Multilocus Enzyme Electrophoresis Study of *Bacillus sphaericus*

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Multilocus enzyme electrophoresis (MLEE) has been used in the study of some Bacillus *species. In this work we applied MLEE and numerical analysis in the study of the* Bacillus sphaericus *group.* B. sphaericus *can be distinguished from other entomopathogenic* Bacillus *by a unique allele (NP-4). Within the species, all insect pathogens were recovered in the same phenetic cluster and all of these strains have the same band position (electrophoresis migration) on the agarose gel (ADH-2). The entomopathogenic group of* B. sphaericus *seems to be a clonal population, having two widespread frequent genotypes (zymovar 59 and zymovar 119).*

Key words: *Bacillus sphaericus* - isoenzyme - taxonomy

Some strains of *Bacillus sphaericus* possess a toxin which is pathogenic for mosquitoes and this bacterium can be used in the control of these vectors. It is very important that these strains, with potential for utilization as bioinsecticides have a well established taxonomic position.

Different techniques have been applied in the study of *B. sphaericus* (Krych et al. 1980, Yousten 1984, De Barjac et al. 1988, Frachon et al. 1991). Among these techniques, DNA homology (Krych et al. 1980) showed clearly the genetic diversity among the *B. sphaericus* species. Althought strains of *B. sphaericus* have high phenetic similarity, in the Krych study the strains analyzed could be divided into five DNA homology groups and the IIA group comprised the entomopathogenic strains.

In our laboratory we have been using MLEE (multilocus enzyme electrophoresis) in the characterization of several organisms including *Vibrio* (Salles et al. 1994), *Leishmania* (Cupolillo et al. 1994) and *Anopheles* (Rosa-Freitas et al. 1992). This technique has three important features: typability, reproducibility and discrimination. In the genus *Bacillus* we found that *B. thuringiensis* is very similar to *B. cereus*. As with other techniques, MLEE could not separate these species into two distinct taxa (Zahner et al. 1989).

The genotypic diversity present among strains of *B. sphaericus* (Krych et al. 1980) and the pathogenicity to mosquito larvae associated with a particular group of strains (Singer 1988), led us to apply MLEE for differentiating among insect pathogenic strains in this group and in trying to find diagnostic alleles for the entomopathogenic group in order to make easier the identification of these strains.

MATERIALS AND METHODS

Strains examined included representatives of all the *B. sphaericus* DNA homology groups (Krych et al. 1980), except group V, and some environmental isolates from Brazilian soils. The strains were studied using MLEE with agarose gels. Thirteen enzymatic loci were analyzed. The enzyme bands were recorded and analyzed by numerical taxonomic procedures for the identification of *B. sphaericus* taxa.

Details of the methods used have been previously described (Zahner et al. 1994b). In this study we have added 30 more strains of *B. sphaericus* (20 entomopathogenic ones and 10 non-entomopathogenic), from CENARGEN/ EMBRAPA/ DF and 30 strains of *B. laterosporus*, a group of weakly entomopathogenic bacteria. The enzyme loci studied were: NP (E. C. 2. 4. 2. 1), ACON (E. C. 4. 2. 1. 3), MDH (E. C. 1. 1.1 .37), LeDH (E. C. 1. 4. 1. 9), ADH (E. C. 1. 4. 1. 1), EST (E. C. 3. 1. 1. 1), PEP-2 (E. C. 3. 4. 11 .1), PEP-3 (E. C. 3. 4. 11), PEP-D (E. C. 3. 4. 13. 9), G6P (E. C. 1. 1. 1. 49), 6PG (E. C. 1. 1. 1. 44), GPI (E. C. 5. 3. 1. 9) and ME (E. C. 1. 1. 1. 40).

RESULTS

Among the 13 enzyme loci studied only nine showed activity in *B. sphaericus:* NP, ACON, MDH, LeDH, ADH, EST, PEP-2, PEP-3 and PEP-D. Bands of enzyme activity were not detected among the strains for the loci G6P, 6PG, GPI and ME. All the enzymic loci were polymorphic except NP and ACON.

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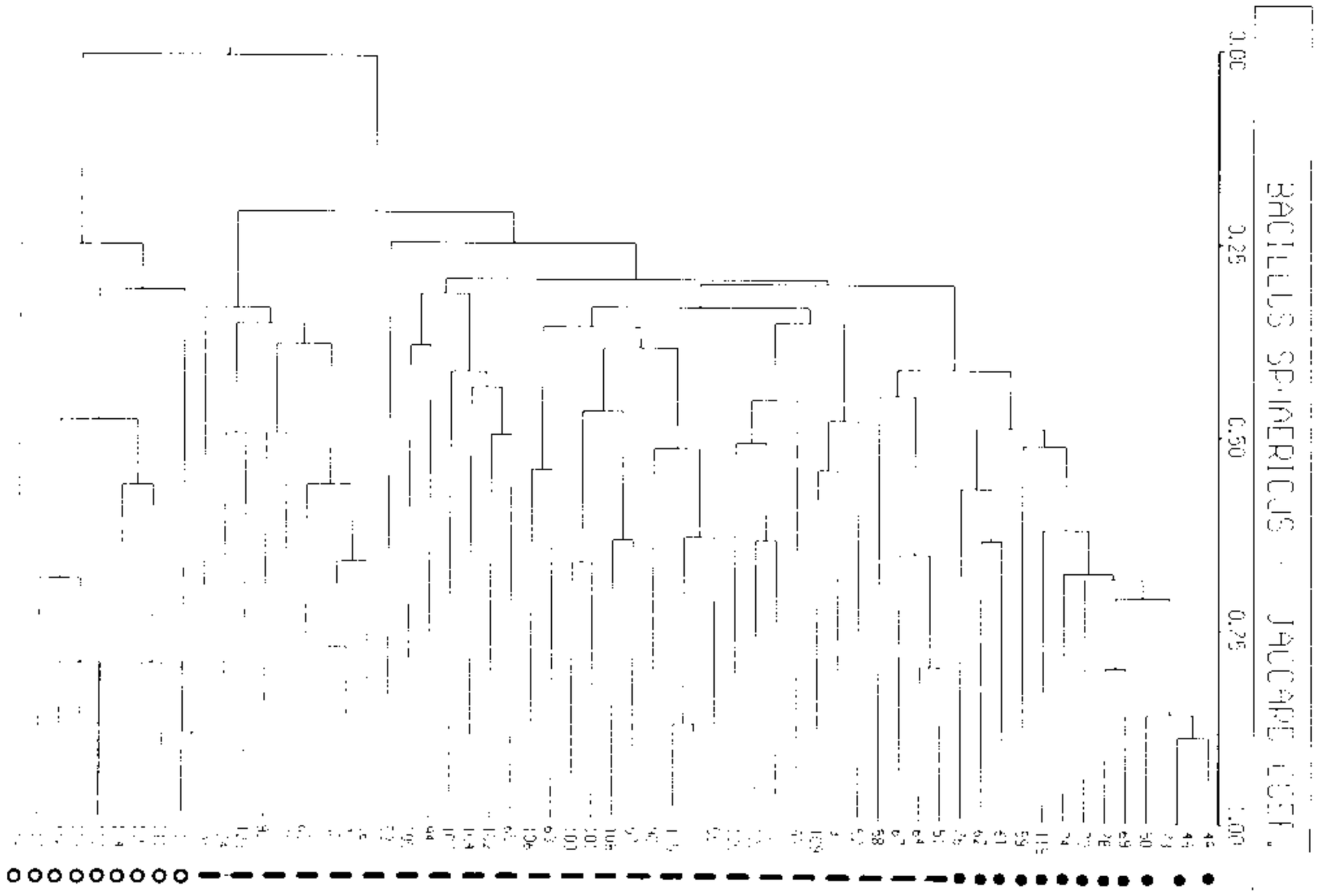

Fig. 1 : phenogram of *Bacillus sphaericus* based on Jaccard's Similarity Coefficient (Sj) and UPGMA. ● *B. sphaericus* entomopathogenic strains, ○ *B. sphaericus* non-entomopathogenic strains, ❐ *B. laterosporus*. The numbers in the phenogram refer to the zymovars designated by Zahner et al. (1994a) where the corresponding strains are listed, excepted for the Brazilian strains isolated by CENARGEN/EMBRAPA group which belong to the zymovars 119, 120, 121, 122, 123, 124 and 125.

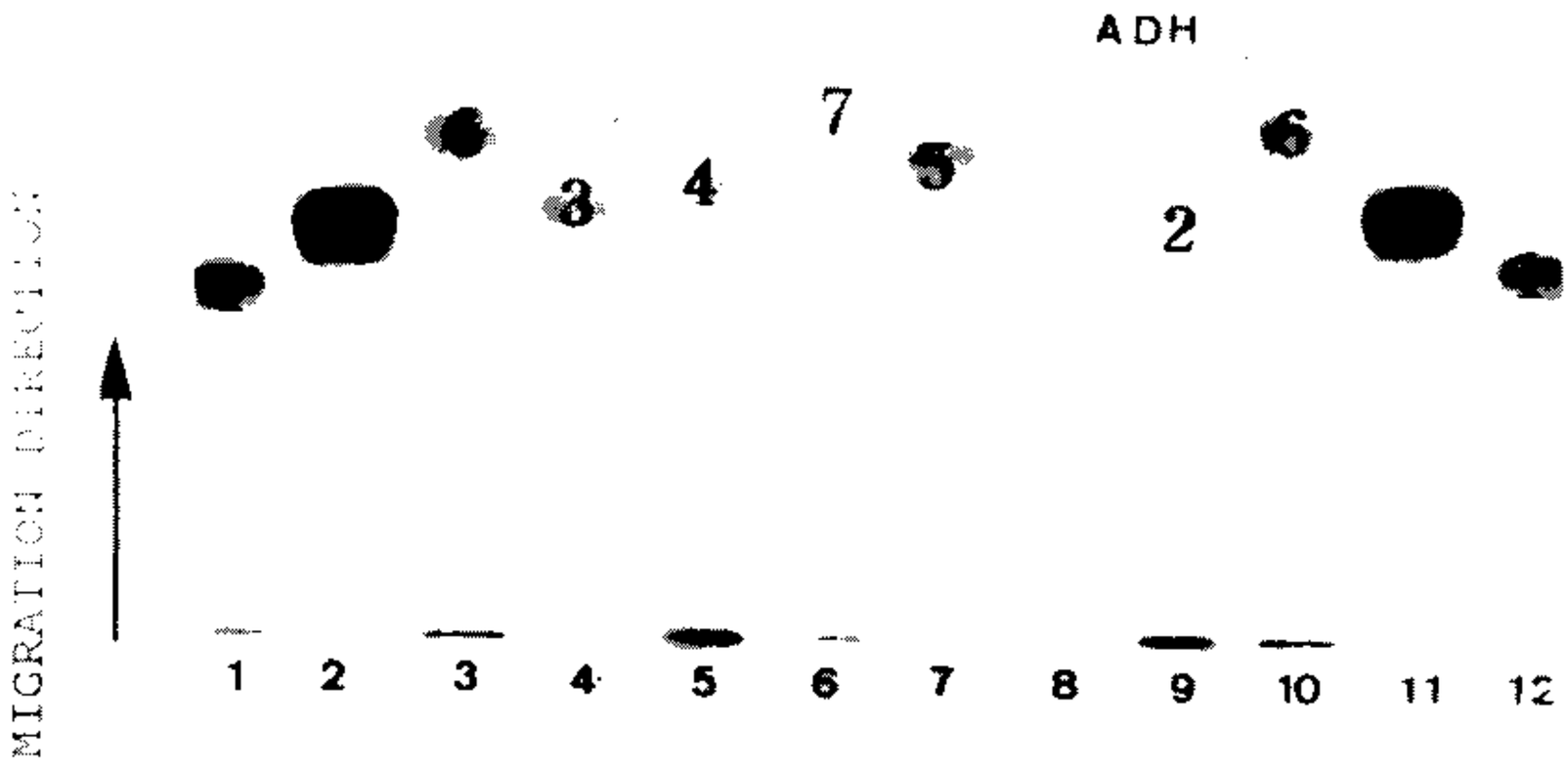

Fig. 2: gel showing ADH (E.C.1.4.1.1) in some *Bacillus sphaericus* strains. Position of strains on the gel: 1) ATCC4978, 2) 1593 (*), 3) 2118, 4) ATCC 145777, 5) ATCC10208, 6) WHO 2115, 7) 2118, 8) 94C, 9) SSII1 (*), 10) 1691, 11) 2297 (*), 12) ATCC 4978. Entomopathogenic strains are labelled with (*). The numbers presented on each band on the gel indicate the position of the allele. Entomopathogenic strains presented ADH-2.

B. laterosporus strains showed activity to all enzymes except for PEP-D, and was also positive for GPI (E.C.5.3.1.9) and ME (E.C.1.1.1.40). They formed a separate cluster, all strains having the isoenzyme profile quite different from *B. sphaericus*. In the numerical analysis using the Jaccard coefficient there is no value of similarity between these groups. In the same zymovar we can find strains with and without pathogenicity to mosquito larvae. In contrast to *B. sphaericus*, no diagnostic allele was found in *B. laterosporus*.

All entomopathogenic strains of *B. sphaericus* could be allocated to the same cluster (Fig. 1 - zymovars represented by bold numbers). All of them have the same electrophoretic mobility on the gel in the ADH enzyme (ADH-2) (Fig. 2).

Some zymovars contain more than one strain - zymovar 59 and 119 for example. Zymovar 59 contains the strains 1881, WHO 1883, SSII-1, 2013-6, Kellen Q, 2317-3, 2362, 2117-2, CIBNIG-14, Braz-1 and CIBGUA-6 (Zahner et al. 1994b) and the zymovar 119 contains all the Brazilians strains isolated by the CENARGEN group.

DISCUSSION

Baptist et al. (1978) demonstrated that isoenzyme electrophoresis with starch gel could be a powerful tool in taxonomic studies on *Bacillus* species. Singer (1988) using different enzymic loci and a different supporting gel (starch and polyacrilamide) had similarly emphasized the utility of MLEE in the study of *B. sphaericus*. The utilization of agarose gel which is very easy to make and has no toxic effects, has a great utility both in diagnosis as well as in the taxonomy of entomopathogenic *Bacillus*.

B. laterosporus is a poor entomopathogen, some strains are toxic to *Culex quinquefasciatus*, *Aedes aegypti* and *Simulium vittatum*. A previous study showed the high Genetic Distance between this species and others *Bacillus* (Zahner 1992). We decided to study this group because in the same form as *B. sphaericus* not all strains are entomotoxic. However the *B. laterosporus* group presented no correlation between pathogenicity and isoenzyme. This species is quite different from *B. sphaericus* (Fig.1), morphological, biochemical and physiological data also show the high divergence between *B. sphaericus* and *B. laterosporus* (Claus & Berkeley 1986).

To study *B. sphaericus* we chose some strains that have already been characterized by other techniques such as DNA homology (Krych et al. 1980), numerical analysis based on phenotypic characters (Alexander & Priest 1990) and serotyping (De Barjac et al. 1988). In each technique all entomopathogenic strains have characteristics that distinguish them from the non-entomopathogenic group, the same findings were demonstrated by MLEE.

As the MLEE results can identify entomotoxic strains, using the diagnostic allele (ADH-2), we suggest that this allele can be utilized as a molecular marker for the *B. sphaericus* entomopathogenic group. The *B. sphaericus* entomopathogenic group is very distinct from the other strains. In Fig. 1 the cluster containing the pathogenic strains has 41% of similarity with the other *B. sphaericus* strains. These results are in agreement with others author who suggested that *B. sphaericus* comprises more than one taxon (Singer 1988, De Muro & Priest 1993).

We have already suggested a low genic flow among *B. sphaericus* insect pathogens as we have recovered in the same zymovar (zymovar 59) eleven toxic strains isolated from different parts of the world (Zahner et al. 1994b). After analysis of the CENARGEN strains we can corroborate the conclusion further as we have analyzed 30 strains, among them 20 entomopathogenic. The strains were recovered from different regions of Brazil and have the same isoenzyme profile and the same serotype H-5 (Dr JM Cabral/CENARGEN, personnal communication). A similar result has been obtained previously with *B. thuringiensis* israelensis serotype H-14 (Zahner et al. 1994a) which appears to be a well adapted genotype with 23 strains isolated from different sources all having the same isoenzyme profile. The results indicate a clonal population structure for this species. According to Orskov and Orskov (1983) a clone in the clonal population conception is a group of strains isolated independently from different sources, in different time and localities showing many common phenotypic and genotypic features.

MLEE has useful practical applications in the diagnosis, genetic diversity and population structure of *Bacillus* strains. Claus and Fritze (1989) have already suggested that the taxonomy of this genera is in evolution, in consequence methods based on more stable characters are being used in the characterization and identification of the species such as pyrolisys mass spectrometry, total protein and isoenzyme electrophoresis.

In isolation programs for example, when dealing with many samples, the presence of ADH-2 indicates that it is probably an insect pathogenic strain. The discovery of this allele (ADH-2) which appears to be diagnostic for this cluster should facilitate the application of this technique for the identification of these strains as not all laboratories have the facilities for bioassays on mosquitoes larvae. The isoenzyme results are reproductible and this technique is fairly cheap.

In addition to the diagnostic potential, the isoenzyme electrophoresis may infer physiological and taxonomic aspects of the strains in study. In this way it is very important that strains with potential in biocontrol have their taxonomic posi-

tion well established. The uncertain taxonomic position of *B. brevis/B. circulans* for example which are toxic to insects, make difficult their utilization in biocontrol (Priest 1989). Also the physiological role of the NP locus should be considered. This locus is monomorphic in different *Bacillus* species studied (Zahner 1992) and is very conserved among species level. All strains of *B. thuringiensis* and *B. cereus* have the position 3 in this locus, *B. circulans* the position 2, *B. laterosporus* position 1 and *B. sphaericus* position 4. Inosine is a metabolite necessary to the *Bacillus* germination and is also the substrate of NP. Maybe this enzyme is connected with germination. The species analyzed by us until now (*B. thuringiensis, B. circulans, B. laterosporus and B. sphaericus*) could have different steps in the germination process, which may be reflected by the different alleles of NP. Alternatively, the intraspecific monomorphism of this locus could indicate a possible selective protection of this important step in the life cycle of these bacteria.

We can conclude that *B. sphaericus* is a complex containing genetically heterogeneous strains which are however similar phenetically when analyzed by biochemical and physiological tests. This complex contains a very closely related and well correlated cluster which includes all the entomotoxic strains. This cluster could in the future be considered as a separate species of *Bacillus*.

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