Bacillus firmus-*Bacillus lentus*: a Series or One Species?

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The 46 strains of *Bacillus firmus* and *Bacillus lentus* in our collection were found to share a group of 17 physiological properties that distinguished them from morphologically similar strains of other species of the genus *Bacillus*. Thirteen other characteristics separated the 46 strains into one group of 20 similar to the nomenclatural type strain of *B. firmus* (ATCC strain 14575) and a second group of eight strains resembling the nomenclatural type strain of *B. lentus* (ATCC strain 10840). The pattern of these 13 additional characteristics of the remaining 18 of the 46 strains fell between the two groups. Guanine plus cytosine content in the deoxyribonucleic acid of 31 of the 46 strains ranged from 39.0 to 48.3 mol% and did not allow separation of the strains into distinct groups. The 46 strains of our collection could not, therefore, be cleanly divided by the properties tested and appeared as a spectrum, or series, of strains.

Bacillus firmus, described and named by Werner (18), is an accepted species of the genus *Bacillus*, and its nomenclatural type strain is the American Type Culture Collection (ATCC) strain 14575 (6). In 1935 Gibson (5) characterized a new species, *Bacillus lentus*, in a description of nine isolates from five different soils. He separated his nine strains of *B. lentus* from strains of *B. firmus* by their production of urease and their failure to decompose casein and gelatin.

In a study of the nutritional requirements of strains of *Bacillus* spp., Proom and Knight (13) examined 20 strains of *B. firmus* and six of *B. lentus*. They reported that the strains of the two species could not be separated by their nutritional requirements; the most exacting strains of *B. firmus* and the least exacting strains of *B. lentus* had the same requirement for amino acids, thiamine, and biotin. This investigation of our 46 strains representing *B. firmus* and *B. lentus* confirmed the spectrum-like relationship of the strains observed by Proom and Knight.

MATERIALS AND METHODS

Organisms. The 46 strains examined were listed by Gordon et al. (7, p. 190) and are also presented in Table 1. The first of these strains (NRS 613) was acquired in 1937, and the last strain (NRS 1576) was added to our collection in 1962.

Resistance to lysozyme. Lysozyme solution was prepared by dissolving 0.1 g of lyophilized lysozyme (6,000 to 10,000 U/mg from Nutritional Biochemicals Corp., Cleveland, Ohio) in 100 ml of distilled water in a volumetric flask and sterilizing the solution by filtration through a 0.2- μ m membrane filter. A 1-ml volume of this filtrate and 99 ml of sterile nutrient

broth were mixed aseptically and dispensed in 2.5-ml amounts in sterile plugged tubes. This broth containing 60 to 100 U of lysozyme per ml and a control tube of nutrient broth without lysozyme were inoculated with a small loopful of a culture in nutrient broth (7 to 14 days old) for each organism tested. Growth was recorded after 7 and 14 days of incubation at 28°C.

Liquefaction of gelatin. Cultures were inoculated into tubes of nutrient gelatin (nutrient broth, 100 ml; Difco gelatin, 12 g; pH 7.0) and incubated at 28°C for 14 days. After they had been cooled to 4°C, they were observed for liquefaction of the gelatin.

Formation of urease. The medium of Collins (3) was modified for detection of urease and as used here contained the following: peptone, 0.1 g; NaCl, 0.5 g; KH_2PO_4 , 0.2 g; 0.04% aqueous solution of phenol red, 2 ml; distilled water, 100 ml. Tubes with 2.5 ml of this broth and a flask containing 100 ml of it were sterilized by autoclaving. A 10-ml volume of a 20% aqueous solution of urea sterilized by filtration was added to the flask and dispensed in 2.5-ml amounts in sterile tubes. Cultures were inoculated into tubes with and without urea, incubated at 28°C, and compared at 1, 3, and 7 days. Alkaline color of the indicator in tubes with urea in contrast to the color of the control tubes was accepted as evidence of the presence of urease.

A culture of *B. lentus* (ATCC 10840), known to be urease positive, and a culture of *B. firmus* (ATCC 14575), known to be urease negative, were used as controls.

Acid production from carbohydrates. The basal ammonium phosphate agar described by Gordon et al. (7) and nutrient agar with 3% NaCl and 0.008% phenol red were used as substrates. Each carbohydrate was sterilized separately by autoclaving (121°C for 20 min) in 10% aqueous solution, cooled, and added immediately in 0.5-ml amounts to the tubes of sterile substrates (7 ml). (In their studies of the genus *Bacillus*, Smith et al. [15, 16] used carbo-

TABLE 1. History of strains of the *Bacillus firmus*-*Bacillus lentus* series

NRS strain ^a no.	Source, strain no., and name when received
613	J. R. Porter, Univ. of Iowa ← G. Brede- mann; <i>B. firmus</i>
670, 1262	T. Gibson, Univ. of Edinburgh, strains 165, 238; <i>B. lentus</i>
854	J. R. Porter, Univ. of Iowa ← G. Brede- mann, strain A-Hamburg; <i>B. firmus</i>
1119	C. E. ZoBell, Scripps Inst., strain 562; <i>B.</i> <i>imomarinus</i>
1131, 1132	W. H. Fuller, Iowa State Univ.; unidenti- fied isolations
1147 to 1149, 1151, 1152	H. W. Reuszer, Tropical Deterioration Lab., U. S. Army, strains 692, 1098, 1124, 1099, 719; unidentified isolations
1329	M. B. Allen, Hopkins Marine Station; <i>B.</i> <i>firmus</i>
1369, 1370	G. Pacheco, Inst. Oswaldo Cruz, strains S, R; <i>B. firmus</i> (Pacheco and Santos, 12)
1560 to 1574	M. Turner, Univ. of Nottingham, SM strains 9, 10, 13, 14, 22, 23, 24, 25, 26, 27, 29, 30, 31, 33, 34; unidentified isolations from a salt marsh (17)
1575, 1576	J. M. Sieburth, Univ. of Rhode Island, strains PJ 1, PJ 3; unidentified isola- tions from sea water

^a Strains from the collection of the late N. R. Smith, U. S. Department of Agriculture.

hydrate solutions sterilized by filtration and by autoclaving. The solutions sterilized by both methods gave the same results.) Cultures on slants of these agars were incubated at 28°C and observed for the acid color of the indicators at 7 and 14 days.

Lysis by bacteriophage. A bacteriophage for ATCC strain 14575 of *B. firmus* was isolated by adding 3 g of garden soil to 100 ml of a nutrient broth culture of ATCC 14575 in the log phase of growth. After incubation at room temperature for 3 days, the culture was filtered through a 0.45- μ m membrane filter. Dilutions (10^{-2} to 10^{-5}) of this filtrate were mixed with 7 ml of culture (in the log phase) and swabbed on plates of nutrient agar. For the preparation of a stock solution of the phage, material from a single isolated plaque from one of the plates was inoculated into a young broth culture and then repeatedly filtered and mixed with more host culture until lysis was observed in a broth culture of ATCC 14575 inoculated with a 10^{-9} dilution of the stock phage.

Sensitivity of the strains to this bacteriophage was demonstrated by smearing the surface of three 60-mm plates of nutrient agar with a sterile swab dipped into an 18- to 24-h culture in nutrient broth (a fresh swab for each plate). A drop of the phage filtrate was placed on the swabbed agar surface of two plates, and a drop of sterile nutrient broth was placed on the surface of the third plate (control). Sensitivity of the strains to the phage was measured by the appearance of plaques or significantly less growth of the host lawn where the phage suspension had been dropped.

DNA base composition. For determination of guanine-plus-cytosine (G+C) content in the deoxyribo-

nucleic acid (DNA) of the strains, cells were grown to the late log phase in shake flasks of Trypticase soy broth (BBL) with or without additional 2% NaCl, depending on a preliminary comparison of growth of the culture in the two media. The DNA was prepared by the method of Marmur (9) and the thermal melting profile of each sample of DNA was determined spectrophotometrically by the procedure of Marmur and Doty (10). Moles percent of G+C was calculated from the midpoint of the profile.

Preparation of spores for scanning electron photomicroscopy. Untreated spores were taken directly from abundantly sporulating cultures on slants of soil extract agar for examination by scanning electron microscopy (SEM). Spore suspensions were also treated with various concentrations of lysozyme (2) or of lysozyme with trypsin. Other spores were shaken with glass beads or subjected to sonic extraction. They were also extracted with an aqueous two-phase polymer system using polyethylene glycol and potassium phosphate (14) or repeatedly washed with distilled water.

Spores were suspended in a drop of sterile distilled water on a cover slip and air-dried. The cover slip was mounted on a stub; the spores were coated with gold and examined in an ETEC Autoscan SEM System (Etec Corp., Hayward, Calif.).

Remaining tests. The other media and methods used were those of Gordon et al. (7, p. 3-14).

RESULTS

Microscopically, cells of 36 of the 46 strains, grown on soil extract agar for 1 or 2 days at 28°C, were approximately 0.7 μ m in diameter with rounded ends and uniformly staining protoplasm. They were gram positive and motile. Their spores were ellipsoidal or cylindrical, central or paracentral, occasionally subterminal, and did not swell the sporangia appreciably. This description also applies to the cells and spores of strains of the subtilis-group (*Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus*) and of some strains of *Bacillus coagulans*.

The 10 strains whose cells did not resemble in size those of the subtilis-group were NRS strains 1149, 1370, and 1565, whose cells were approximately 1.0 μ m in diameter, and 7 of 17 strains isolated from a salt marsh or seawater whose cells were of the same diameter as that of cells of the subtilis-group but were longer and often filamentous. The spores of these 10 strains, like those of the subtilis-group, were ellipsoidal or cylindrical, central or paracentral, and swelled the sporangia only slightly if at all.

Cultures of 17 strains referred to above (NRS 1560 to 1576) were received in 1962 as isolations from a salt marsh or from seawater (17). When first cultivated on soil extract agar without added NaCl, they produced pleomorphic rods,

approximately $1.2\ \mu\text{m}$ in diameter, filaments, and shadow forms. The cells resembled those of nutritionally unsatisfied cultures. Upon continued cultivation on soil extract agar, the cells gradually decreased in diameter. After 14 years, cells of 9 of the 17 strains resembled cells of the subtilis-group in diameter and length; cells of 7 strains, as previously stated, had the same diameter but were longer than typical cells of the subtilis-group; and cells of 1 strain (NRS 1565) were approximately $1.0\ \mu\text{m}$ in diameter. Cultures of all 17 strains sporulated during the first years of cultivation on soil extract agar. After 14 years, however, only 10 of the 17 continued to sporulate.

Spore surfaces of 9 of the 46 strains (NRS 749, 783, 855, 858, 860, 861, 883, 1070, and 1131) were found to be smooth (Fig. 1) in accordance with the observations of Bradley and Franklin (1). Various attempts to remove the coating apparently covering all the spores were ineffective, and our best photographs were of spores taken directly from the agar slants.

Macroscopically, growth of the 46 cultures on agar was soft, smooth, and usually nonpigmented, but the 17 isolations from a salt marsh or seawater and NRS strain 749 exhibited yellow, pale orange, or salmon pink pigment.

The 46 strains examined shared a distinctive pattern of physiological reactions (Table 2). Positive characteristics of a typical strain (for example, ATCC 14575) were formation of catalase, hydrolysis of starch, production of acid from glucose and mannitol (basal ammonium phosphate agar plus the carbohydrate), growth in 7% NaCl, and growth at 35°C . Negative characteristics were the V-P and egg-yolk reactions, anaerobic growth, growth on Sabouraud dextrose agar and/or broth, resistance to lysozyme, utilization of citrate and propionate, decomposition of tyrosine, production of dihydroxyacetone, and growth at 50°C . The most useful of these properties for the separation of strains of the *B. firmus*-*B. lentus* series from strains of the subtilis-group are presented in Table 3.

Other tests applied to the 46 strains were acid

FIG. 1. Scanning electron photomicrograph of spores of NRS strain 749, a *B. firmus*-*B. lentus* intermediate.

production from arabinose, mannose, melibiose, raffinose, sorbitol, sucrose, and xylose (with nutrient agar containing 3% NaCl and phenol red as the substrate) and sensitivity to a bacteriophage for ATCC strain 14575. Gibson's (5) three tests for separating *B. firmus* and *B. lentus* (decomposition of casein and gelatin and production of urease) were also used as well as reduction of nitrate to nitrite and deamination of phenylalanine (Table 4). The nomenclatural type strain of *B. firmus* (ATCC 14575) and 19 other strains produced acid from only one (sucrose) of the seven carbohydrates; they were

lysed by the phage; and they decomposed casein. Other properties were variable for the 20 strains.

The nomenclatural type strain of *B. lentus* (ATCC 10840) and seven other strains formed acid from arabinose, mannose, melibiose, raffinose, sucrose, and, with the exception of NRS 783, xylose. The eight strains were not lysed by the bacteriophage and, with the exception of NRS strain 1571, they did not decompose casein. Their reactions to the other tests were variable. According to the data presented in Table 4, however, the 20 strains of *B. firmus* and the eight strains of *B. lentus* are connected by 18 intermediate strains. Except for a negative reaction on arabinose and, with the exception of NRS strain 1148, a positive reaction on casein, the 18 intermediate strains exhibit a gradual progression from the comparatively nonsaccharolytic, bacteriophage-sensitive strains of *B. firmus* to the more saccharolytic, phage-resistant strains of *B. lentus*.

The G+C content of the DNA of eleven strains of *B. firmus* (Table 5) ranged from 39.1 to 44.7 mol%, with a mean of 42.5 mol%; the G+C content of seven strains of *B. lentus* varied from 39.0 to 48.3 mol%, averaging 42.5 mol%; and the G+C content of 13 intermediate strains varied from 40.5 to 47.8 mol%, with a mean of 43.6 mol%.

DISCUSSION

According to the data presented here, strains of *B. firmus* and *B. lentus* share a distinctive group of morphological and physiological properties. Other physiological reactions and sensitivity to a bacteriophage for ATCC strain 14575 of *B. firmus*, however, divide our strains into two groups and connect the two groups by a significant number of intermediate strains in confirmation of Cowan's (4) observation that "The different kinds of bacteria are not sepa-

TABLE 2. Some physiological properties of strains of the *Bacillus firmus*-*Bacillus lentus* series

Property	46 strains (% positive)	Typical strain
Production of:		
Acetoin	0	—
Catalase	100	+
Dihydroxyacetone	0	—
Hydrolysis of starch	100	+
Anaerobic growth	0	—
Egg-yolk reaction	0	—
Acid from:		
Glucose	100	+
Mannitol	93	+
Growth in:		
7% NaCl	85	+
Sabouraud dextrose broth	0	—
Sabouraud dextrose agar	0	—
Resistance to lysozyme	0	—
Utilization of:		
Citrate	15	—
Propionate	4	—
Decomposition of tyrosine	9	—
Growth at:		
35°C	98	+
50°C	0	—

TABLE 3. Some criteria for separating strains of the *Bacillus firmus*-*Bacillus lentus* series from morphologically similar strains

Property	Percent positive for:				
	<i>B. firmus</i> - <i>B. lentus</i> , 46 strains	<i>B. licheniformis</i> , ^a 27 strains	<i>B. subtilis</i> , ^a 71 strains	<i>B. pumilus</i> , ^a 27 strains	<i>B. coagulans</i> , ^a 22 strains
Production of acetoin	0	100	97	96	100
Hydrolysis of starch	100	100	100	0	100
Anaerobic growth	0	100	0	0	95
Growth on Sabouraud dextrose agar and/or broth	0	100	100	100	100
Utilization of citrate	15	100	93	96	18
Growth at 50°C	0	100	72	59	100

^a Data from Gordon et al. (7).

TABLE 4. *Spectrum-like arrangement of strains of the Bacillus firmus-Bacillus lentus series based on physiological properties and bacteriophage sensitivity^a*

Strain	Acid from:							SB	C	G	U	N	P
	Ar	Ma	Me	Ra	So	Su	Xy						
<i>B. firmus</i>													
ATCC													
8247	—	—	—	—	—	+	—	+	+	+	—	+	+
14575	—	—	—	—	—	+	—	+	+	+	—	+	+
NRS													
613	—	—	—	—	—	+	—	+	+	+	—	+	+
853	—	—	—	—	—	+	—	+	+	+	—	+	+
854	—	—	—	—	—	+	—	+	+	±	—	+	+
855	—	—	—	—	—	+	—	+	+	+	—	+	+
858	—	—	—	—	—	+	—	+	+	±	—	+	+
860	—	—	—	—	—	+	—	+	+	+	—	+	+
861	—	—	—	—	—	+	—	+	+	+	+	+	+
1070	—	—	—	—	—	+	—	+	+	+	+	+	+
1119	—	—	—	—	—	+	—	+	+	+	—	+	+
1131	—	—	—	—	—	+	—	+	+	+	—	+	+
1132	—	—	—	—	—	+	—	+	+	+	—	+	—
1152	—	—	—	—	—	+	—	+	+	+	—	—	—
1560	—	—	—	—	—	+	—	+	+	+	—	—	+
1561	—	—	—	—	—	+	—	+	+	—	—	—	+
1563	—	—	—	—	—	+	—	+	+	—	—	—	+
1567	—	—	—	—	—	+	—	+	+	+	—	—	—
1568	—	—	—	—	—	+	—	+	+	+	—	—	+
1576	—	—	—	—	—	+	—	+	+	+	—	—	+
Intermediate													
NRS													
1369	—	—	—	—	—	±	—	—	+	+	—	—	—
1329	—	—	—	—	—	+	—	—	+	+	+	—	—
1370	—	—	—	—	—	—	—	—	+	+	—	—	—
1148	— ^b	+	—	—	—	+	—	+	—	+	—	—	—
1573	—	+	—	—	—	—	—	—	+	+	—	—	—
749	—	+	—	—	—	+	—	v^c	+	±	+	+	—
1147	—	+	—	—	—	+	—	—	+	+	—	—	—
1564	—	+	—	—	—	+	—	—	+	±	—	—	+
1151	—	—	—	—	—	+	+	—	+	—	—	+	—
1575	—	—	—	+	—	+	—	+	+	+	—	—	+
1572	—	—	+	+	—	+	—	+	+	+	—	—	—
1562	—	+	+	—	—	+	—	—	+	±	—	—	—
1574	—	+	+	—	—	+	+	—	+	±	—	—	—
1149	—	+	+	±	—	+	—	+	+	+	—	—	—
1570	—	+	+	—	—	+	—	—	+	±	—	—	—
1569	—	+	+	—	+	+	+	—	+	+	—	+	—
1565	—	+	+	+	—	+	±	—	+	+	—	—	+
1566	—	+	+	+	—	+	+	—	+	+	—	—	—
<i>B. lentus</i>													
ATCC													
10840	+	+	+	+	—	+	+	—	—	—	+	—	+
10841	+	+	+	+	—	+	+	—	—	—	+	—	—
NRS													
670	+	+	+	+	+	+	+	—	—	±	+	—	+
769	+	+	+	+	+	+	+	—	—	—	+	+	—
783	+	+	+	+	+	+	—	—	—	+	—	+	—
883	+	+	+	+	—	+	+	—	—	—	+	—	—
1262	+	+	+	+	—	+	±	—	—	—	+	—	—
1571	+	+	+	+	—	+	+	—	+	—	—	+	—

^a Abbreviations: Ar, arabinose; Ma, mannose; Me, melibiose; Ra, raffinose; So, sorbitol; Su, sucrose; Xy, xylose; SB, sensitive to bacteriophage; C, decomposition of casein; G, decomposition of gelatin; U, production of urease; N, reduction of nitrate to nitrite; P, deamination of phenylalanine.

^b Substrate medium for carbohydrates inoculated with strain 1148 was nutrient agar without 3% NaCl.

^c Sensitivity of strain 749 to bacteriophage was variable, sometimes positive, and other times negative.

TABLE 5. DNA base composition of some strains of the *Bacillus firmus*-*Bacillus lentus* series

Strain ^a	Mol% G+C	
	Laboratory 1 ^b	Laboratory 2 ^c
<i>B. firmus</i>		
ATCC		
8247	43.2, 40.7	
14575	43.7	43.7
NRS		
853	42.7	
854	44.6	
855	40.0	
860	45.4	
1070	44.4	
1119	41.7	
1132	39.0	42.9
1152	40.0	
1561	42.2	
Intermediate		
NRS		
1329	43.9	
749		47.8
1147	42.2	
1564		43.2, 43.7
1151	42.9	
1575		45.6
1572		44.1
1562		43.4
1574		44.1
1149	42.4	
1570	40.5	
1565	42.4	
1566	43.7	
<i>B. lentus</i>		
ATCC 10840	42.2	
NRS		
670	39.0, 39.0	
769	40.7	
783	47.3	48.3
883	42.2	
1262	42.4	42.9
1571	42.9	

^a Arranged in same order as in Table 4.^b Determinations made in the laboratory of W. E. Kloos, North Carolina State University, Raleigh, N. C.^c Determinations made in this laboratory.

rated by sharp divisions but by slight and subtle differences in characters so that they seem to blend into each other and resemble a spectrum." A microbial series, or spectrum, is an informal category in taxonomy (8) between subgenus and species. Because we were unable to find a group of properties by which the strains of *B. firmus* and *B. lentus* could be cleanly divided, we believe that these strains provide a good illustration of a microbial series. Connected by intermediate strains, *B. firmus* and *B. lentus* merge and form a spectrum, or

series, of strains.

Bacterial spectra, needless to say, are difficult to classify and lead to endless controversy among taxonomists. The examination of one or two strains from each end of a spectrum, or series of strains, will show distinct differences between them, differences that fade into insignificance when more strains are observed. (Beware of the representation of a species by one or two strains!) The intermediate strains cannot be ignored; they exist and must be incorporated in a classification.

Our present knowledge of our 46 strains of the *B. firmus*-*B. lentus* series supports a lumper's recommendation that all 46 strains be assigned to the same species and that the species *B. firmus* (the oldest species name represented) be defined by the following group of characteristics: gram-positive, motile rods; ellipsoidal, central or paracentral spores that do not swell the sporangia appreciably; G+C content of the DNA varies from 39.0 to 48.3 mol%. The cultures are catalase positive and sensitive to lysozyme. They hydrolyze starch, form acid from glucose and mannitol, and grow in 7% NaCl broth and at 35°C. They do not grow anaerobically, nor on Sabouraud dextrose agar and/or broth, nor at 50°C. Their egg-yolk reaction is negative; they do not utilize citrate or propionate, do not decompose tyrosine, and do not form acetoin or dihydroxyacetone. Variations in one or two of these properties by a strain would not prevent the strain's assignment to *B. firmus*.

The study of spore surfaces of strains of 20 species of *Bacillus* by Bradley and Franklin (1) suggested that the surface configurations might be useful in characterizing some species. The examinations reported here did not offer any means of separating strains of the *B. firmus*-*B. lentus* spectrum and did not contradict our belief that these strains belong to one species.

In contrast to the G+C content of the DNA (as determined by the thermal melting point) of strains of many species of microorganisms, the range of 39.0 to 48.3 mol% observed for 31 of our 46 strains seems unusually wide. For strains of *Bacillus cereus*, *Bacillus stearothermophilus*, and *B. subtilis*, however, equally wide ranges have been reported (11).

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REPRINT REQUESTS

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