DNA Relatedness of *Bacillus brevis* Migula 1900 Strains and Proposal of *Bacillus agri* sp. nov., nom. rev., and *Bacillus centrosporus* sp. nov., nom. rev.

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Studies were carried out to determine the underlying reasons for the phenotypic heterogeneity of *Bacillus brevis* Migula 1900. DNA reassociation measurements demonstrated that 31 of 37 strains segregated into seven groups of genetically related organisms. The members of the seven groups were not related genetically to selected presently recognized *Bacillus* species that are strictly aerobic and form oval spores. The results of the DNA relatedness studies and phenotypic comparisons suggested that two of these groups have not been described previously and that the five other groups probably represent previously, but inadequately, described species. Two of the five groups are proposed as new species and are given the revived names *Bacillus agri* and *Bacillus centrosporus*. Thus, the phenotypic heterogeneity of *B. brevis* sensu lato results from the inclusion of genetically unrelated organisms in the taxon and not from the inherent variability of genetically related strains.

Bacillus brevis, a strictly aerobic species, was first described by Migula in 1900 (8). The discovery of gramicidin production heightened interest in this species and fostered the isolation of numerous strains, many of which varied significantly from the standard strains. Eventually, the heterogeneity among the strains engendered controversy concerning the definition of the species. For example, the gramicidin-producing isolates of Gause and Brazhnikova (5) grew optimally at 60°C but were considered not to be different from the mesophilic strains of Dubos and Hotchkiss (2); thus, a precedent was established for including thermophiles in the species *B. brevis*. Other unusual traits ascribed to strains of *B. brevis* are molluscicidal activity (14) and hyperproduction of proteins (18).

The wide discrepancies in growth temperatures, the occurrence of unusual characteristics in selected strains, and the wide range of G+C values (47 to 55 mol% [18]) suggested that *B. brevis* is a phenotypically heterogeneous species, an observation in keeping with the results of the numerical taxonomic comparisons of Priest et al. (12). Furthermore, in a previous study, only 25 of 87 strictly aerobic strains tentatively classified as *B. brevis* were found to exhibit high levels of DNA relatedness to the type strain of this taxon (10). The present study was undertaken to determine by using DNA reassociation measurements the taxonomic relationships of the strains misclassified as *B. brevis*. The findings of this study suggest that at least seven genetically unrelated species have been erroneously classified as *B. brevis*.

MATERIALS AND METHODS

Bacterial strains. The *B. brevis* strains used in this study are listed in Table 1. Also included were strains NRRL NRS-663, NRRL NRS-1347, NRRL NRS-1351, and NRRL B-1172, the type strains of *Bacillus badius* Batchelor 1919, *Bacillus alginolyticus* Nakamura 1987, *Bacillus chondroitinus* Nakamura 1987, and *Bacillus stearothermophilus* Donk 1920, respectively. These strains are maintained in the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research. Each NRRL designation includes the prefix B-, which identifies strains that were obtained directly from individuals or strains that were

isolated at the National Center for Agricultural Utilization Research, or the prefix NRS-, which identifies strains obtained from the *Bacillus* collection of N. R. Smith. All of the strains bearing the NRS- prefix were deposited in the Agricultural Research Service Culture Collection by R. E. Gordon.

Working stocks were cultured at 30°C on soil extract agar (6) until sporulation occurred and were stored at 4°C.

Characterization. Strain characterization was carried out as described previously (10).

DNA isolation, G+C contents, and DNA reassociation. The procedures used for DNA isolation and purification by hydroxylapatite chromatography and for estimating DNA reassociation values spectrophotometrically have been described previously (11). Reassociation values were calculated by the equation of De Ley et al. (1).

G+C contents of the DNAs were estimated from their buoyant densities as determined by CsCl density gradient centrifugation in a model E ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) (13). *Micrococcus luteus* (synonym, "*Micrococcus lysodeikticus*") DNA with a buoyant density of 1.724 g/cm³ (17) was purchased from Sigma Chemical Co., St. Louis, Mo., and was used as an internal standard.

Numerical analyses. Clustering for DNA relatedness was based on the unweighted-pair-group arithmetic average algorithm (16). The TAXAN program of David Swartz, University of Maryland, College Park, was used to carry out the analyses with a DTK PC-AT personal computer.

RESULTS

The 37 strains examined in this study were strains with G+C contents of 45 to 54 mol% that previously (10) had been shown to be distantly related genetically to *B. brevis* sensu stricto. DNA reassociation determinations performed with all 37 strains (i.e., each strain was compared with all of the other strains) established that 31 of the strains identified as *B. brevis* could be separated into seven distinct DNA relatedness groups (Fig. 1). Relegated to an eighth group were six strains (strains NRRL NRS-751, NRRL NRS-761, NRRL NRS-779, NRRL NRS-857, NRRL NRS-1222, and NRRL NRS-1366)

TABLE 1. *B. brevis* strains compared in this study^a

Strain	Received as strain:	Source ^b	G+C content (mol%)	History
Group 1				
NRRL B-1157		1	54	H. R. Curran strain Penn-O, from A. Bondi, clinical isolate
NRRL B-1158		1	53	H. R. Curran strain Penn-S, from A. Bondi, clinical isolate
NRRL NRS-641	NRS-641	2	52	J. R. Porter, " <i>B. ventricosus</i> ," from G. Bredemann
NRRL NRS-873	NRS-873	2	53	J. R. Porter, " <i>B. hollandicus</i> " H1, from G. Bredemann
NRRL NRS-874	NRS-874	2	54	J. R. Porter, " <i>B. hollandicus</i> " H2, from G. Bredemann
NRRL NRS-1002	NRS-1002	2	54	J. R. Porter, " <i>B. ventricosus</i> ," from G. Bredemann
NRRL NRS-1028	NRS-1028	2	53	A. Bondi strain Schotte
NRRL NRS-1060	NRS-1060	2	55	C. T. Townsend and T. L. Zuck strain A
NRRL NRS-1219	NRS-1219	2	53	C. Lamanna, " <i>B. agri</i> " 13
NRRL NRS-1373	NRS-1373	2	52	W. G. Walter strain 141
Group 2				
NRRL NRS-376	NRS-376	2	49	N. R. Smith, from soil
NRRL NRS-378	NRS-378	2	49	N. R. Smith, from soil
NRRL NRS-379	NRS-379	2	49	N. R. Smith, from soil
NRRL NRS-380	NRS-380	2	50	N. R. Smith, from soil
Group 3				
NRRL NRS-135	NRS-135	2	51	W. C. Bohrer strain NCA 11773-2, from spinach
NRRL NRS-136	NRS-136	2	50	W. C. Bohrer strain NCA 11773-8, from spinach
NRRL NRS-628	NRS-628	2	51	J. R. Porter, " <i>B. pallidus</i> ," from G. Bredemann
NRRL NRS-631	NRS-632	2	50	J. R. Porter, " <i>B. rarus</i> ," from G. Bredemann
NRRL NRS-664	NRS-664	2	49	B. S. Henry strain 120, " <i>B. centrosporus</i> "
NRRL NRS-925	NRS-925	2	51	J. R. Porter, " <i>B. pallidus</i> " P2, from G. Bredemann
Group 4				
NRRL B-541	WRRL 550	3	48	R. J. Dubos
NRRL NRS-780	NRS-780	2	51	R. J. Dubos strain TC, from Turkish cheese
NRRL NRS-815	NRS-815	2	53	J. R. Porter, " <i>B. bellus</i> " B1, from G. Bredemann
Group 5				
NRRL NRS-1206	NRS-1206	2	53	H. W. Heuszer Army strain 39
NRRL NRS-1207	NRS-1207	2	53	H. W. Heuszer Army strain 723
NRRL NRS-1208	NRS-1208	2	48	H. W. Heuszer Army strain 842
Group 6				
NRRL NRS-863	NRS-863	2	45	J. R. Porter, " <i>B. formosus</i> " F12, from G. Bredemann
NRRL NRS-948	NRS-948	2	50	J. R. Porter, " <i>B. rufescens</i> " R8, from G. Bredemann
NRRL NRS-1210	NRS-1210	2	47	H. W. Heuszer Army strain 1035
Group 7				
NRRL NRS-818	NRS-818	2	52	J. R. Porter, " <i>B. borstelensis</i> "
NRRL NRS-1029b	NRS-1029b	2	54	G. M. Eisenberg, " <i>Bacillus schuykilliensis</i> "
Group 8				
NRRL NRS-751	NRS-751	2	53	R. J. Dubos gramicidin strain, from soil (= ATCC 8185) ^c
NRRL NRS-761	NRS-761	2	49	L. T. Leonard, from soil
NRRL NRS-779	NRS-779	2	48	R. J. Dubos strain oka, from oka cheese (= ATCC 8186)
NRRL NRS-857	NRS-857	2	50	J. R. Porter, <i>Bacillus firmus</i> d-Utliberg F6, from G. Bredemann
NRRL NRS-1222	NRS-1222	2	50	D. H. Rose, from an orange
NRRL NRS-1366	NRS-1366	2	49	R. Y. Stanier strain 13

^a Names in quotation marks are not on the Approved Lists of Bacterial Names (15) and have not been validly published since 1 January 1980.

^b 1, N. R. Smith collection maintained by R. E. Gordon, Rutgers University, New Brunswick, N.J.; 2, N. R. Smith, Agricultural Research Center, Beltsville, Md.; 3, Western Regional Research Center, Albany, Calif.

^c Designations in parentheses are equivalent strain designations.

that were distantly related to each other and to the other groups. The G+C contents of the members of groups 1 through 7 were 52 to 55, 49 to 50, 49 to 51, 48 to 53, 48 to 53, 45 to 50, and 52 to 54 mol%, respectively. The G+C contents of the six unrelated strains ranged from 48 to 53 mol%.

Previously recognized strictly aerobic species that have G+C contents of 44 to 52 mol% and produce oval spores include *B. badius*, *B. chondroitinus*, *B. alginolyticus*, and *B. stearothermophilus*. DNA complementarity values of 30% or less were measured between the reference strains of the groups and the type strains of the previously recognized species (Table 2).

The phenotypic characteristics of the seven relatedness groups and *B. brevis* sensu stricto are shown in Table 3. In general, whereas *B. brevis* sensu lato exhibited pronounced

phenotypic heterogeneity, each of the groups was highly homogeneous. In contrast, the six unrelated strains formed a phenotypically heterogeneous group. The members of all of the groups were strictly aerobic, produced oval spores in swollen sporangia, were catalase positive, and produced negative responses in tests for growth at 50°C, starch hydrolysis, acetylmethylcarbinol synthesis, urease activity, egg yolk lecithin decomposition, hydrogen sulfide production, indole production, propionate utilization, and fermentation of arabinose, lactose, melibiose, salicin, sorbitol, and xylose.

DISCUSSION

A previous study of 87 strains identified as *B. brevis* revealed that 80 of these strains had G+C contents of 45 to

FIG. 1. Dendrogram showing the levels of DNA relatedness among the *B. brevis* strains studied, determined by using the simple matching coefficient and the unweighted-pair-group arithmetic average algorithm.

56 mol%, a range that included the G+C content (49 mol%) of the type strain (10). Seven strains had G+C values of 41 mol% or less; these strains were probably not *B. brevis* strains. Only 24 of the 80 organisms with G+C contents of 45 to 56 mol% formed a tight genetic cluster with the type strain (10) and thus could be considered members of the species

sensu stricto. In this study 37 of the remaining 55 strains were examined to determine their taxonomic relationships.

On the basis of DNA reassociation data, seven distinct groups of genetically related organisms were identified among the 37 strains formerly identified as *B. brevis*. According to the data in Table 3, each of the groups tends to be phenotypically homogeneous and can be readily distinguished from the other groups, as well as from *B. brevis*. These data established that the seven groups are genetically and phenotypically distinct entities.

The range of G+C contents (45 to 56 mol%) for the seven DNA relatedness groups encompasses the range of DNA base contents for previously described strictly aerobic, oval-spored species, including *B. alginolyticus* Nakamura 1987, *B. chondroitinus* Nakamura 1987, and *B. stearothersophilus* Donk 1920. An additional species that can be considered is *B. badius*, whose members have G+C contents of 44 to 47 mol%, a range of values just outside the range exhibited by the seven relatedness groups. Low DNA relatedness values (30% or less) (Table 2) indicate that none of the seven groups is related to the four previously described species mentioned above.

Phenotypically, the inability of the members of all seven groups to grow at 50°C eliminates classification as *B. badius* or *B. stearothersophilus* (Table 4). Furthermore, growth in the presence of 5% NaCl and an inability to ferment glucose differentiate *B. badius* from the seven groups. Minimal growth temperatures of 30 to 45°C, an acid pH in Voges-Proskauer broth, hydrolysis of starch, and an inability to degrade tyrosine are other characteristics that are exhibited by *B. stearothersophilus* but not by the members of the seven groups. Negative responses for alginate and starch hydrolysis, as well as for the fermentation of arabinose and xylose, indicate that the seven groups are phenotypically distinct from *B. alginolyticus* and *B. chondroitinus*.

DNA relatedness measurements and the results of phenotypic studies have demonstrated that the majority of strains studied have been erroneously classified as *B. brevis*. According to the results of such studies, 37 erroneously classified strains were members of seven genetically unrelated groups; these groups probably represent seven distinct species. Two of the groups represent species that have not been described previously, but some members of the remaining five groups have been classified as species other than *B. brevis* by previous researchers. It is interesting that the previous workers considered the phenotypic differences significant enough to give the organisms separate names. For example, strains in group 1 were received as "*Bacillus hollandicus*" Meyer and Stapp 1920, "*Bacillus ventricosus*" Bredemann and Heigener 1935, and "*Bacillus agri*" Laubach and Rice 1916; strains in group 3 were received as

TABLE 2. Levels of DNA relatedness between the seven group reference strains and selected *Bacillus* type strains

DNA from:	G+C content (mol%)	% Reassociation with DNA from strain ^a :						
		NRRL NRS-1206	NRRL B-1157	NRRL NRS-135	NRRL B-541	NRRL NRS-818	NRRL NRS-863	NRRL NRS-376
<i>B. badius</i> NRRL NRS-663 ^{Tb}	45	25	20	18	22	23	27	15
<i>B. alginolyticus</i> NRRL NRS-1347 ^T	48	26	18	17	12	16	13	11
<i>B. chondroitinus</i> NRRL NRS-1351 ^T	49	26	29	21	21	12	26	17
<i>B. stearothersophilus</i> NRRL B-1172 ^T	52	30	22	25	19	18	20	30

^a Reassociation values are averages of two determinations. The maximum variation observed was 6%.

^b T = type strain.

TABLE 3. Phenotypic characteristics of the *B. brevis* DNA relatedness groups

Characteristic	% of positive strains in ^a :								<i>B. brevis</i> (n = 25) ^c
	Group 1 (n = 10) ^b	Group 2 (n = 4)	Group 3 (n = 6)	Group 4 (n = 3)	Group 5 (n = 3)	Group 6 (n = 3)	Group 7 (n = 2)	Group 8 (n = 6)	
Anaerobic growth	0	0	0	0	0	0	0	0	0
Growth in the presence of 3% NaCl	0	0	0	0	0	0	0	0	0
Growth at pH 5.6	100	100	0	100	100	100	100	75	100
Growth at 50°C	0	0	0	0	0	0	0	0	0
Growth in the presence of 0.001% lysozyme	0	0	0	0	0	100	0	12	0
Casein hydrolysis	100	0	0	100	0	100	0	43	100
Starch hydrolysis	0	0	0	0	0	0	0	0	0
Nitrate reduced to nitrite	0	0	33	100	0	100	0	57	100
Voges-Proskauer test ^d	0	0	0	0	0	0	0	0	0
Urease	0	0	0	0	0	0	0	0	0
Egg yolk lecithinase	0	0	0	0	0	0	0	0	0
H ₂ S production	0	0	0	0	0	0	0	0	0
Catalase	100	100	100	100	100	100	100	100	100
Oxidase	0	0	0	0	0	0	0	0	100
Indole	0	0	0	0	0	0	0	0	0
pH in Voges-Proskauer broth of >7.0	100	100	100	100	0	100	100	100	100
Utilization of citrate	0	100	33	100	0	66	0	57	100
Utilization of propionate	0	0	0	0	0	0	0	0	0
Fermentation of:									
Arabinose	0	0	0	0	0	0	0	0	0
Cellobiose	50	0	100	100	100	0	0	57	100
Fructose	100	100	0	0	100	100	100	43	100
Galactose	0	0	0	0	0	0	0	0	100
Glucose	100	100	100	100	100	100	100	100	100
Lactose	0	0	0	0	0	0	0	0	0
Maltose	100	100	100	100	100	100	100	86	100
Mannitol	100	100	100	100	100	0	0	71	64
Mannose	0	0	0	0	0	0	0	0	64
Melibiose	0	0	0	0	0	0	0	0	0
Rhamnose	0	100	100	0	0	0	0	0	0
Ribose	100	100	100	67	100	0	100	100	100
Salicin	0	0	0	0	0	0	0	0	0
Sorbitol	0	0	0	0	0	0	0	0	0
Sucrose	100	100	100	100	0	0	0	57	100
Trehalose	100	100	100	100	0	0	0	57	100
Xylose	0	0	0	0	0	0	0	0	0

^a The members of groups 1 through 7 are shown in Fig. 1. Group 8 contained the miscellaneous unrelated organisms.

^b n is the number of strains in the group.

^c Data from reference 10.

^d Test for acetylmethylcarbinol synthesis.

"*Bacillus centrosporus*" Ford 1916, "*Bacillus pallidus*" Bredemann and Heigener 1935, and "*Bacillus rarus*" Bredemann and Werner 1933; strains in group 4 were received as "*Bacillus bellus*" Bredemann and Heigener 1935; strains in group 6 were received as "*Bacillus formosus*" Bredemann and Heigener 1935 and "*Bacillus rufescens*" Bredemann and Stuhk 1935; and strains in group 7 were received as "*Bacillus borstelensis*" Bredemann and Stuhk 1935. Because the genetic distinctiveness and phenotypic distinctiveness of groups 1 and 3 have been based on studies of adequate numbers of strains, these groups should be considered for proposal as the new species *Bacillus agri* and *Bacillus centrosporus*. In contrast to the description of Ford et al. (4), in this study no predilection by *B. centrosporus* to form spores centrally was observed. However, inasmuch as these are the oldest names used for members of these groups, the names *B. agri* and *B. centrosporus* are revived and used for the same taxa for which they were used originally, in accordance with Rules 27 and 28a of the *International Code of Nomenclature of Bacteria* (7). Strains NRRL NRS-1219

and NRRL NRS-664 are the type strains of *B. agri* and *B. centrosporus*, respectively. Descriptions of the species are given below.

Also present in this study were a number of strains which were not genetically related *B. brevis* sensu stricto or to the seven relatedness groups and displayed widely varied characteristics. Consequently, the phenotypic heterogeneity of *B. brevis* sensu lato was not due to the inherent variability of genetically related strains but was derived from the inclusion of a number of unrelated organisms in the taxon.

Description of *Bacillus agri* sp. nov., nom. rev. *Bacillus agri* (ag'ri. Gr. n. *agrus*, field; L. gen. n. *agri*, of a field).

Rods that are 0.5 to 1.0 by 2.0 to 5.0 µm. Gram positive. Motile. Ellipsoidal spores are produced in swollen sporangia.

Agar colonies are nonpigmented, translucent, thin, smooth, circular, entire, and about 2 mm in diameter.

Catalase positive. Oxidase is not produced.

Strictly aerobic.

TABLE 4. Characteristics that differentiate groups 1 through 7 from *B. badius*, *B. alginolyticus*, *B. chondroitinus*, and *B. stearotheophilus*

Characteristic	Group							<i>B. badius</i> ^a	<i>B. alginolyticus</i> ^b	<i>B. chondroitinus</i> ^b	<i>B. stearotheophilus</i> ^a
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7				
pH in Voges-Proskauer broth	>7.0	>7.0	>7.0	>7.0	<7.0	>7.0	>7.0	>7.0	>7.0	>7.0	<7.0
Maximum growth temp (°C)	35–40	35–40	35–40	35–40	35–40	35–40	35–40	45–50	35–40	35–40	65–75
Minimum growth temp (°C)	5–10	5–10	5–10	5–10	5–10	5–10	5–10	10–15	5–10	5–10	30–45
Growth in the presence of 0.001% lysozyme	– ^c	–	–	–	–	+	–	–	+	–	–
Growth at pH 5.6	+	+	–	+	+	+	+	–	+	+	–
Growth in the presence of 5% NaCl	–	–	–	–	–	–	–	+	–	–	v
Fermentation of:											
Arabinose	–	–	–	–	–	–	–	–	+	+	v
Glucose	+	+	+	+	+	+	+	–	+	+	+
Mannitol	+	+	+	+	+	–	–	–	+	+	v
Xylose	–	–	–	–	–	–	–	–	+	+	v
Alginate hydrolysis	–	–	–	–	–	–	–	–	+	+	–
Casein hydrolysis	+	–	–	–	–	+	–	+	–	–	v
Starch hydrolysis	–	–	–	–	–	–	–	–	+	+	+
Tyrosine hydrolysis	+	+	+	+	+	+	+	+	–	–	–
Citrate utilization	–	–	v	+	–	v	–	+	–	–	+
Nitrate reduction	–	–	v	+	–	+	–	–	–	–	v
G+C content (mol%)	52–55	49–50	49–51	48–53	48–53	45–50	49–53	44–47	47–49	47–48	44–52

^a Physiological characteristics taken from reference 6; G+C values taken from reference 3.^b Data taken from reference 9.^c –, negative reaction; +, positive reaction; v, variable reaction.

Acetylmethylcarbinol, hydrogen sulfide, and indole are not produced.

The pH in Voges-Proskauer broth (test for acetylmethylcarbinol production) is higher than 7.0.

Nitrate is not reduced to nitrite.

Starch, urea, and egg yolk lecithin are not hydrolyzed. Casein is hydrolyzed.

Citrate and propionate are not utilized.

Growth occurs at pH 5.6 or 5.7. Growth is inhibited by 3% NaCl, 0.001% lysozyme, and 50°C.

The optimum growth temperature is 28°C, the maximum growth temperature is 40°C, and the minimum growth temperature is 10°C.

Acid but no gas is produced from fructose, glucose, maltose, mannitol, ribose, sucrose, and trehalose. Variable acid production occurs with cellobiose. Arabinose, galactose, lactose, mannose, melibiose, rhamnose, salicin, sorbitol, and xylose are not fermented.

The DNA buoyant density is 1.7055 to 1.7084 g/cm³; the G+C content is 52 to 55 mol%.

Isolated from soil and clinical material.

The type strain, strain NRRL NRS-1219, has been deposited in the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research, Peoria, Ill.

Description of *Bacillus centrosporus* sp. nov., nom. rev. *Bacillus centrosporus* (cen.tro.spor'us. L. n. *centrum*, the center; M. L. n. *spora*, spore; M. L. adj. *centrosporus*, with a central spore).

Rods that are 0.5 to 1.0 by 2.0 to 6.0 µm. Gram positive. Motile. Ellipsoidal spores are produced in swollen sporangia.

Agar colonies are nonpigmented, translucent, thin, smooth, circular, entire, and 2 to 3 mm in diameter.

Catalase positive. Oxidase is not produced.

Strictly aerobic.

Acetylmethylcarbinol, hydrogen sulfide, and indole are not produced.

The pH in Voges-Proskauer broth (test for acetylmethylcarbinol production) is higher than 7.0.

Nitrate is reduced to nitrite by some strains.

Casein, egg yolk lecithin, starch, and urea are not hydrolyzed.

Citrate, but not propionate, is utilized by some strains.

Growth is inhibited at pH 5.6 or 5.7 and by 3% NaCl, 0.001% lysozyme, and 50°C.

The optimum growth temperature is 28°C, the maximum growth temperature is 40°C, and the minimum growth temperature is 10°C.

Acid but no gas is produced from cellobiose, glucose, maltose, mannitol, rhamnose, ribose, sucrose, and trehalose. Arabinose, fructose, galactose, lactose, mannose, melibiose, salicin, sorbitol, and xylose are not fermented.

The DNA buoyant density is 1.7025 to 1.7045 g/cm³; the G+C content is 49 to 51 mol%.

The type strain, strain NRRL NRS-664, has been deposited in the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research, Peoria, Ill.

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