

Isolation and genetic characterization of *Lysinibacillus sphaericus* strains found in mosquito larvae (Diptera: Culicidae)

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Introduction: *Lysinibacillus sphaericus* is a highly effective and specific bioinsecticide used for the control of Culicidae larvae.

Objective: This study aimed to identify and characterize *L. sphaericus* strains isolated from *Culex quinquefasciatus* larvae in Brazil.

Methods: *C. quinquefasciatus* larvae were collected from streams in the urban area of São Paulo state. *L. sphaericus* strains were identified through cytomorphology, biochemical, and physiological analyses. Qualitative bioassays were performed to evaluate the toxicity of the strains against *C. quinquefasciatus*. The crystal compound protein pattern of *L. sphaericus* strains was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Five reference strains were used as standards in all tests performed. Repetitive extragenic palindromic elements-polymerase chain reaction (REP-PCR) was utilized in an attempt to differentiate pathogenic and nonpathogenic isolates.

Results: Twenty-one strains were isolated. Only one presented toxic activity against *C. quinquefasciatus*. REP-PCR results identified 23 patterns among the 26 strains used in the study, and the fragment analysis showed low similarity (16%) between *L. sphaericus* isolates and the five reference strains.

Conclusion: Comparison of strains isolated in this study using REP-PCR showed a low similarity to other strains, demonstrating the high intraspecific variability for *L. sphaericus*.

Keywords: *Lysinibacillus sphaericus*, Culicidae, SDS-PAGE, qualitative bioassays, REP-PCR, mosquitoes, entomopathogenic bacteria

Introduction

Lysinibacillus sphaericus, previously described as *Bacillus sphaericus*, is an obligately aerobic, mesophilic, round-spore shaped bacterium that naturally occurs in soil.¹ It is characterized by the lack of carbohydrate fermentation when evaluated using standard phenotypic tests for *Bacillus* identification.² Some strains may be highly toxic to some species of mosquito larvae, such as *Culex* sp., *Anopheles* sp., and *Psorophora* sp., but show low toxicity against *Aedes* sp. larvae.³ However, strains of *L. sphaericus* can be found as part of the natural microbiota of these larvae without causing any disease. Moreover, the specificity of *L. sphaericus* for mosquito larvae eliminates the risks for nontarget organisms and vertebrates such as fish, birds, and mammals.³ For these reasons, this bacterium has been used for mosquito biocontrol.⁴

L. sphaericus is widely used for the control of *Culex quinquefasciatus* in the states of Pernambuco and São Paulo, Brazil, and for *Anopheles* spp. in the Amazon region. Other countries in Latin America, such as Colombia, Mexico, Guatemala, Nicaragua, and Honduras, have been subject to extensive tests of products based on

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L. sphaericus strain 2362 to control the vectors of malaria, obtaining promising results, with a >90% reduction rate of anopheline populations.⁴

The mosquitocidal characteristics of *L. sphaericus* are attributed to the presence of two different types of toxins. The binary toxin (Bin) is expressed as a parasporal body divided into two polypeptides of 42 and 51 kDa, and is known as BinA and BinB, respectively. Both of these polypeptides are required for high toxicity to mosquito larvae. Some toxic strains also produce the 100, 32, and 35 kDa toxins encoded, respectively, by *mtx1*, *mtx2*, and *mtx3* genes. Strains considered highly toxic are those that produce the binary toxin and also harbor *mtx* genes.³ The mode of action of these crystal toxins is very similar to the Cry toxins of *Bacillus thuringiensis* serovar israelensis in mosquito larvae.⁵ The aim of this study was to identify and characterize several strains of *L. sphaericus* isolated from Culicidae larvae in Brazil.

Methods

Collection of larvae and species identification

Culex sp. and *Aedes* sp. larvae were collected from rivers in São Paulo state, Brazil, and transferred, on ice, to the laboratory where they were submitted to sterilization and heat treatment according to Alves⁶ and Cavados et al.⁷

Strains of *L. sphaericus* were identified using cytology, biochemistry, and physiological analyses, and the results were analyzed with the support of a dichotomous key.²

Qualitative bioassays

Qualitative bioassays were performed to evaluate the toxicity of isolated strains against *C. quinquefasciatus* Say (Diptera: Culicidae) larvae under laboratory conditions. As toxicity references, lineages of *L. sphaericus* nonmosquitocidal SSII-1 (LFB-FIOCRUZ 847) and *L. sphaericus* mosquitocidal 2362 (LFB-FIOCRUZ 736) were used. All lineages were tested in duplicate and larvae without bacterial suspensions were used as negative controls. The biological activity was evaluated according to Oliveira et al⁸ and Araújo da Silva et al.⁹

Analyses of *L. sphaericus* crystal protein profile

The protein profile of the crystal components of *L. sphaericus* strains was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), following the procedure described by Laemmli and Favre.¹⁰ Some modifications were made, such as growth in nutrient yeast extract salt media

and use of Tris-EDTA (TE) buffer (Tris-HCl 1 M, pH 8.0; EDTA 0.5 M, pH 8.0) in centrifugation. Electrophoresis was carried out on a 10% resolving gel (pH 8.0) and a 5% stacking gel (pH 6.8) at 90 V for 45 minutes.

The following were used as standards in all tests performed: 1) pathogenic *L. sphaericus* LFB-FIOCRUZ 736, serotype H5a5b, strain 2362; 2) pathogenic LFB-FIOCRUZ 735, serotype H5a5b, strain 1593; 3) nonpathogenic LFB-FIOCRUZ 847, serotype H2a2b, strain SS II-1; 4) nonpathogenic LFB-FIOCRUZ 742, ATCC 14577 (homology group I); and 5) nonpathogenic LFB-FIOCRUZ 751, serotype H26a26b, strain 2315.

Repetitive extragenic palindromic elements polymerase chain reaction (REP-PCR)

In this study, REP-PCR was used in an attempt to differentiate pathogenic and nonpathogenic *L. sphaericus* isolates. DNA extraction was performed according to Nunes et al.¹¹ The REP-PCR was performed as previously described by Reyes-Ramirez and Ibarra.¹² The selection of primers was based on the genetic relationship between genus *Bacillus* and genus *Lysinibacillus*.

Results

Results of REP-PCR were transformed into binary matrices and evaluated by the NTSYSpc (Version 2.1) numerical analysis program. Profile analysis was performed based on the calculation of the Sørensen–Dice coefficient, used to establish the similarity matrix. This matrix was transformed into a dendrogram (Figure 1), using the unweighted pair group method according to Rohlf.¹³ A cophenetic matrix was constructed using the similarity matrix to test the validity of the generated clusters. Correlation (Mantel *t*-test) between the cophenetic and similarity matrices was determined using the MXCOMP module.¹⁴

Twenty-one strains were isolated during the study and identified by phenotypic features as *L. sphaericus*. In the qualitative bioassays, only the strain LFB-FIOCRUZ 1513 demonstrated toxicity against *C. quinquefasciatus* larvae. This result is in agreement with the protein profile obtained with SDS-PAGE, which showed that lineage LFB-FIOCRUZ 1513 shared the same protein pattern, with strain LFB-FIOCRUZ 736, flagellar serotype H5a5b, strain 2362 showing the two polypeptides of the binary toxin. Strain 2362 was used as active ingredient in *L. sphaericus*-based insecticides.

Based on the results obtained with REP-PCR, 23 different profiles were found among the 26 strains in the study. Pathogenic and nonpathogenic *L. sphaericus* strains could not be distinguished based on this criterion. Analysis of

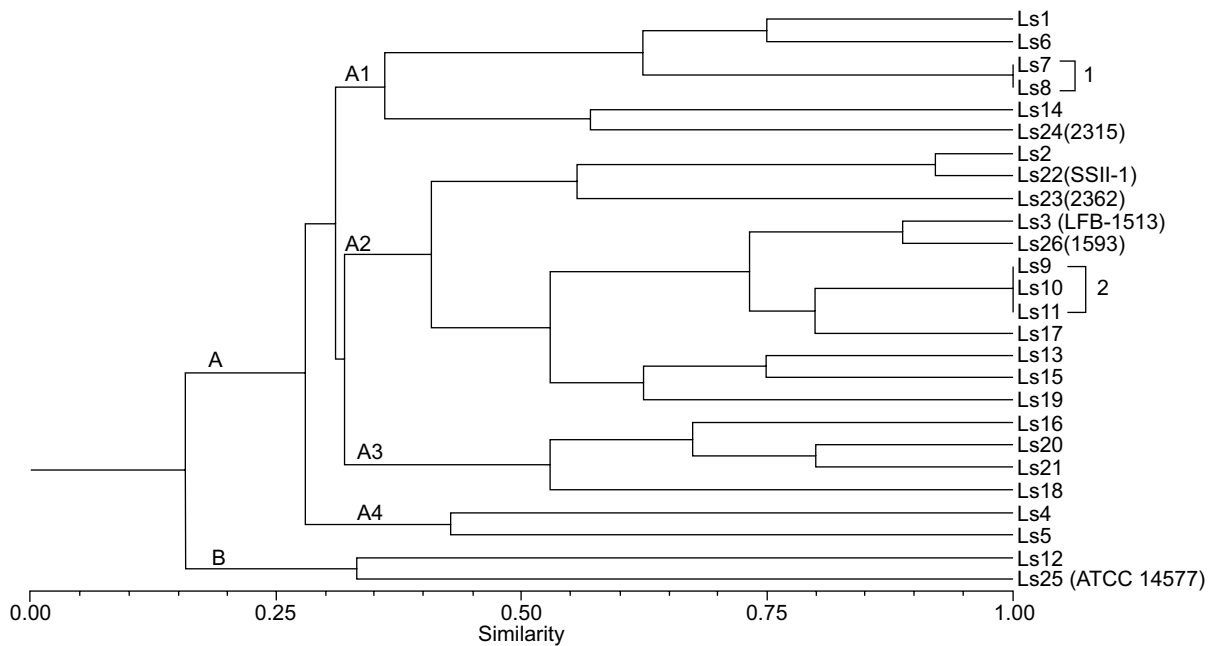


Figure 1 Dendrogram illustrating group A (with subgroups A1, A2, A3, and A4) and group B with increasing levels of similarity. Groups 1 and 2 comprise strains with 100% similarity.

Abbreviations: Ls, *Lysinibacillus sphaericus*; ATCC, American Type Culture Collection.

fragment profiles generated by REP-PCR demonstrated low genetic similarity (16%) among the *L. sphaericus* isolates and the five reference strains. This result may be related to the high levels of genetic variability within this species. These strains were divided into two groups (A and B). Group A was split into subgroups (A1, A2, A3, and A4) with increasing levels of similarity between them; on the other hand, group B consisted of two *L. sphaericus* strains with only 34% similarity. PCR reactions were performed twice to validate the obtained data. The coefficient of cophenetic correlation in the dendrogram was relatively high (0.77), indicating the validity of the generated groups.¹⁴

Discussion

Miteva et al¹⁵ performed a comparative study of 15 strains, representing the five homologous groups of *L. sphaericus*, by applying two PCR methods: random amplified polymorphic DNA (RAPD) and REP-PCR fingerprinting. These molecular methods demonstrated highly variable patterns and allowed differentiation of the strains studied. Similarity matrix analysis revealed a low level of affinity among the different homology groups, and within groups I and III, which is evidence of the high genetic heterogeneity in *L. sphaericus* species.

Woodburn et al¹⁶ analyzed pathogenic and nonpathogenic strains of *L. sphaericus* with RAPD-PCR to determine their genetic relationships. These authors demonstrated, by the Jaccard coefficient, that pathogenic strains belonging to

DNA homology group IIA were similar to strains belonging to nonpathogenic homology groups, with an average level of similarity of 6.3%. This technique has been used to differentiate several related species as well as strains of *L. sphaericus*. These results obtained using RAPD fingerprinting indicated that there is heterogeneity within *L. sphaericus*.

Among *L. sphaericus* strains, RAPD and REP-PCR revealed considerable genetic heterogeneity, which was confirmed by phylogenetic studies. The differences in pathogenicity can also assist taxonomic studies of the *Lysinibacillus* genus.^{15,17}

Araújo da Silva et al⁹ performed REP-PCR and BOX-PCR with 30 pathogenic strains of *L. sphaericus* to evaluate genetic diversity among strains isolated from different sources and localities in Brazil. DNA fingerprinting revealed five groups of similar strains by BOX-PCR and eight groups by REP-PCR. The cluster analysis indicated a high degree of diversity among the studied strains.

This paper is only a preliminary study of *Bacillus* species and species in associated genera isolated from healthy and diseased Culicidae larvae. Further isolation of new strains and use of molecular techniques will help in the characterization and population analysis of new bacterial strains. The use of REP-PCR with this pair of primers was apparently unsuccessful in distinguishing pathogenic and nonpathogenic *L. sphaericus* strains. This is due to the high intraspecific genetic variability for these regions amplified by the set of primers which appear to be unrelated to pathogenicity. It is worthwhile to note that this study was the first to use a pair of

primers specific to the genus *Bacillus* to analyze the closely related genus *Lysinibacillus*. A literature search did not find any other article describing the utilization of this pair of primers or, even specific primers for *Bacillus* and related genera.

Conclusion

The present study is in agreement with data reported by other authors, where the genetic variability in the *L. sphaericus* population was analyzed and showed low similarity between the strains.

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Disclosure

The authors report no conflicts of interest in this work.

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