DNA Similarities among Mosquito-Pathogenic and Nonpathogenic Strains of *Bacillus sphaericus*

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***Bacillus sphaericus* strains isolated on the basis of pathogenicity for mosquito larvae and strains isolated on the basis of a reaction with a *B. sphaericus* DNA homology group IIA 16S rRNA probe were analyzed for DNA similarity. All of the pathogens belonged to homology group IIA, but this group also contained nonpathogens. It appears inappropriate to designate this homology group a species based solely upon pathogenicity.**

Aerobic bacilli that form spherical endospores are common in soil and water and are usually classified as *Bacillus sphaericus*. There are few useful phenotypic tests for identification of these bacteria. Spore morphology combined with negative reactions in tests for fermentation products and extracellular enzymes have been the basis for taxonomic placement. The species was found to be comprised of at least five distinct homology groups, each sufficiently separated from the others to merit species status (5). Representative strains of the homology groups have also been examined by rRNA gene restriction fragment length polymorphisms analyses (ribotyping), and these analyses confirmed that there are distinct groups within the *B. sphaericus* complex (2). Recently, randomly amplified polymorphic DNA analysis has also clearly distinguished the groups originally identified by DNA similarity analysis (9). These five groups have not been designated separate species because of the lack of readily utilizable phenotypic tests to distinguish them.

In the original study of Krych et al. (5), group II was divided into two subgroups based on levels of DNA similarity and DNA heteroduplex stability. It was of considerable interest that all of the isolates in group IIA were pathogenic for mosquito larvae. No mosquito pathogens were found in any other group. These bacteria are pathogenic because they produce one or more of four toxins, a binary toxin composed of two distinct proteins and three additional toxins designated Mtx, Mtx2, and Mtx3 (6–8). Strains that produce the binary toxin are highly toxic (50% lethal concentrations, around 10^2 to 10^3 cells ml⁻¹), and strains that produce only toxins Mtx, Mtx2, and Mtx3 have low toxicity (50% lethal concentrations, about 10^5 to 10^7 cells ml⁻¹). It appeared that the group IIA mosquito pathogens might be designated a separate species. However, only seven pathogenic isolates were available at the time of the original DNA similarity study. Now, many more pathogenic isolates from many geographic locations are available, and although they have been referred to as group IIA strains on the basis of ribotyping data, DNA similarity studies have never actually been performed with them. In this paper we report DNA similarity results for a large number of strains from diverse geographic locations.

Bacteria and DNA isolation. The strains of *B. sphaericus* used in this study are listed in Table 1. The bacteria were grown in NY broth (Difco nutrient broth supplemented with 0.05% yeast extract) at 30°C with shaking at 150 rpm. Cells were recovered by centrifugation, suspended in 20 ml of pH 8.0 buffer (10 mM Tris, 1.0 mM EDTA, 0.35 M sucrose, 0.1 mg of

TABLE 1. Strains used in this study

Strain	Source	Geographic origin	Serotype
ATCC 14577 ^{1a}	— ^b	NA ^c	NA
1593 ^d	—	Indonesia	5a5b
ATCC 7055	—	NA	NA
NRS 592	—	NA	NA
NRS 400	—	NA	NA
NRS 1198	—	NA	NA
Kellen Q ^d	—	United States	1a
SSII-1 ^d	—	India	2a2b
1404 ^d	—	The Philippines	2a2b
1881 ^d	—	El Salvador	5a5b
1691 ^d	—	El Salvador	5a5b
1885 ^d	Singer	Israel	2a2b
LP12-AS ^d	Priest	Singapore	3
LP1-G ^d	Priest	Singapore	3
LP7-A ^d	Priest	Singapore	3
IAB881 ^d	Ofori	Ghana	3
2362 ^d	Weiser	Nigeria	5a5b
BSE18 ^d	Priest	Scotland	5a5b
2013-6 ^d	Singer	Romania	5a5b
2317-3 ^d	Singer	Thailand	5a5b
2500 ^d	Singer	Thailand	5a5b
TS-1 ^d	Gaixin	People's Republic of China	5a5b
JI21 ^d	Ibarra	Mexico	5a5b
JI42 ^d	Ibarra	Mexico	5a5b
2117-2 ^d	Singer	The Philippines	5a5b
RS1A ^d	deSouza	Brazil	NA
RS2A ^d	deSouza	Brazil	NA
S2 ^d	Vilarinhos	Brazil	5a5b
TG148 ^d	Guaycurus	Brazil	5a5b
TG229 ^d	Guaycurus	Brazil	5a5b
CIB14 ^d	Orduz-Peralta	Nigeria	NA
IAB59 ^d	Ofori	Ghana	6
IAB460 ^d	Ofori	Ghana	6
IAB871 ^d	Ofori	Ghana	6
31-2 ^d	Cokmus	Turkey	9a9c
2297 ^d	Wickremsinghe	Sri Lanka	25
TG365 ^d	Guaycurus	Brazil	25
2173 ^d	Amonkar	India	26a26b
2377 ^d	Amonkar	India	26a26b
G4a	Priest	Brazil	NA
Gt1-a	Priest	Brazil	6
Gt1-d	Priest	Brazil	2
R1e	Priest	Brazil	6
R4a	Priest	Brazil	26a26b

^a T = type strain.

^b —, see reference 5.

^c NA, not applicable or not available.

^d Strain reported to be pathogenic for mosquitoes.

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TABLE 2. Levels of DNA similarity among strains of *B. sphaericus*

Unlabeled strain	% Similarity with:						Strain Gt1-a
	Group I strain ATCC 14577 ^T	Group IIa strain 1593	Group IIb strain ATCC 7055	Group III strain NRS 592	Group IV strain NRS 400	Group V strain NRS 1198	
ATCC 14577 ^T	100	26	27	26	28	29	26
1593	26	100	71	45	41	29	90
ATCC 7055	26	74	100	54	46	31	75
NRS 592	28	46	43	100	53	30	46
NRS 400	28	47	44	32	100	33	50
NRS 1198	29	31	28	57	31	100	52
Kellen Q	26	91	69	48	43	30	90
SSII-1	27	93	72	45	43	30	91
1404	26	91	71	45	43	30	90
1881	26	100	71	46	44	30	93
1691	32	98	69	44	45	30	92
1885	31	91	73	46	44	29	91
LP12-AS	27	92	76	46	43	30	91
LP1-G	31	88	68	48	45	30	92
LP7-A	26	93	71	47	45	30	90
IAB 881	26	90	70	47	40	30	89
2362	33	98	70	45	45	30	90
BSE 18	33	99	66	45	41	30	89
2013-6	26	99	71	46	44	30	93
2317-3	26	100	69	46	41	30	89
2500	27	100	71	47	44	30	92
Ts-1	26	93	69	47	43	29	90
JI 21	31	98	70	45	43	30	89
JI 42	27	98	70	46	43	30	85
2117-2	26	98	71	48	43	30	92
RS1A	31	99	72	47	43	30	92
RS2A	26	98	68	45	43	29	90
S2	25	95	66	48	39	29	88
TG 148	26	100	70	48	44	29	91
TG 299	26	97	71	43	42	30	92
CIB NIG 14	33	98	71	47	43	30	93
IAB 59	27	91	70	48	43	29	90
IAB 460	26	84	70	45	40	30	89
IAB 871	26	93	70	46	42	30	92
31-2	26	90	69	47	43	29	88
2297	30	89	70	47	44	29	88
TG 365	29	93	69	47	44	31	90
2173	27	91	72	45	45	28	92
2377	27	91	68	45	44	31	89
G4a	63	91	73	51	46	33	91
Gt1-a	27	90	71	47	44	32	100
Gt1-d	28	92	72	47	42	30	91
R1e	26	90	71	48	42	29	92
R4a	26	89	70	42	41	30	89

lysozyme per ml), and incubated at 37°C for 30 min. A 20-ml portion of lysing solution (100 mM Tris, 20 mM EDTA, 0.3 M NaCl, 2% [wt/vol] sodium dodecyl sulfate, 2% [vol/vol] β -mercaptoethanol, 100 μ g of proteinase K per ml) was added to each preparation, and the mixture was incubated at 55°C for 1 h. Protein was removed by multiple phenol-chloroform extractions, and DNA was precipitated with 0.6 volume of isopropanol. The DNA was dried and suspended in 20 ml of TE, 250 μ l of an RNase solution (1 mg of RNase A per ml, 4,000 U of RNase T₁ per ml) was added, and the preparation was incubated 1 h at 37°C. The DNA was chloroform extracted and precipitated with ethanol. The precipitated DNA was dissolved in 3 ml of TE and frozen.

DNA homologies. DNA was sheared in a French pressure cell and labeled with ¹²⁵I, and a hybridization analysis was performed by using the S1 nuclease method (4). DNA samples were heated for 5 min at 60°C before they were used. Reaction tubes containing 10 μ l of labeled DNA (0.4 mg/ml), 50 μ l of

unlabeled DNA (0.4 mg/ml), and 50 μ l of buffer (13.2 $\times$ SSC, 5 mM HEPES; pH 7.0 [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) were incubated at 60°C for 24 h to allow reassociation. Following this incubation, 1 ml of buffer (0.3 M NaCl, 0.05 M acetic acid, 0.5 mM ZnCl₂), 100 U of S1 nuclease, and 50 μ l of denatured salmon sperm DNA (0.4 mg/ml) were added to each reaction mixture, and the mixture was incubated for 1 h at 50°C. Then 0.5 ml of HCl buffer (1 M HCl, 1% Na₄P₂O₇, 1% NaH₂PO₄) and 50 μ l of native salmon sperm DNA (1.2 mg/ml) were added to the reaction mixture, and the preparation was incubated for 1 h at 4°C to precipitate the DNA. The precipitated DNA was collected on Whatman glass fiber filters and counted with a gamma counter.

The strains used as reference strains for the homology groups were the same as those used in the study of Krych et al. (5). An additional strain, strain Gt1-a, was labeled and also used as a reference. Also, six of the seven pathogenic strains included in group IIA in the original study were analyzed

again. A total of 27 additional mosquito pathogens from a variety of geographic locations were included in the study. These pathogens had been isolated on the basis of their ability to kill mosquito larvae. Each of these isolates, regardless of its level of toxicity, was found to be a member of homology group IIA (Table 2). This suggests that the four genes that have been identified as being responsible for toxicity in these bacteria have not been transferred beyond this genetically defined group. As long as isolations were made on the basis of mosquito pathogenicity, it appeared that homology group IIA might contain only these distinctive pathogenic bacteria.

Jahnz et al. (3) utilized an oligonucleotide probe based on a specific region of 16S rRNA from group IIA strains (1) to isolate group IIA strains not on the basis of pathogenicity but on the basis of membership in homology group IIA. These authors recovered 20 strains from Brazilian soil that produced ribotype and isozyme patterns typical of group IIA. However, these strains lacked mosquito pathogenicity, and probes for the binary toxin and Mtx toxin revealed that the genes for these toxins were absent. We included five of these strains in this study (strains G4a, Gt1-a, Gt1-d, R1e, and R4a) and utilized Gt1-a as a labeled reference strain. The high levels of homology of these strains to 1593, the group IIA reference strain, leaves no doubt that they are in fact members of homology group IIA. In addition, the group IIA pathogens exhibited high levels of homology to Gt1-a.

Therefore, it appears that although all of the pathogens belong to homology group IIA, this homology group also contains nonpathogens. It is interesting that Jahnz et al. (3) recovered only nonpathogens when they used their probe. These authors suggested that the nonpathogens may, in fact, be more

common in soil than the homology group IIA pathogens. Whether a pathogen or a nonpathogen is isolated may simply depend on the method used for selection (i.e., pathogenicity or response to the group IIA probe). In view of this, it does not seem appropriate to utilize mosquito pathogenicity as the sole characteristic for defining a new species based on homology group IIA.

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