Screening for *traG/D*- and *mob*-like sequences in *Lysinibacillus sphaericus* strains

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Abstract

Lysinibacillus sphaericus is used in bioremediation of soils which are contaminated with toxic metals. Furthermore, *L. sphaericus* is used in biological control of some species like mosquitoes *Culex quinquefasciatus*, which is a vector of some viruses, like denge fever and West Nile fever, and some parasites like Plasmodium and filariasis. Conjugation is an effective mechanism of horizontal transfer of genetic material that bacteria has. In gram-positive bacteria, conjugation system appear to be a subgroup of type IV secretion system. By multiple alignment were found conservative sequences that allow to make primers for *traA* and *mob* genes, at the same time primers for *traG/D* gene were mad. By PCR was found that several strains of *L. sphaericus* possess sequences *traG/D*-like, and this belong to a *TraM* recognition site of *TraD* and *TraG*.

Keywords: *traG/D*-like sequences, *L. sphaericus*, conjugation, T4SS.

1. Introduction

Lysinibacillus sphaericus is used in bioremediation of soils which are contaminated with toxic metals like cobalt, copper, chromium and lead, and organic compounds (Lozano & Dussán, 2013). As it has been shown that this bacterium has the ability to adsorb metals such as Cr (IV) in both, dead and living cells (Velásquez & Dussan, 2009).

Furthermore, *L. sphaericus* is used in biological control of some species like mosquitoes *Culex quinquefasciatus*, which is a vector of some viruses, like denge fever and West Nile fever, and some parasites like Plasmodium and filariasis. The mechanism that strains of *L. sphaericus* use in larvicidal activity determinate it toxicity. The mosquitocidal toxins (*Mtx*) is synthesized in vegetative cells for high and low toxicity strains, and the binary toxin coded by *binA* and *binB* genes is produced for high toxicity strains in sporulation states. Besides, the lavicidal toxicity may be explained due to expression of *Cry48/Cry49* toxin and S-layer protein (Lozano, *et al*., 2011).

Recently, has been reported the genome sequence of two strains from this genus: *L. sphaerius* OT4b.31 and *L.sphaericus* CBAM5. (Peña-Montenegro & Dussán, 2013; Peña-Montenegro et al., 2014)

Conjugation is an effective mechanism of horizontal transfer of genetic material that bacteria has, because the extrachromosomal genetic material shared can provide certain desirable characteristics, the most known is antibiotic resistance (Grohmann, *et al.* 2003). Although, it has been found that some genes coding for proteins and enzymes that are involved in the proses of resistant and/or biosorption of toxic metals, such as arsenic and mercury. As well as essential enzymes for the degradation of organic compounds such as phenol, xylene, benzene, ethylbenzene, toluene and naphthalene (Karpagam & Lalithakumari, 1999; Zídková, *et al.* 2013; Janto, *et al.* 2011; He, *et al.* 2010).

Conjugation in gram-negative bacteria has been highly studied; it is known that the first models of bacterial horizontal transfer were developed in *Escherichia coli*. The main elements of the conjugation are encoded on the plasmid such as proteins. In gram-positive bacteria, conjugation system appear to be a subgroup of type IV secretion system that have evolved the capacity to translocate DNA-protein complexes. Among the

proteins used in this system is found TraG, which is described as a coupling protein (Lu, *et al.* 2008). DNA relaxases use mobilization (Mob) proteins and/or Tra proteins in the imitation of conjugative plasmids transfer, which contain the motifs I and III that are found in all conjugative DNA relaxases (Grohmann, *et al.* 2003).

Conjugation genes have not been highly reported in *Lysinibacillus sp,* thus it is necessary a bioinformatic study of these genes in bacilli gram-positive, such as *Bacillus thuringiensis* and *Geobacillus stearothermophilus,* which is phylogenetically close to genus *Lysibacillus.*

Since *L. sphaericus* is a bacteria with desired characteristic both environmental and vector control, the objective of this study is to identify if the strains of *L. sphaericus* and *Bacillus sp*. possess the genes required for conjugation.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Several strains of *L. sphaericus* were used as: 2362, 1593, OT4b.25, OT4b.26, OT4b.31, OT4b.32, OT4b.48, OT4b.49, OT4b.56, OT4b.58, SB.2.15, SB.2.94, III(3)7, III(2)4, III(2)7, IV(3)13, IV(4)10, CH2.17, CBAM5, NE.2.1, NE.2.2 SA.2.4 (Dussán, 2006; Lozano, 1998; Villegas-Torres *et al.,* 2011) In vitro Conjugant, Conjugant from oil sludge of °API: 32, Conjugant from oil sludge of °API: 28 and Conjugant from oil sludge of °API: 14 (Rojas, 2015). As positive control of some primers was used *Bacillus thuringiensis DH1* (donated by Jairo Cerón Salamanca from IBUN Instituted of Biotechnology at Universidad Nacional de Colombia).

Bacteria were grown in nutrient agar for 18 h at 30 °C, and in Luria Broth (LB) 24 h for 18 h at 30 °C.

2.2. Primers design

Primers for *traG/D* gene were made in two ways. First, in order to obtain conserved sequences for primers design, multiple alignments were performed using MAFFT program of different genes in *Bacillus* and *Geobacillus* species. Primers designed were FBtraG/D (TGCAGAAGAGATGGCGAGAC) and RBtraG/D (GCATTCATCTGTGCTGCTCG).

Second, through *traG/D* gene of strain *L. sphaericus C3-41 (*reported in the National Center of Biotechnology Information), FtraG/D (ATTGGTGCTGAGGGTGAAGA) and RtraG/D (TCTTTATTAGGGCCCCGTCC).

Moreover, in order to obtain conserved sequences for primers design for *traA* and *mob* genes, multiple alignments were performed using MAFFT program of different genes in *Bacillus* species. For *traA* gene the following primers were obtained FtraA (GCTGTTGCTTCGGCTTCATA) and RtraA (CCCCAACTCCCATCTTCGTT). Since multiple alignments for *mob* gene created large gaps, sequences were divided in two groups, and for each group it was design a pairs of primers. First Fmob1 (ACATTTATCGACGCAGCGGT) and Rmob1 (CTCTAAGGGCTTCCGACGAC); second Fmob2 (GCAGCAACAAGGTGTCGAAG) and Rmob2 (GGCACTTGATCCGTAGCACT).

2.3. Amplifications

Amplifications were made from reactions of 25 µL, each reaction contains 2.5 mM MgCl2, 1X PCR Buffer, 0.2 mM dNTPs, 0.3 µM of each primer, 1.5 U Taq polymerase (Thermo Scientific), and 0.9 µL of whole DNA extract. The following PCR program was performed: a hot start for denaturing step at 95 °C for 5 min, 35 cycles of denaturing at 95 °C for 30 s, annealing at temperature indicated by each pair of primers for 30 s and extension at 72 °C for 30 s, and a last extension at 72 °C for 5 min. The products were visualized in agarose gel at 1 % using Gel Red,

subsequently purified using the kit Wizard® SV Gel ad PCR Clean-Up System (Promega), and sequenced in the sequencing laboratory of Universidad de los Andes. The sequences were align using BLASTn with the data base of Nucleotide collection.

3. Results and Discussion

3.1. Amplifications from Bacillus *traG/D, traA* and *mob* primers

As positive control of this primers a strain of *Bacillus thuringiensis DH1* was used. It was possible to obtain an amplification weighing approximately 300 bp in the control strain, but in none of *L. sphaericus* strains was able to obtain an amplification. This indicates that *traG/D* gene differs from *Bacillus* to *Lysinibacillus* strains. Same result were obtained with traA and mob2 primers.

In contrast, with mob1 primers it was able to find a band weighing approximately 900 bp in *L. sphaericus CBAM5*. Product sequence is located in chromosome.

3.2. Amplifications from traG/D primers

As shown in Fig 1 to 3, from 22 strains studied 15 present the expected band weighing approximately 400 bp. Therefore, amplifications were sequenced and all had 99% of identity with *traG/D* of *L. sphaericus C3-41*, this indicated that strains may possess the gene.

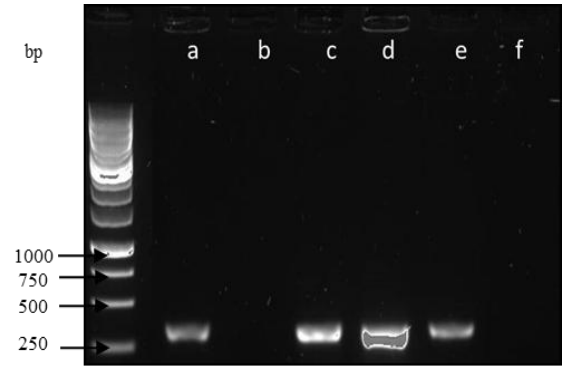

Fig 1. Amplifications with primers *traG/D.* a) OT4b.31 b) CBAM5 c) OT4b.49 d) III(3)7 e) 2362 f) OT4b.49

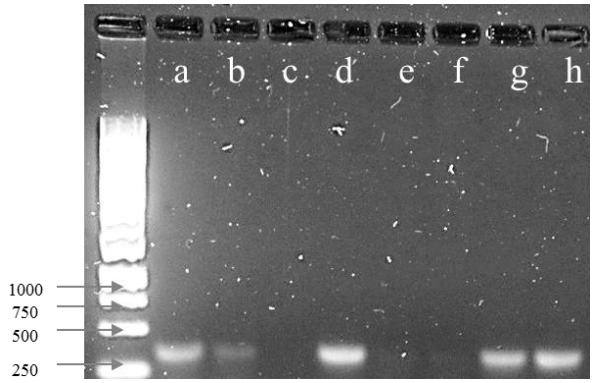

Fig 2. Amplifications with primers *traG/D.* a) IV(4)10 b) SA.2.4 c) III(2)4 d) OT4b.48 e) III(3)7 f) IV(3)13 g) 1593 h) OT4b.32

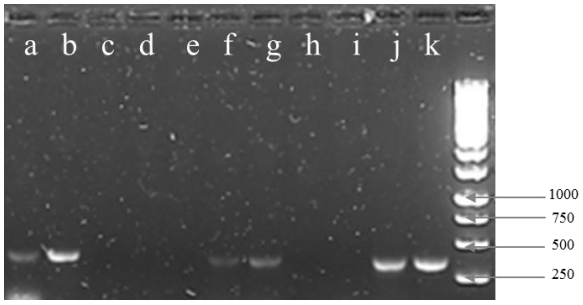

Fig 3. Amplifications with primers *traG/D.* a) OT4b.56 b) OT4b.58 c) SB.2.15 d) CBAM5 e) CH217 f) NE.2.2 g) SB.2.94 h) NE.2.1 i) OT4b.26 j) 2362 k) III(2)7

In order to create a phylogenetic tree, a multiple alignment was performed using Clustal Omega algorithms. From this alignment was obtained a consensus sequence, it was translate into the following protein sequence:

EGGVLLVNTSLAELDELSLMFGQFFIRQFQSAIFRRPQEGRIPI FFYIDEFPLYVNEAFERILTLGRSYNVGAVIAMQSIGQLEGVK AGYQDIIW

At Pfam data base it was found that this protein sequences belongs to a TraM recognition site of TraD and TraG. But at the same time, with less identity, this sequence has a match with Type IV secretion-system (T4SS) coupling protein DNA-binding domain. This evidence how conjugation system and T4SS are joined. In gram positive bacteria, conjugation system appears to be a subgroup of T4SS. This system has the capacity to translocate DNA-protein complexes. Some proteins involved are VirB1, VirB4, VirB6, VirB11 and VirD4 which have a similar role to

TraG, TrwB, TraD that are described as coupling proteins (Grohmann, *et al.* 2003).

Furthermore, since the region that covers T4SS is larger than the one for *TraM* recognition site, it can be infer an evolutionary process from T4SS that leads to conjugation system.

In Fig. 4 is showed a Phylogenetic tree made by Bayesian statistic. There are 4 clades grouping strains of *L. sphaericus* differently than would be grouped by place of isolation or pathogenicity.

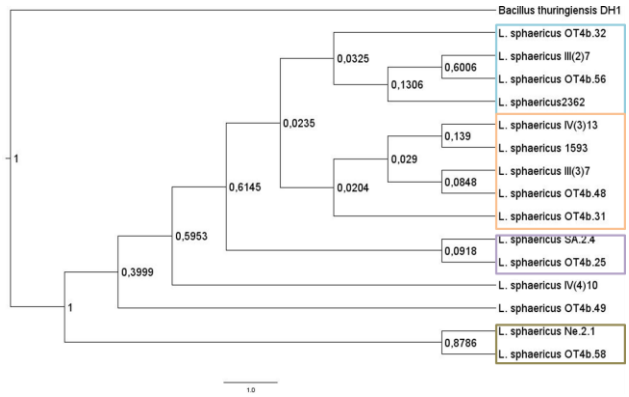

Fig 4. Phylogenetic tree made by Bayesian statistic.

From work made by Rojas *et al.* (2014), it was took conjugative cells made with strains *OT4b.31* and *CBAM5* in 1 and 2 hours of conjugation. In Fig. 5 it could be seen the expected band weighing approximately 400 bp.

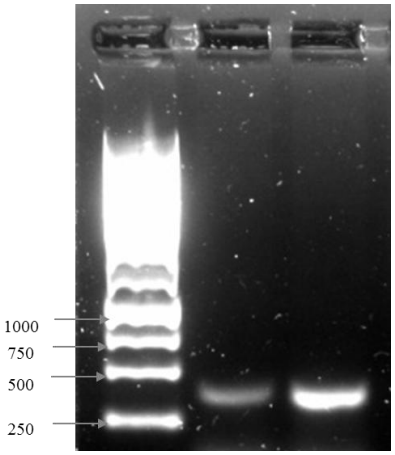

Fig 5. Amplifications with primers *traG/D*. Second lane: Conjugative cell found in 1 h of conjugation. Third lane: Conjugative cell found in 2 h of conjugation.

In addition, conjugative cells obtained by exposure to different concentrations oily sludge show the expected band weighing approximately 400 bp (Fig. 6). This means that during conjugation is not lost the plasmid that possess region of traG/D amplified, indicating the presence of this plasmid in conjugants.

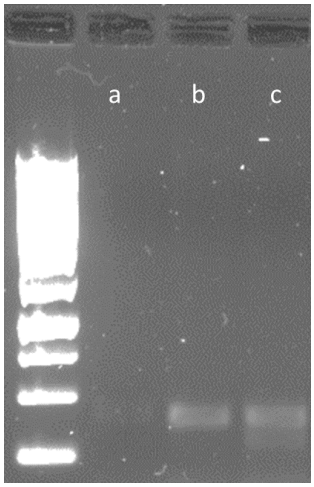

Fig 6. Amplifications with primers *traG/D*. a) Conjugant from oil sludge of °API: 32 b) Conjugant from oil sludge of °API: 28 c) Conjugant from oil sludge of °API: 14

4. Conclusions

According to multiple alignment of traG/D gen, species of *Bacillus* and *Geobacillus* genes do not have a great similarity with *Lysinibacillus* gen. this is also evidenced amplifications with primers.

There is molecular evidence shows that strains of *L. sphaericus* can perform bacterial conjugation.

L. sphaericus might possess T4SS genes involved in a coupled system that would transport DNA.

Found groups of phylogenetic closeness in *Lysinibacillus* strains, these differ according to origin and characteristics.

5. Future Studies

Acknowledge of *traG/D*-like sequences in *Lysinibacillus* strains might help in plasmid sequencing.

Amplifications with traG/D can be used as a protocol of molecular marked in conjugation.

Results found here leads to a molecular characterization of plasmids in *L. sphaericus* through T4SS proteins.

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7. References

Dussán, J. (2006) Diversidad de *Bacillus sphaericus* en diferentes hábitat y regiones geográficas, Tesis de Doctorado, Universidad de los Andes, Bogotá, Colombia.

Grohmann, E., Muth, G., & Espinosa, M. (2003). Conjugative Plasmid Transfer in Gram-Positive Bacteria. Microbiology and molecular biology reviews, (67-2), 277–301.

Janto, B.; Ahmed, A.; Ito, M.; Liu, J.; Hicks, D.B.; Pagni, S.; Fackelmayer, O.J.; Smith, T.A.; Earl, J.; Elbourne, L.D.; Hassan, K.; Paulsen, I.T.; Kolsto, A.B.; Tourasse, N.J.; Ehrlich, G.D.; Boissy, R.; Ivey, D.M.; G., Li; Xue, Y.; Ma, Y.; Hu, F.Z.; Kru. (2011). Genome of alkaliphilic *Bacillus pseudofirmus* OF4 reveals adaptations that support the ability to grow in an external pH range from 7.5 to 11.4. Environ Microbiol., 3.

Karpagam, S., & Lalithakumari, D. (1999). Plasmid-mediated degradation of o- and p-phthalate by *Pseudomonas fluorescens*. World Journal of Microbiology & Biotechnology (15), 565-569.

Lozano, L. (1998). Diferenciación de cepas nativas de Bacillus sphaericus patógenas y no patógenas para larvas de mosquitos por amplificación azarosa del ADN. Universidad de los Andes, Bogotá, Colombia.

Lozano, L., Ayala, J. & Dussán, J. (2011). *Lysinibacillus sphaericus* S-layer protein toxicity against *Culex quinquefasciatus*. Biotechnol Lett (33), 2037-2041.

Lozano, L., & Dussán, J. (2013). Metal tolerance and larvicidal activity of *Lysinibacillus sphaericus*. World J. Microbiol Biotechnol (29), 1383–1389.

Lu, J., Wong, J.J.W., Edwards, R.S., Manchak, J., Frost, L.S., Glover, J.N.M. (2008). Structural basis of specific TraD–TraM recognition during F plasmid-mediated bacterial conjugation. Molecular Microbiology (70), 89-99.

Peña-Montenegro et al. (2014).Genome sequence and description of the mosquitocidal and heavy metal tolerant strain Lysinibacillus sphaericus CBAM5.

Peña-Montenegro et al. (2013).Genome sequence and description of the heavy metal tolerant bacterium Lysinibacillus sphaericus strain OT4b.31. Standards in Genomic Sciences, (9) 42-56

Rojas, P. (2015). Conjugation between *Lysinibacillus sphaericus* strains OT4b.31 and CBAM5 in microcosms with oils sludges. Universidad de los Andes, Bogotá, Colombia.

Velásquez, L., & Dussan, J. (2009). Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus*. Journal of Hazardous Materials (167), 713–716.

Zídková, L., Szököl, J., Rucká, L., Pátek, M., & Nesvera, J. (2013). Biodegradation of phenol using recombinant plasmid-carrying *Rhodococcus erythropolis* strains. International Biodeterioration & Biodegradation (84), 179-184.