleicher and Schuell Type B-6, 25 mm) were embedded with 5 μg of nonradioactive, heat-denatured (100 C, 15 min) DNA. Embedded filters were dried at room temperature for 4 hr and then placed at 60 C for 12 hr. For annealing, DNA-embedded filters were preincubated in 20-ml scintillation vials containing 1 ml of preincubation mixture (2) at 65 C for 6 hr. Heat-denatured ^{3}H-DNA (0.5 μg of DNA in 0.1 ml of 5 × SSC, sonically treated for 45 sec in a Branson ST25 Sonifier at maximum output) was added, and incubation at 65 C was continued for 16 hr. The filters were washed on both sides with 40 ml of 6 × SSC, dried overnight at 60 C, and counted (2). Control filters (i.e., filters not embedded with DNA) taken through the entire procedure retained less than 0.3% of the input radioactivity.

Buoyant density (ρ) determination. DNA of unknown density and *Escherichia coli* K-12 DNA (reference marker, ρ = 1.710 g/ml) were diluted in a cesium chloride solution [Harshaw optical grade, dissolved in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, *p*H 8.0] to a final concentration of 2.5 μg/ml. The solution (average ρ = 1.704 g/ml) was centrifuged at 25 C in an analytical ultracentrifuge (Spinco Model E) for 22 to 24 hr at 44,000 rev/min. The positions of the DNA bands at equilibrium were measured by ultraviolet absorption (265 nm) and plotted as a function of radial distance (5). Buoyant densities and base compositions were calculated by the equations described by Schildkraut, Marmur, and Doty (8).

Transformation. The procedures for extraction of DNA and performing DNA mediated transformation have been described (10).

RESULTS AND DISCUSSION

Classification. B-3275 was compared with seven strains of *B. pumilus* and five strains of *B. subtilis*. The cultural and biochemical tests are based on the scheme used by Smith, Gordon, and Clark (9) for the classification of species of the genus *Bacillus*. In all instances, the characteristics of B-3275 were similar to those of known strains of *B. pumilus*. The characteristics in which the *B. subtilis* strains differed from those of *B. pumilus* are indicated with an asterisk.

Morphology. B-3275 is a highly motile, gram-positive, spore-forming rod. By phase microscopy, the cells and spores of B-3275 are indistinguishable from those of *B. pumilus* or *B. subtilis*. On nutrient agar, *B. pumilus* formed rough, dry colonies which ranged from beige to slightly yellow depending on the strain. Occasionally smooth variants were observed. On the same medium *B. subtilis* formed brown, rough colonies.

Sporangia. No swelling by spores.

NaCl broth. Moderate growth in 7%. Scant to moderate growth in 10%.

Glucose-nutrient agar. Growth heavier and softer than on nutrient agar.

Tyrosine agar. Growth same as on nutrient agar.

Glucose-asparagine agar. Growth abundant.

*** Hydrolysis of starch.** *B. pumilus*, negative; *B. subtilis*, positive.

Acetylmethylcarbinol produced.

Fermentation tests. Acid without gas from arabinose, xylose, sucrose, and mannitol. No acid from lactose.

Citrate utilized.

Hydrolysis of gelatin. Positive.

*** Reduction of nitrate to nitrite.** *B. pumilus*, negative; *B. subtilis*, positive.

Anaerobic production of gas from nitrate. Negative.

*** Biotin requirement.** *B. pumilus*, required; *B. subtilis*, not required.

Three characteristics distinguish *B. pumilus* from *B. subtilis*: starch hydrolysis, nitrate reduction, and a biotin requirement (4, 9). We could not confirm the previous report (1) that B-3275 hydrolyzed starch.

The %GC of B-3275 DNA was 43.4% as determined by thermal denaturation (Table 1). This is within the range obtained for the GC content of DNA of the *B. pumilus* strains (41.2 to 43.9%) and the *B. subtilis* strains (42.9 to 45.6%). The %GC of B-3275 DNA calculated from its buoyant density was 41.8% (Table 2), which is similar to the values obtained for two *B. pumilus* strains (41.8 and 42.9%) but below the value obtained for *B. subtilis* 168 (44.9%). It is concluded that the DNA base composition of B-3275 cannot be used as a major criterion for distinguishing this organism from known strains of *B. pumilus* or *B. subtilis*.

DNA hybridization. Labeled DNA from B-3275 and *B. subtilis* 168 were annealed with a 10-fold excess of unlabeled DNA isolated from the bac-

TABLE 1. *Thermal denaturation and hybridization studies*

Source of DNA	T_m (C)[a]	%GC	DNA hybridization	
			B. pumilus NRRL B-3275	*B. subtilis* 168
			%[b]	%[b]
B. pumilus				
NRRL B-3275	87.1 (±0.1)	43.4	100	16
ATCC 945	87.0 (±0.1)	43.2	99	22
ATCC 14884	87.0 (±0.1)	43.2	87	20
ATCC 1	86.6 (±0.2)	42.2	75	16
BD 2002	86.7 (±0.2)	42.4	75	18
ATCC 70	86.2 (±0.2)	41.2	73	18
NCIB 8982	87.3 (±0.3)	43.9	60	14
ATCC 7061	86.9 (±0.3)	42.9	58	14
B. subtilis				
ATCC 6633	86.9 (±0.2)	42.9	24	70
W-23	88.0 (±0.2)	45.6	21	89
168 I^-	87.3 (±0.2)	43.9	18	100
var. *niger* ATCC 7972	87.2 (±0.2)	43.7	12	70
var. *aterrimus* ATCC 7060	86.9 (±0.2)	42.9	7	77
B. licheniformis FDO-12	88.7 (±0.2)	47.3	9	24
B. amyloliquefaciens H	ND[c]		5	11
E. coli K-12	90.4 (±0.1)	51.5	4	4

[a] Average of three determinations with average deviation.

[b] Percentage of input DNA bound in homologous reactions: *B. subtilis* 168, 25%; *B. pumilus* NRRL B-3275, 21%. Results shown are averages from 5 or 10 membrane filters. Data expressed as per cent of homologous reaction.

[c] Not done.

TABLE 2. *Buoyant densities of four DNA preparations in CsCl*

Source of DNA	Buoyant density	GC
	g/ml	%
B. pumilus NRRL B-3275	1.701	41.8
B. pumilus ATCC 945	1.701	41.8
B. pumilus ATCC 14884	1.702	42.9
B. subtilis 168 I^-	1.704	44.9

terial strains listed in Table 1. The percentage of hybridization of B-3275 DNA (relative to the homologous reaction) with the DNA isolated from strains of *B. pumilus* ranged from 58% (ATCC 7061) to 99% (ATCC 945). Hybridization with the DNA from any of the *B. subtilis* strains did not exceed 24% and hybridization with *B. amyloliquefaciens* H and *B. licheniformis* FDO-12 DNA was 9 and 5%, respectively. When ^{3}H-DNA from *B. subtilis* 168 was used for annealing, approximately reciprocal results were obtained with the DNA from the *B. pumilus* and *B. subtilis* strains. The percentage of hybridization of both labeled DNA types with *E. coli* K-12 DNA (4%) is taken as an indication of the amount of binding occurring with the DNA from an unrelated bacterium. We conclude that DNA from B-3275 shares more regions of homology with DNA from the *B. pumilus* strains than with those of the other species examined.

Transformation. Attempts to transform two amino acid auxotrophic markers (*trp*-2 and *met* B10) in *B. subtilis* 168 to prototrophy by using a high concentration of B-3275 DNA were unsuccessful (Table 3). However, the DNA of B-3275 competed with the transforming activity of the DNA of *B. subtilis* 168 when a mixture containing 2 μg/ml of both DNA types was used for transformation. Lowering the concentration of both DNA types in the mixture to 0.002 μg/ml allowed full expression of the transforming activity of the *B. subtilis* DNA present in the mixture. These data suggest that the inability of DNA from B-3275 to transform competent cells of *B. subtilis* 168 is not due to degradation of the DNA by an associated nuclease. Based on the low in vitro hybridization between the DNA of the two species (Table 1), it is probable that the lack of transformation is due to genetic nonhomology.

The data presented demonstrate that B-3275 is not a strain of *B. subtilis*. This organism appears closely related to *B. pumilus*. We suggest that B-3275 be hereafter referred to as *B. pumilus* NRRL B-3275.

TABLE 3. *Effect of B. pumilus NRRL B-3275 DNA on B. subtilis 168 transformation*[a]

Source of DNA[b]	Concn	Transformants (per ml)		Viable count (per ml)
		Trp[c]	Met[c]	
	μg/ml			
B. subtilis	2	930 × 10^3	720 × 10^3	1.0 × 10^8
B. pumilus	2	<0.01 × 10^3	<0.01 × 10^3	1.1 × 10^8
B. subtilis + *B. pumilus*	2 + 2	190 × 10^3	82 × 10^3	1.3 × 10^8
B. subtilis	0.002	3.6 × 10^3	4.8 × 10^3	1.0 × 10^8
B. pumilus	0.002	<0.01 × 10^3	<0.01 × 10^3	1.1 × 10^8
B. subtilis + *B. pumilus*	0.002 + 0.002	2.5 × 10^3	5.2 × 10^3	1.3 × 10^8

[a] Recipient: *B. subtilis* 168 strain BR151 (*trp*-2, *lys*-3, *met*B10).

[b] Donor: *B. pumilus* NRRL B-3275; *B. subtilis* 168 strain BC 467 (*his*A1, *arg*C4, *ura*-1, *met*D1 and *gta*-C67).

[c] Selected trait.

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