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ORIGINAL ARTICLE

Isolation, characterization and evaluation of mosquitocidal activity of *Lysinibacillus* strains obtained from *Culex pipiens* larvae

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Abstract Three strains—C107, C207, and C307—of spore-forming Gram-positive motile rod-shaped bacteria were isolated from dead larvae of *Culex pipiens* in La Plata city, Argentina. The three bacterial strains have different phenotypic and molecular characteristics. A comparative analysis of their 16S rRNA gene sequences and phylogenetic analysis demonstrated that isolates C107 and C207 were related to reference strains of *Lysinibacillus sphaericus*, while C307 was related to *Bacillus licheniformis*. The cytomorphology, biochemical characterization, and phylogenetic relatedness corroborated these respective group assignments. The isolated bacterial strain exhibited the same PCR amplified pattern for the *binA*, *binB*, and *mtx* genes as did the reference strains used. These bacterial strains presented different pathogenic actions among the following mosquito species tested: *Culex pipiens*, *Aedes aegypti*, *Culex dolosus*, *Culex apicinus*, *Ochlerotatus albifasciatus*, and *Anopheles albitarsis*. Only isolates C107 and C207 exhibited mosquitocidal activity. *Culex pipiens* was the species most susceptible to C107 (LC_{50}, 4×10^4 spores/ml), while *O. albifasciatus* was most susceptible to C207 (LC_{50}, 3.4×10^6 spores/ml).

Keywords Mosquito larvae · *Culex pipiens* · *Lysinibacillus sphaericus* · Biological control · 16S rRNA gene

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Introduction

Mosquitos pose a major human health problem worldwide. Efforts aimed at mosquito control have traditionally involved pesticides, but this approach has become harmful to the environment and has also resulted in insect resistance. At the present time, for the comprehensive control of insects, environmentally friendly pesticides together with biological control agents are strongly recommended (Federici et al. 2007; Lacey et al. 2007). *Lysinibacillus sphaericus* (Meyer and Neide) is an aerobic mesophilic spore-forming bacterium that has been used with great success in mosquito control programs worldwide (Charles et al. 1996). Based on their biochemical and genetic characteristics, *Bacillus sphaericus* (Neide) and *Bacillus fusiformis* (Priest) were recently reassigned to the genus *Lysinibacillus* as *L. sphaericus* and *L. fusiformis*, respectively (Ahmed et al. 2007). The presence of binary toxins (41.9 and 51.4 kDa) with mosquitocidal activity, the inability to ferment carbohydrate, and the production of spore resistance are among the more significant physiological features of this genus.

Species of *Lysinibacillus* have been isolated from soil and from infected mosquito larvae. Although most strains of *L. sphaericus* are not pathogenic to insects, strains that are in fact mosquitocidal can be exploited as important tools in pest-control programs (Xu et al. 1992). The

mosquitocidal activity of *L. sphaericus* and the persistence of its spores in the environment would make this species a suitable candidate for inoculation of mosquito breeding sites as an effective and ecologically friendly biological control agent (Mulla et al. 1984; Siegel et al. 2001). More than 380 *L. sphaericus* strains belonging to at least three genera (*Culex*, *Aedes*, *Anopheles*) have been identified that are toxic to the larvae of mosquitos (de Barjac et al. 1988; Baumann et al. 1991). These strains have been subdivided according to their exhibited toxicity as (1) low toxicity (50% lethal concentration (LC_{50}), 10^5 cells per ml), and (2) high toxicity (LC_{50}, 10^2 to 10^3 cells per ml) strains (Baumann et al. 1991; Porter et al. 1993).

The mosquitocidal properties result from the action of both a binary toxin (Bin proteins) with molecular weights 41.9 and 51.4 kDa that forms crystalline inclusions during sporulation (Broadwell et al. 1990) and a 100-kDa toxin (the Mtx protein) produced during vegetative growth (Priest et al. 1997). The genes encoding the binary toxin (*binA* and *binB*) are distributed among the high-toxicity strains, while the gene encoding the 100 kDa toxin (the *mtx* gene) is widely present among both low- and high-toxicity strains (Thanabalu et al. 1991).

Isolates of *L. sphaericus* can be grouped into 49 serotypes on the basis of flagellar-binding specificity, but relatively few biochemical and morphological tests have been used to define *L. sphaericus* as a species. Over the last decade, the taxonomy of the *Lysinibacillus* genus has been revised on the basis of analyses of 16S rRNA gene sequences and other chemotaxonomic data (Woodburn et al. 1995; Logan and Berkley 2000; Nakamura 2000). Because of this heterogeneity, we undertook a screening approach to search for local strains of *L. sphaericus*-like microorganisms having high toxicities for a wide range of hosts.

We report here the isolation of *Lysinibacillus* sp. with different phenotypic characteristics and mosquitocidal activity against a range of mosquito-host species, and also describe the pattern obtained from PCR amplification of the *bin* and *mtx* genes of these isolates. Finally, we use phylogenetic relationships based on 16S rRNA gene sequences in combination with biochemical and physiological methods to determine the taxonomic status of the local isolates of *L. sphaericus*-like microorganisms that we obtained.

Materials and methods

Lysinibacillus sphaericus strain

Lysinibacillus sphaericus strains 2362 and SPH 88, and *L. fusiformis* strain K7865 were kindly supplied by the Pasteur institute (France) and National Institute of Agricultural Biotechnology, Seoul, Korea, respectively. Strains of infected *Culex* larvae were isolated from breeding sites located in the city of La Plata, Buenos Aires, Argentina.

Collection of mosquito larvae

Several *Culex pipiens* breeding sites were sampled weekly during a period of 3 years in La Plata, Buenos Aires, Argentina. Samples were collected from drainage ditches (25×0.40×0.20 m, width × length × depth), with a 300-ml dipper, passed through a fine-mesh net (100 μm), and transported to the laboratory in plastic containers along with water from the same site.

Mosquito species were identified by their morphological characteristics, according to the criteria of Darsie (1985) and Lane (1953). Once in the laboratory, specimens were observed under a stereoscopic microscope in order to detect symptoms produced by the presence of pathogens. On one occasion, in March 2006, 100% (N=2,536) mortality of the *C. pipien*s larvae was observed 6 h after their collection.

Isolation of bacterial strains colonizing mosquito larvae

The dead larvae were sterilized by first placing them in sterile distilled water for 20 s followed by two 20-s washes with 70% (v/v) aqueous alcohol and a final 20-s wash with sterile distilled water. They were then homogenized in 10 ml sterile physiological saline solution according to Días et al. (1992); after dilution with 10 ml physiological saline containing sterile glass beads, the homogenate was vortexed for 1 min. After pasteurization at 65°C for 30 min and cooling on ice for 5 min, 0.5 ml of the suspensions from each sample were diluted to 10 ml with sterile distilled water and 100 μl of the dilution were spread on 9 cm diameter Petri dishes containing nutrient agar. The dishes were incubated at 25°C in the dark for 72 h (Lacey and Brooks 1997). Finally, colonies were picked after single-cell seeding on nutrient yeast extract salt medium (NYSM) agar (10 g/l glucose, 5 g/l NaCl, 0.3 g/l meat extract, 0.5 g/l yeast extract, 0.203 g/l $MgCl_2$, 0.102 g/l $CaCl_2$, 0.01 g/l $MnCl_2$, pH 7) (Yousten et al. 1985) for phenotypic characterization.

Phenotypic characterization

Cytomorphological analyses of strains were conducted with cultures 10–15 days old, under an optical microscope, using Gram stain for free bacteria (Thiery and Frachon 1997) and malachite green for spores. For a first characterization of the isolates, biochemical and physiological testing was performed to assess their capacity to grow in minimal medium with either glucose, arabinose, mannitol, xylose, or starch as the sole carbon source; in gelatin (Britania Laboratories, Buenos Aires, Argentina); casein; acetoin (3-hydroxybutanone); tyrosine; or lecithin (Thiery and Frachon 1997).

The isolates were also cultivated in NYSM agar as above but with different concentrations of antibiotics added: streptomycin, 100 and 200 μg/ml; erythromycin, 1 and 2 μg/ml; tetracycline, 2 and 5 μg/ml; and chloramphenicol, 8 μg/ml. Likewise, varying concentrations of NaCl: 5, 7, and 10% (w/v); or boric acid: 50, 60, and 75 μg/ml were tested. The catalase activity of the isolates was measured as described by Thiery and Frachon (1997), their ability to hydrolyze urea evaluated by their growth in test tubes containing 5 ml Christensen agar (Britania) in the presence of 5 ml 40% (w/v) urea, and their hemolytic capacity assessed in NYSM agar with 5% sterile sheep blood (Britania).

To study the fermentation of 49 carbohydrate substrates, we used the API 50 CH (bioMérieux, Marcy L'Etoile, France) according to the instructions of the manufacturer. Suspensions of the reference strains and the bacterial strains isolated were inoculated in CHB/E medium (bioMérieux) with a McFarland turbidity value equal to 2 and equivalent to 6×10^8 colony forming units (CFU)/ml. The cultures were then incubated for 24 and 48 h at 30°C. The carbohydrate catabolism produces organic acids that change the color of the phenol-red indicator from red to yellow.

DNA preparation

Total genomic DNA was extracted from the bacterial isolates as described by Aguilar et al. (2001) with some modifications. In brief, a loop of cells grown in NYSM agar (Yousten et al. 1985) was suspended in 0.5 ml 1 M NaCl for a period of 15–40 min. Cells were pelleted by centrifugation (11,000 *g*, resuspended in 0.5 ml sterile distilled water, and then re-centrifuged under the same conditions. The pellets were finally resuspended in 150 μl 6% (w/v) Chelex resin for the preparation of total DNA by means of the IntaGene DNA Purification Matrix (Bio-Rad) according to the manufacturer's instructions.

Sequencing of the 16S rRNA gene

To determine the sequence of the 16S rRNA gene, a 1.3-kb fragment was amplified by PCR from the genomic DNA of the samples using universal eubacteria-specific primers: 16S rP3 (5′-TACGGHTACCTTGTTACGACTT-3′) and 16S fD1 (5′-AGAGTTTGATCMTGGCTCAG-3′) as described by Lane (1991).

PCR amplification and sequencing of the 16S-rRNA gene were carried out as described by Weisburg et al. (1991). The PCR was performed in a final volume of 50 μl containing 10 μl (5X) commercial buffer, 0.5 μM of each oligonucleotide, 200 μM dNTPs, 2 mM $MgCl_2$, 1.0 U *Taq* polymerase and 100 ng total DNA. The PCR conditions used for initial denaturation were 95°C for 5 min, followed by 35 1-min denaturation cycles at 94°C, an annealing at 58°C for 1 min, an initial chain extension at 72°C for 2 min, and a final extension at 72°C for 3 min. The amplified 16S rRNA gene PCR products from the three bacterial isolates were sequenced at Macrogen (Seoul, Korea). The primers used to obtain the partial sequence of the 16S rRNA gene from the isolates were the same as for the PCR amplification.

Sequence analysis

Standard chromatographic curves of forward and reverse sequences were edited using the program Bioedit (Hall 1999). The sequences obtained from strains C107 and C207 were compared to 16S rRNA gene sequences available in the databases of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) by BLASTN homology search, as described by Altschul et al. (1997).

Phylogenetic analysis included the 16S rRNA gene sequences of the local isolates C107 and C207 and the reference strains *L. sphaericus*, and *L. fusiformis* obtained from GenBank (see accession numbers in Fig. 1). *Alycyclobacillus cycloheptanicus* was used as outgroup (Nakamura 2000). Sequences were aligned using CLUSTALW (Thompson et al. 1994) and adjusted manually. Our complete dataset included 1,445 aligned nucleotide positions. Genetic distances were estimated using the Kimura two parameters model (Kimura 1980). The phylogenetic tree was inferred with the neighbor joining (NJ) algorithm (Saitou and Nei 1987) using PAUP 4.0 (Swofford 2003). Cluster support was assessed through 1,000 bootstrap replicates (Felsenstein 1985). The partial 16S rRNA gene nucleotide sequences of the isolates C107 and C207 described in this study have been deposited into GenBank under the accession numbers HM125961 and HM125962, respectively.

Screening for the presence of *L. sphaericus* toxin genes by PCR

Primers designed by Otsuki et al. (1997) for detection of the binary-toxin operon as well as the individual *bin* genes [*bsn1*/*bsn2* and *bs1*/*bs2* (*bin* B), *bsn3*/*bsn4* and *bs3*/*bs4* (*binA*)] and the *mtx*1 gene (100.1/100.2) were used as previously described by the authors. Supernatant (1 or 2 μl) of the Chelex-resin resuspension (50 ng DNA) were transferred to a 20-μl reaction mixture containing 4 μl buffer (5X), 1 μM of each primer, 200 μM dNTPs, 2.5 mM $MgCl_2$, and 1.25 U *Taq* polymerase.

The reaction was performed under the following amplification conditions: initial denaturation at 94°C for 3 min, followed by 35 30-s denaturation cycles at 94°C, annealing at 55°C for 30 s, an initial chain extension at 72°C for 30 s, and a final extension at 72°C for 7 min.

After the first PCR with the pairs of primers BSN1/BSN2 (5′-CACGGAATGGTTATGGTT-3′/5′-AGGTGCATTAG

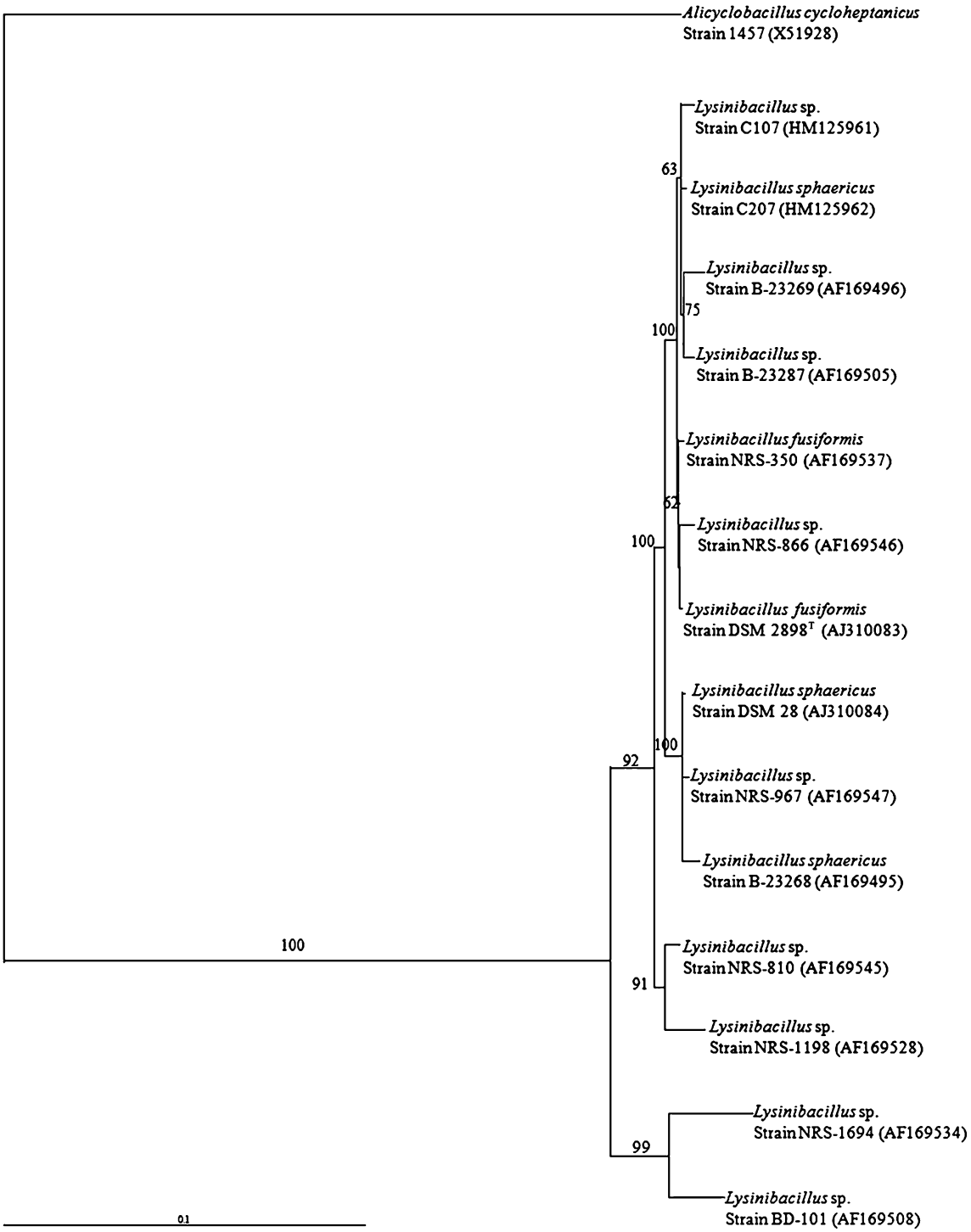

Fig. 1 Phylogenetic relationships based on 16S rRNA gene sequence analysis of members of *Lysinibacillus* isolated from *Culex pipiens* larvae. Evolutionary distances were calculated using the Kimura two parameters method, and the topology was inferred using the neighbor-joining (NJ) method. Numbers above branches represent percentage bootstrap values based on 1,000 replicates. The *scale bar* represents 0.1 substitutions per site. The 16S rRNA gene sequences of *Alycyclobacillus cycloheptanicus* was arbitrarily chosen as the out-group. Accession numbers are between parentheses

GATACGA-3′) and BSN3/BSN4 (5′-GTACATTCGCGT TATGG-3′/5′-GTATCATAGGTGAACC-3′), 1 μl of the above reaction was used as template for the nested PCR with the inner primers BS1/BS2 (5′-GTAGGGCGCTTGACAG TAGG-3′/5′-GGCCTATTTAGCCCCCTTG-3′) and BS3/ BS4 (5′-GGCATAATGGGTCCGT-3′/5′-GAGCGCGGAC CACATGC-3′), using the same conditions as the first PCR. These primers direct the amplification of internal fragments

from the *binB* and *binA* genes, respectively. The products of amplification were separated in a 1% (w/v) agarose gel containing 0.5 % TBE (Tris-Boric acid-EDTA) buffer and 0.5 μg/ml ethidium bromide.

For detection of *mtx* genes, the primer pair used was 100.2 /100.2 (5′-CCAGGGGGAATTCGTC-3′/5′-GAGC TACTGTTCTCAC-3′), previously designed by Otsuki et al. (1997).

Biological characterization: host range

Bioassays were conducted to determine the varieties of mosquitos susceptible to the isolates. Dilutions of bacterial cultures were made after 10–15 days of incubation in nutrient agar. A 1-ml aliquot (about 10^9 cells) was placed in tubes with 10 ml Tween 80 [0.01% (w/v) sodium polysorbate] and glass beads to release the spores of the isolates. The number of spores was counted in a Neubauer hemocytometer. Seven different concentrations (10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8) were then obtained by serial dilution. This concentration range was used to infect *C. pipiens* larvae and subsequently isolate the pathogen according to Koch's postulates. The host range of bacterial isolates and the reference strains (SPH 88 and K7865) was evaluated in third larval instars of six different mosquito species: *C. pipiens* (field and laboratory specimens), *Culex dolosus* (Lynch Arribalzaga), *Culex apicinus* (Philippi), *Anopheles albitarsis* (Lynch Arribalzaga), *Ochlerotatus albifasciatus* (Macquart), and *Aedes aegypti* L. (field and laboratory specimens). The biossay was conducted at 25°C in 8-cm-diameter plastic containers containing 100 ml distilled water plus 1 ml each bacterial concentration and the mosquito larvae host to be tested. A container with larvae but without bacteria was used as a control. Nine replicates plus three control containers were used for each combination of bacterial concentration and mosquito species. Per each container, 25 *C. pipiens* laboratory specimens, 25 *C. pipiens* field specimens, 15 *C. dolosus*, 25 *C. apicinus*, 10 *A. albitarsis*, 15 *O. albifasciatus*, 25 *A. aegypti* field specimens and 25 *A. aegypti* laboratory specimens were used. No source of food was added. The number of dead larvae in each container was registered after 48 h. The LC_{50} was calculated by PROBIT Analysis (Chi 1997).

Results

Isolation of bacteria from infected larvae and their phenotypic characterization

The presence of bacilli was detected by phase-contrast microscopy in slides of dissected infected larvae (N=2,536) collected on one sampling date (March 2006). Three bacterial strains were isolated after cultivation: C107, C207, and C307. These strains were Gram-positive and lacked parasporal bodies, while C107 and C207 had mobile cells with a terminal spherical sporangium measuring between 0.6 and 2.5 μm along with abundant free spherical spores. Strain C307 exhibited mobile cells with a non-deformed cylindrical spore-containing sporangium located subterminally and measuring 0.6–3.8 μm.

Table 1 summarizes the biochemical tests performed. Isolate C307 is a member of *Bacillus licheniformis* (Weigmann), while isolations C107 and C207 are members of *L. sphaericus*. Nevertheless, the presence of differing features between both these *L. sphaericus* strains prompted us to continue with their molecular and biological characterization. The phenotypic differences between C107 and C207 lay in their resistance or sensitivity to different antibiotics, their degree of tolerance to boric acid or NaCl, and the exclusive hemolytic capacity of strain C207. The combination of these features in strain C207 was similar to that described by From et al. (2005) and Ahmed et al. (2007) for *L. fusiformis*. With SPH 88—the reference strain of *L. sphaericus*—a slightly positive hydrolysis reaction was observed with urea.

Through their ability to ferment the 49 sugars of the API 50 CH gallery, we determined the biochemical profile of the *Lysinibacillus* strains isolated and of the reference strains. *L. sphaericus* strains SPH 88 and 2362 and the bacterial isolates C107 and C207 were able to metabolize both glycerol and N-acetylglucosamine, while *L. fusiformis* K7865 was positive for N-acetylglucosamine alone.

16S rRNA gene sequence analysis

Sequencing of the genes encoding the 16S rRNA subunit in DNA from the isolated bacterial strains C107 and C207 by means of BLASTN (NCBI) analysis indicated a 99% similarity between the nucleotides of strain C107 and those of *L. sphaericus* (e.g., strains RG-1, PRE16, and C3-41). A similar result was obtained with the same *L. sphaericus* strains and C207. Some strains of *L. fusiformis* (e.g., *Lysinibacillus fusiformis* X-9, WH22, and X-25 plus the reference strain DSM 2898^T for this species), however, also shared a 99% nucleotide-sequence identity with both strains C107 and C207. Next, we determined the degree of relatedness of our isolates to different *L. sphaericus* and *L. fusiformis* species through a phylogenetic analysis.

The NJ tree shows a close relationship between the strains isolated in the present study and Group I of Nakamura (strains B-23269 and B-23287; Fig. 1). The reference strain DSM 2898^T clustered with Nakamura's Group II, containing strains of *L. fusiformis*; while reference strain DSM 28 grouped with Nakamura's Group II, comprising *B. sphaericus* strains, as expected (Fig. 1).

Table 1 Biochemical and physiological characterization of the bacterial strains C107, C207 and C307 and their reference strains SPH 88 and K7865

Biochemical and physiological characteristics	*Lysinibacillus* sp. C107	*Lysinibacillus* sp. C207	*Lysinibacillus sphaericus* strain SPH 88	*Lysinibacillus fusiformis* strain K7865	*Bacillus* sp. C307
Growth in:					
Glucose	-[a]	-	-	-	+
Arabinose	-	-	-	-	-
Mannitol	-	-	-	-	-
Xylose	-	-	-	-	+
Starch	-	-	-	-	+
Gelatin	+	+	+	+	+
NaCl 5%	+	+	+	+	+
NaCl 7%	±	+	+	+	+
NaCl 10%	-	±	-	+	-
Boric acid 50	+	+	+	+	+
Boric acid 60	+	-	+	-	-
Boric acid 75	±	-	±	-	-
Streptomycin 100[b]	+	+	+	+	+
Streptomycin 200[b]	+	+	+	+	+
Erythromycin 1[b]	+	+	+	+	+
Erythromycin 2[b]	-	+	-	+	ND[c]
Tetracycline 2[b]	-	-	-	-	-
Tetracycline 5[b]	-	-	-	-	-
Chloramphenicol 8[b]	+	-	+	-	-
Acetoin	-	-	-	-	+
Citrate	+	+	+	+	+
Tyrosine	-	-	-	-	-
Lecithinase	-	-	-	-	+
pH of medium	<6	<6	<6	<6	>6
Enzymatic reactions					
Hemolysis	-	+	-	+	+
Catalase	+	+	+	+	+
Hydrolysis of urea	±	±	±	±	-
API test. Ability to metabolize:					
Glycerol	+	+	+	-	ND
N-acetylglucosamina	+	+	+	+	ND

[a] + Growth, − no growth, ± little growth

[b] Antibiotic concentrations expressed in μg/ml

[c] Not determined

Screening for the presence of *L. sphaericus* toxin genes

Primers BS1/BS2 and BSN1/BSN2 gave rise to fragments of 523- and 1.053-bp, respectively, both corresponding to the 51.4-kDa toxin gene; whereas primers BS3/BS4 and BSN3/BSN4 amplified 478- and 720-bp fragments, respectively, both belonging to the 41.9-kDa toxin protein. The set of primers 100.1/100.2, homologous to the *mtx* gene, yielded a 700-bp PCR product (Fig. 2a–e).

Biological characterization: host range

Culex pipiens larvae from a laboratory colony were susceptible to strain C107 at an LC_{50} of 4×10^4 spores/ml, compared to a value of 1×10^5 spores/ml for the reference strain SPH 88. By contrast, *C. pipiens* larvae collected in the field exhibited respective LC_{50} values of 1.1×10^5 and 2.2×10^5 spores/ml for these two bacterial strains.

Colony-bred *C. pipiens* larvae showed greater susceptibility to the strain C207 at an LC_{50} of 1×10^7 spores/ml than

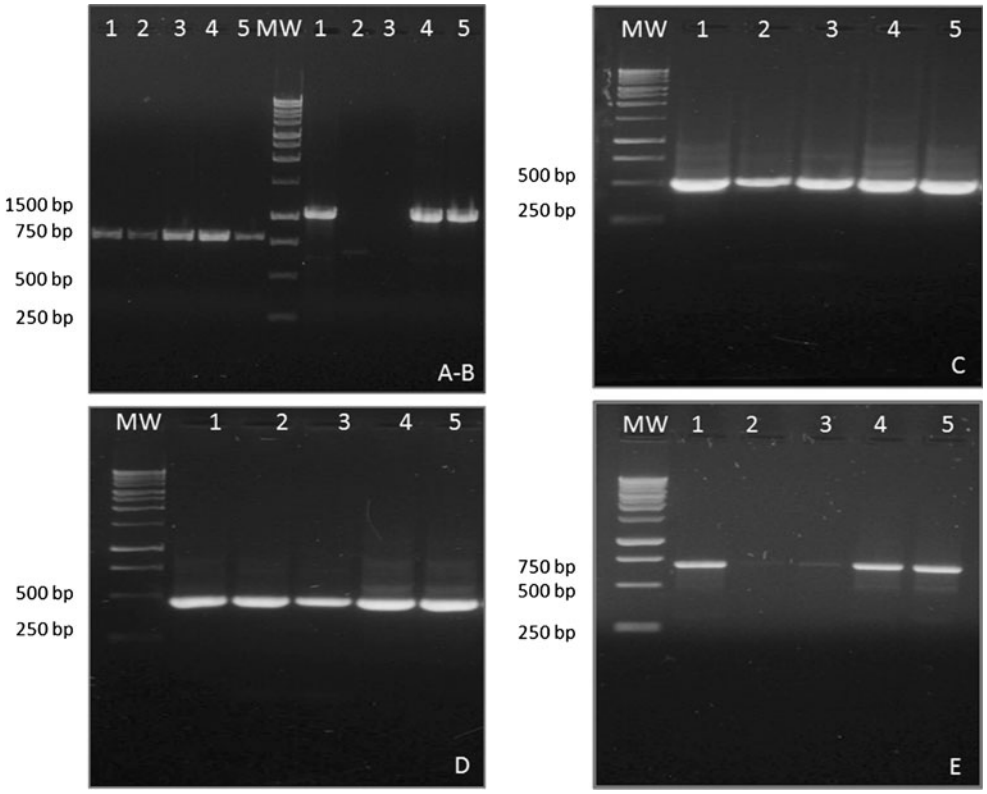

Fig. 2 Representative 1% agarose-gel electrophoresis of nucleic-acid-amplification products from *Lysinibacillus sphaericus* with the following primers: **a** BSN3/BSN4, **b** BSN1/BSN2, **c** BS3/BS4, **d** BS1/BS2, **e** 100.1/100.2. Lanes: *MW* 1Kd molecular weight DNA ladder (10,000–250 bp), *1* C107, *2* C207, *3* K7865, *4* SPH8, *5* 2362

to the reference strain, where the LC_{50} was some three orders of magnitude higher at 1×10^{10} spores/ml. Field-collected *C. pipiens* larvae, however, exhibited a lower or comparable sensitivity to these strains with LC_{50} values of 1×10^{8} for C207 and 9.9×10^{8} for *L. fusiformis* K7865 (Table 2).

Culex apicinus larvae showed similar susceptibilities to the bacterial strains isolated and their reference strains; the LC_{50} for C107 and SPH 88 were 2.1×10^{6} and 3.1×10^{6} spores/ml, respectively, and about 10^{7} spores/ml for C207 and K7865 (Table 2). By contrast, *C. dolosus* larvae exhibited a higher mortality upon exposure to C107 or to SPH 88, with resulting LC_{50} values of 2.6×10^{5} and 8.2×10^{3} spores/ml, respectively. This mosquito species, however, evinced a lower susceptibility to strain C207 as well as to strain K7865, with the LC_{50} for both being in the order of 10^{8} spores/ml (Table 2).

Aedes aegypti larvae showed susceptibility neither to isolates C107 and C207 nor to strain SPH 88. *Ochlerotatus albifasciatus*, however, was equally sensitive to C107 and to SPH 88—the LC_{50} for both strains was in the order of 1×10^{7} spores/ml—but exhibited differing sensitivities to C207 and K7865 at respective LC_{50} values of 3.4×10^{6} and 2.8×10^{9} spores/ml (Table 2). The susceptibility of *Anopheles albitarsis* to the reference strains SPH 88 and K7865 was so low that the LC_{50} values could not be calculated. When, however, third instar larvae were exposed to strains C107 and C207, the respective LC_{50}s obtained were 6.6×10^{6} and 1.4×10^{7} spores/ml (Table 2).

Discussion

In this study we isolated the strains C107 and C207 with mosquitocidal activity. The strain C107 was toxic to all species of *Culex* tested, with *C. pipiens* being the most highly susceptible of the species tested. These results are similar to those of Wraight et al. (1987) for *L. sphaericus* strains and different target species of *Culex*.

Strain C207 exhibited lower toxicity for the *Culex* species than did strain C107; but *O. albifasciatus* proved a more susceptible host species to C207, exhibiting an LC_{50} of 3.4×10^{6} spores/ml upon infection with that strain. Although *L. sphaericus* had been reported as nontoxic for the genus *Ochlerotatus* (Federici et al. 2006, 2007), several studies with different strains of *L. sphaericus* had indicated toxicity to species of this genus (Wraight et al. 1987; Park et al. 2008).

The isolates C107 and C207 did not produce mortality in any *Aedes aegypti* larvae.

Federici et al. (2006, 2007) regarded *L. sphaericus* as a toxic species against *Anopheles* immature stages, but the bacterial strains we isolated and investigated showed only a low toxicity for *A. albitarsis* at an LC_{50} of 6.6×10^{7} spores/ml for C107 and 1.4×10^{9} spores/ml for C207.

Strain C307 had no mosquitocidal activity and therefore was not studied genetically. The taxonomic status of this strain was determined using morphological and biochemical techniques. We speculated that this strain had been isolated after secondary infection of the *C. pipiens* larvae

Table 2 Lethal concentration 50 (LC_{50}) values for strains C107 and C207 expressed in spores/ml against third-instar larva of different mosquito species. The data were analyzed with Pearson Chi-square goodness on probit model (α=0.05)

Mosquito species	Bacterial strain	Intercept	Slope	LC_{50} (spores/ml)	X^2 (*df*)	*P*
Culex pipiens (laboratory specimens)	Strain C107	2.633	$0.51\pm2.19\times10^{-2}$	4.4×10^{4} (1.2×10^{4}-1.4×10^{5})	17.77 (6)	0.006
	Strain C207	2.82	$0.31\pm1.9\times10^{-2}$	1.03×10^{7} (2.3×10^{5}-1.1×10^{8})	20.87 (5)	0.001
	L. sphaericus SPH 88	1.47	$0.56\pm2.3\times10^{-2}$	1.1×10^{5} (7.5×10^{5} -5.4×10^{6})	7.00 (3)	0.071
	L. fusiformis K7865	3.1	$0.19\pm1.59\times10^{-2}$	1.5×10^{10} (6.1×10^{8}-1.6×10^{14})	12.16 (4)	0.016
Culex apicinus	Strain C107	2.81	$0.35\pm1.6\times10^{-2}$	2.1×10^{6} (7.3×10^{5}-7.2×10^{6})	28.36 (4)	1.054^{-05}
	Strain C207	2.82	$0.31\pm2.3\times10^{-2}$	2.4×10^{10} (4.8×10^{9}-2.4×10^{11})	2.4 (2)	0.301
	L. sphaericus SPH 88	2.59	$0.36\pm3.01\times10^{-2}$	3.1×10^{6} (1.9×10^{6}-5.4×10^{6})	0.64 (3)	0.887
	L. fusiformis K7865	2.17	$0.26\pm3.7\times10^{-2}$	4.3×10^{10} (3.7×10^{9}-2.3×10^{12})	1.57 (4)	0.814
Culex dolosus	Strain C107	2.56	$0.45\pm4.16\times10^{-2}$	2.6×10^{5} (1.4×10^{5}-4.6×10^{5})	1.443 (4)	0.837
	Strain C207	1.97	$0.37\pm3.72\times10^{-2}$	1.5×10^{8} (1.0×10^{8} -2.4×10^{8})	3.55 (5)	0.615
	L.. sphaericus SPH 88	3.7	$0.33\pm2.8\times10^{-2}$	8.2×10^{3} (3.2×10^{3} – 1.8×10^{4})	3.58 (6)	0.733
	L. fusiformis K7865	3.46	$0.19\pm2.48\times10^{-2}$	1.1×10^{8} (4.6×10^{7}- 3.2×10^{8})	1.1 (6)	0.981
Aedes aegypti (laboratory specimens)	Strain C107	NC[a]	NC	NC	NC	NC
	Strain C207	NC	NC	NC	NC	NC
	L. sphaericus SPH 88	NC	NC	NC	NC	NC
	L. fusiformis K7865	NC	NC	NC	NC	NC
Anopheles albitarsis	Strain C107	1.89	$0.34\pm1.2\times10^{-2}$	6.6×10^{6} (2.4×10^{6}-3.1×10^{7})	21.1(4)	1.14^{-04}
	Strain C207	2.03	0.32 ± 0.11	1.4×10^{9} (3.5×10^{8}- 2.4×10^{10})	2.27 (3)	0.518
	L. sphaericus SPH 88	1.49	0.19 ± 7.10^{-2}	NC	0.2 (3)	0.977
	B. fusiformis K7865	1.61	$0.14\pm8.6\times10^{-2}$	NC	0.12 (3)	0.989
Ochlerotatus albifasciatus	Strain C107	2.99	$0.18\pm2.9\times10^{-2}$	7.7×10^{7} (2.1×10^{7}- 2.4×10^{9})	3.32 (5)	0.651
	Strain C207	2.47	$0.39\pm3.33\times10^{-2}$	3.4×10^{6} (2.2×10^{6}-5.2×10^{6})	2.65 (4)	0.618
	L. sphaericus SPH 88	1.58	$0.48\pm4.43\times10^{-2}$	1.07×10^{7} (4.8×10^{6}-2.5×10^{7})	3.3 (4)	0.509
	L. fusiformis K7865	2.55	$0.26\pm3.5\times10^{-2}$	2.8×10^{9} (6.0×10^{8}-2.8×10^{10})	3.87 (5)	0.568
Aedes aegypti (field)	Strain C107	NC	NC	NC	NC	NC
	Strain C207	NC	NC	NC	NC	NC
	L. sphaericus SPH 88	NC	NC	NC	NC	NC
	L. fusiformis K7865	NC	NC	NC	NC	NC
Culex pipiens (field)	Strain C107	2.69	$0.45\pm3.5\times10^{-2}$	1.12×10^{5} (4.5×10^{4}-2.7×10^{5})	5.97 (5)	0.309
	Strain C207	2.35	$0.29\pm4.1\times10^{-2}$	1.10^{8} (4.1×10^{7}-3.7×10^{8})	0.79 (4)	0.939
	L. sphaericus SPH 88	3.3	$0.41\pm3.1\times10^{-2}$	1.2×10^{5} (6.9×10^{4}-2×10^{5})	2.24 (6)	0.896
	L. fusiformis K7865	2.28	$0.7\pm4\times10^{-2}$	9.1×10^{9} (1.4×10^{9}-3.2×10^{11})	1.63 (4)	0.803

[a] Not calculated

because the species that the strain most resembled is a saprophytic bacterium found commonly in soil as an opportunistic pathogen.

The toxicity of *L. sphaericus* against immature stages of mosquitos results from the expression of the binary toxins (41.9 and 51.4 kDa) encoded by the *binA* and *binB* genes, which are loci expressed at the start of sporulation. The presence of both gene products comprising this toxin is necessary to produce mortality in mosquito larvae (Broadwell et al. 1990; Thanabalu et al. 1992; Davidson 1990). Another toxin of 100 kDa, called Mtx, is present in *L. sphaericus* strains of both low and high toxicity. According to Thanabalu et al. (1991), the presence of the *mtx* genes does not per se confer toxicity to this bacterial strain against mosquito larvae. Therefore, the low toxicity in certain strains could result from either low expression or short-lived stability of the binary toxins during sporulation. The presence of the genes that encode the binary toxin and the Mtx protein has been proved in the strains C107 and C207, and the presence of the two gene products were similar to those of the reference *L. sphaericus* strains 2362 and SPH 88.

This work thus suggests that there is no direct relationship between the presence of the *bin* and *mtx* genes within the *L. sphaericus* strains and phenotype of the latter with respect to mosquito toxicity and host range.

A classic feature used in the description of the *L. sphaericus* species is the inability to use pentoses and hexoses as the sole carbon source as a result of the absence of the *pgi* genes encoding enzymes for degrading and transporting those sugars (Hu et al. 2008). Nevertheless, in some *L. sphaericus* strains, we did observe the ability to metabolize N-acetylglucosamine. In addition, the presence of glucose carriers, 6-phosphofructokinase activity, enzymes involved in metabolism of those compounds, and operons responsible for their transport within the bacterial genome have been verified with different strains of *L. sphaericus* (Logan and Berkley 1984; Alice et al. 2003; Hu et al. 2008). These observations would argue for a reassessment of this character as having taxonomic and physiological relevance.

The biochemical profile of fermentation of various sugars within the API test indicates that the strains C107 and C207, along with the reference strain, *L. sphaericus* 2362, are able to ferment N-acetylglucosamine and glycerol. This ability suggests the existence of variability among *L. sphaericus* strains as an adaptation to survival in the environment.

16S rRNA gene sequencing provides, in many instances, useful information at the species level. Because of the high 16S rRNA gene similarity, DNA–DNA hybridization should be useful to elucidate taxonomic positions (Stackebrandt and Goebel 1994). In this work, the BLASTN method was not precise enough to differentiate among closely related species. Therefore, we performed a phylogenetic analysis including the sequences of the isolated bacterial strains.

The study of 16S rRNA gene sequences by Nakamura (2000) indicated that the *L. sphaericus* species, sensu lato, is a heterogeneous taxon formed by seven groups. This author admits that the divisions between groups 1 (strains with mosquitocidal activity) and 2 (*L. fusiformis*) have not been borne out statistically. Accordingly, on the basis of their biochemical, morphologic, and genetic features, strains belonging to groups 1 and 2 should be considered as members of the same group or species. According to Nakamura (2000), our phylogenetic analysis indicates that strains C107 and C207 are both closely related to the *L. sphaericus* group, although the cytomorphology, host range, and some physiological and biochemical features suggest the existence of variability between these two bacterial strains.

The high larval toxicity found in some *L. sphaericus* strains, such as 2362 (Weiser 1984) and IAB59 (de Barjac et al. 1988), has led to the commercial use of these strains as biopesticides against populations of mosquitos. Nevertheless, because of the variability found among the *L. sphaericus* strains and the specific mechanism of action of the binary toxins, we concluded that a search for new *L. sphaericus* strains of high mosquitocidal activity that were, moreover, ecologically adapted to the environment had become necessary. The strains isolated and identified in this study thus represent good candidates for use in the control of mosquito-breeding sites within an environment to which both the host and the parasite are equally well adapted.

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