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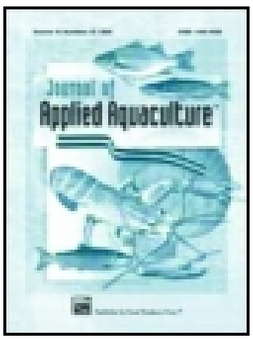

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Genetic manipulation of native *Bacillus cereus*: a biotechnological tool for aquaculture

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ABSTRACT

This work aimed to perform the genetic manipulation of the native *B. cereus* #25 strain and to evaluate the colonization in the intestinal tract of *Litopenaeus vannamei*. The expression of the GFPmut1, absence of amylase activity, and growth in spectinomycin plates confirmed the transformation of the strain (BC25GM). In the shrimp bioassay, its addiction in the feed showed non-difference for water quality, growth performance, and toxic effects for *L. vannamei* (survival 100%). The presence of cells expressing GFPmut1 in the midgut of the animals was observed, suggesting that the genetically engineered strain colonized the shrimp intestines. The relative fluorescence was 35% higher in the midgut of the shrimps feed with BC25GM. In conclusion, the present work showed that it is possible to manipulate a native *B. cereus* strain with the nonspecific pSG1154 plasmid. We reported the possibility to implement native strains as a biotechnology tool applied in aquaculture, especially to deliver beneficial molecules for shrimps.

KEYWORDS

Bacillus; GFPmut1; probiotics; beneficial molecules; *Litopenaeus vannamei*

Introduction

The culture of aquatic organisms is growing in the last years, mainly concerning crustaceans, where the shrimp *Litopenaeus vannamei* is the most produced species in the world (FAO 2018). However, one of the main challenges of carciniculture has been the occurrence of viral diseases, which often cause significant economic and social repercussions (Lafferty et al. 2015). Therefore, it is necessary to develop innovative technologies promoting actions to reduce the health problems observed in shrimp farming, minimizing economic losses. In this way, it has been reported the benefits of probiotic bacteria and their application in aquaculture, causing sanitary improvements in culture systems (Lakshmi, Viswanath, and Gopal 2013).

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Genetic manipulation of microorganisms has promoted the possibility to produce beneficial molecules to immunology and nutrition of crustaceans. Some studies, involving the treatment of viral infections in shrimp, have demonstrated the production of antiviral molecules through the genetic manipulation of strains of bacteria such as *Escherichia coli* (Attasart et al. 2013; Taju et al. 2015) and *Bacillus subtilis* (Nguyen et al. 2014). As examples, we can mention the double-stranded RNA (dsRNA) that can activate the interference RNA mechanism (Taju et al. 2015) and antiviral proteins (Fu et al. 2011; Nguyen et al. 2014). The use of gram-positive bacteria as bioreactors of beneficial molecules applied to immunology of shrimp can represent an interesting tool to mitigate some problems faced on the development of this sector.

Although there are many *Bacillus* strains commercially used in aquaculture, several of them with established genetic manipulation protocols, there are no studies (to our knowledge) that address manipulation for native *Bacillus* species isolated from crustaceans. As follows, the objective of this work was to perform the genetic manipulation of the native strain *B. cereus* #25 isolated from the crab *Ucides* sp. and to evaluate its presence and colonization in the intestinal tract of *Litopenaeus vannamei* under culture conditions.

Material and methods

Ethics statement

No ethical approval was required in this study.

Bacillus cereus #25

Bacillus cereus #25 was obtained from the Laboratory of Environmental Microbiology and Fish at the Institute of Marine Sciences – LABOMAR/UFC (Brazil). This strain was isolated from the intestinal tract of the crab (*Ucides* sp.) from the Pacoti River (Ceará, Brazil) and characterized as a potential probiotic bacterium. Biochemical analysis indicated catalase activity, antagonism to *V. harveyi*, and no activity to the virulence factors elastase, gelatinase and lipase (data not shown). The characterization involved the identification of catalase activity and antagonism to the *Vibrio harveyi*. The genome of this strain was previously sequenced (Costa Filho et al. 2018).

Genetic manipulation

The integration vector pSG1154, obtained from the Bacillus Genetic Stock Center (BGSC), was used for genetic manipulation of *B. cereus* #25. The vector has two recombination sites for the amylase gene (*amyE*), spectinomycin

resistance, and the green fluorescent protein (GFPmut1) gene controlled by the P_{xyl} promoter, which is induced by xylose. *Escherichia coli* One Shot TOP10 Electrocomp (Invitrogen, Brazil) was used for cloning and the isolation of the plasmids was performed with the Plasmid prep Mini Spin kit (GE HealthCare, Brazil), following the manufacturer's protocol. The transformation of *B. cereus* #25 was performed according to the electrocompetent method (Xue, Johnson, and Dalrymple 1999). Detailed transformation protocol can be found at https://www.protocols.io/view/transformation-protocol-for-bacillus-subtilis-4uigwue/abstract. Transformed strain was inoculated into LB medium containing spectinomycin (250 $\mu g.mL^{-1}$) and xylose (0.2%). Aliquots of the culture were used for visualization of *B. cereus* #25 expressing GFPmut1 on the Olympus IX81 epifluorescence microscope.

To test the occurrence of homologous recombination of pSG1154 plasmid on the amylase gene, transformed *B. cereus* #25 colonies were incubated for 48 h at 37°C in LB agar (1% starch). Colonies were covered with lugol solution (iodine crystals 5%; potassium iodate 10%). The absence of the transparent halo indicated no amylase activity and confirmation of the integration.

Feed supplemented with genetically modified *B. cereus* #25

A genetically modified *B. cereus* #25 (hereafter named BC25GM) inoculum was incubated in Luria-Bertani medium containing xylose and spectinomycin. 10 ml of the culture remained at 37°C and agitation at 250 rpm until the optical density of 1.0 (600 nm). After centrifugation (5,000 g for 5 minutes) the pellet was resuspended in 1 ml of sterile water from the experimental tanks and then sprayed on 10 grams of the feed (40% of crude protein and 7.5% of ether extract). For the control group was used only water. The feed was incubated for 1 hour and 30 minutes at 37°C, stored at 4°C, and used within two days. The final concentration of BC25GM was 10^8 bacteria.g^{-1} of the feed. The amount of feed supplied to the shrimps was standardized to 8% of the biomass and divided into three times daily.

Bioassays with *Litopenaeus vannamei*

Juveniles of *Litopenaeus vannamei* (1.7 ± 0.1 g) were obtained from the Marine Aquaculture Station (EMA/FURG, Brazil) and transported to the Institute of Biological Sciences (ICB/FURG, Brazil). The first experimental culture started after 10 days of acclimatization and lasted for 15 days. Two treatments were tested in triplicate: one group received only the regular feed (control) and another with BC25GM supplementation. The inclusion of wild strain was non-necessary, since the GFP is only used as a reporter for gene expression (Chalfie et al., 1994), and promotes non-significant effects on the strain. A closed system with polyethylene tanks of 0.33 m^2 and a volume of 50 L

was used. Each triplicate contained 20 shrimps, totaling 60 per treatment. Water renewal was conducted daily to control nitrogen compounds. Dissolved oxygen, temperature, salinity, total ammonia, non-ionized ammonia, and pH were checked daily.

Biometric was performed during the experiment to adjust feed quantity. The performance parameters evaluated were weight gain (mean final weight – mean initial weight), specific growth rate ([ln final weight – ln initial weight/ days of cultivation] * 100), apparent feed conversion (feed offered/weight gain) and survival ([initial number of animals – final number of animals/initial number of animals)] * 100). The data from the performance and water quality of the triplicates were analyzed with the SAS program (version 9.0), using the t-test ($P < .05$).

BC25GM colonization in the *L. vannamei* midgut

At the end of the experiment, GFPmut expression of C25GM strain indicated the colonization in shrimp. For relative fluorescence analysis, 10 shrimps per group were fasted for 24 hours to obtain a more satisfactory quality of the samples, with non-food remains. Subsequently, the midguts were dissected (Figure 1), sonicated with 300 µL of phosphate-buffered saline, for 30/10 seconds on/off, 50% amplitude, for 3 minutes and 30 seconds, using the Qsonica Sonicators. The fluorescence of GFPmut1 was measured on the FilterMax F5 fluorimeter (485 nm excitation and 535 nm emission). The proteins obtained from the extract were quantified with the Qubit® Protein Assay kit (Invitrogen, Brazil). Relative fluorescence was estimated by the ratio between the reading obtained on the fluorimeter and the proteins observed ($\mu g.mL^{-1}$).

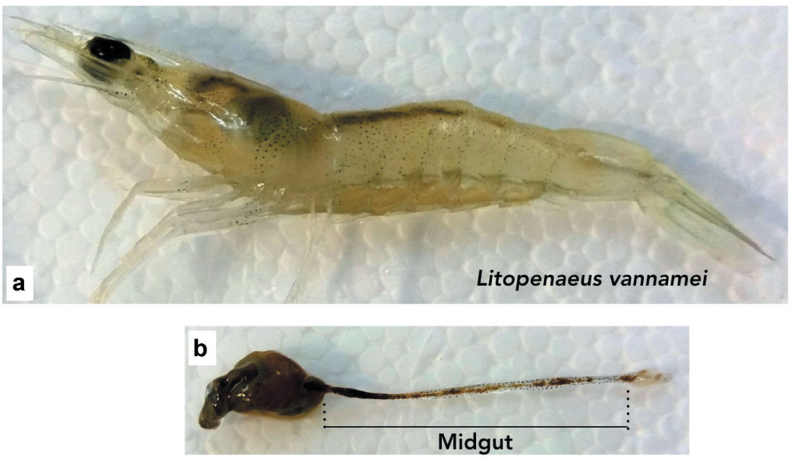

Figure 1. (A) Juvenile of *Litopenaeus vannamei* used in the present study. (B) Dissected shrimp intestinal tract, evidencing the midgut region used for histological and fluorescence analyses.

For ensuring the presence of BC25GM in the midgut of the shrimp, histological slides were prepared for analysis in epifluorescence microscopy. The tissues were fixed in paraformaldehyde 4%, included in paraplast and cut into slices with 6 μm of thickness. No coloring method was used.

Results

Genetic manipulation of *B. cereus* #25

The successful genetic manipulation of *B. cereus* # 25 was confirmed by its three new characteristics: (a) expression of the GFPmut1 fluorescent protein, whose gene present in the plasmid pSG1154 is activated by xylose 0.2% (Figure 2A-B), (b) absence of amylase activity (Figure 2C-D), and (c) growth in medium containing spectinomycin since pSG1154 provides resistance to this antibiotic.

Bioassays with *Litopenaeus vannamei*

Water quality indicators (Table 1) and growth parameters (Table 2) determined during the bioassay indicated no difference between BC25GM treatment and untreated control.

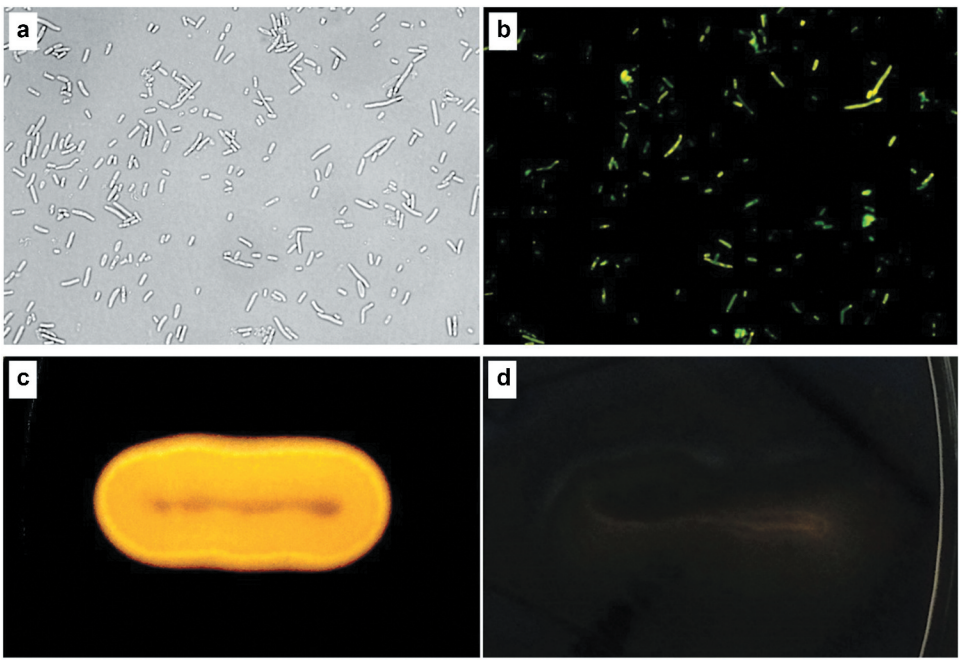

Figure 2. Phenotypic characteristics of genetically modified *B. cereus* #25. BC25GM under white light (A) and epifluorescence microscopy, evincing the expression of GFPmut1 (B). Amylase activity showed by the presence of a clear halo around the wild type *B. cereus* #25 colony (C), which was non-observed in the BC25GM (D).

Table 1. Values (mean ± standard deviation) of the water quality indicators during 15 days of the experiment.

Parameters	Treatments		*P-values
	Feed with BC25GM	Regular feed	
DO ($mg.L^{-1}$)	5.6 ± 0.3	5.4 ± 0.1	.311
Temperature (°C)	27.8 ± 0.2	27.9 ± 0.1	.398
Salinity ($g.L^{-1}$)	20.2 ± 0.05	20.3 ± 0	.116
Total ammonia ($mg.L^{-1}$)	0.41 ± 0.04	0.41 ± 0.05	.942
NH_3 ($mg.L^{-1}$)	0.02 ± 0	0.01 ± 0.005	.373
pH	7.6 ± 0	7.5 ± 0.05	.116
Daily renewals (%)	85.6	85.6	-

*No difference between the treatments by the t-test ($P < .05$).

Table 2. Values (mean ± standard deviation) of the growth parameters during 15 days of the experiment.

	Treatments		
Parameters	Feed with BC25GM	Regular feed	* *P*-vales
Initial weight (g)	1.8 ± 0.1	1.7 ± 0.1	.496
Final weight (g)	3.1 ± 0.3	3.1 ± 0.2	1.000
Weight gain (g)	1.3 ± 0.2	1.4 ± 0.1	.565
SGR	3.5 ± 0.2	3.9 ± 0.1	.088
AFC	2.1 ± 0.3	2.1 ± 0.3	.970
Survival (%)	100	100	-

SGR – specific growth rate; AFC – apparent feed conversion; * No difference between the treatments by the t-test ($P < .05$).

BC25GM midgut colonization in *L. vannamei*

Figure 3 shows the BC25GM presence in the midgut of the shrimps through epifluorescence microscopy. In all images was not seen bacterial aggregates just disperse cells. Suggesting that the genetically engineered strain colonized the shrimp intestines. GFPmut1 was detected revealing viable cells expressing this gene. Figure 4 shows the relative fluorescence in the midguts of the shrimps. This result shows that GFPmut1 can be measured in the intestinal tract of the shrimps supplemented with BC25GM, increasing the fluorescence 35% ($P < .05$) comparing to the basal fluorescence observed in the midgut of the shrimps fed only with the regular feed.

Discussion

In the present work, a native strain *B. cereus* #25 was used for genetic manipulation using the plasmid pSG1154 from the Bacillus Genetic Stock Center (BGSC). This vector was developed for integration on the amylase gene of *B. subtilis*. Two amylase genes were identified in the genome of *B. cereus* #25. The first one has 1,584 bp and encodes for a protein with 527

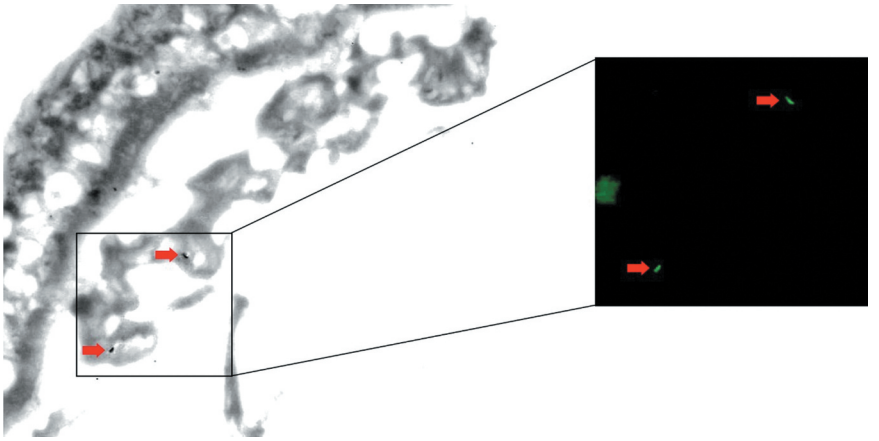

Figure 3. (A) The absence of the GFP cells in the midgut. (B) White light and epifluorescence microscopy evincing the presence of transgenic *B. cereus* #25 (red arrows) expressing GFPmut1 in the midgut of the *L. vannamei*.

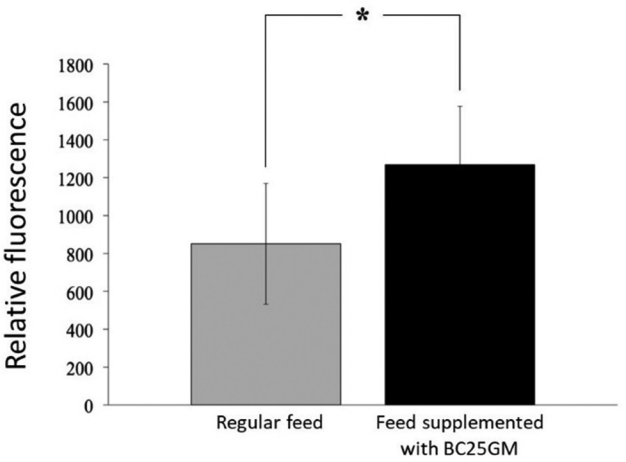

Figure 4. Relative fluorescence in the midgut of the shrimps receiving regular feed and BC25GM supplementation.

amino acids. The other one is shorter, with 1,356 bp and the protein contains 451 amino acids. However, in contrast with the amylase gene from the *B. subtilis*, in *B. cereus* the amylase genes are shorter, lacking the starch binding domain (CBM26). Even with a more accurate investigation throughout *B. cereus* #25 genome, the CBM26 was non-encountered. The native strain has a functional amylase system even without the CBM26 (Figure 2C).

The use of the pSG1154 vector for integration in another *Bacillus* specie was applied in this work. The alignment of the amylase genes from the *B. cereus* #25 and *B. subtilis* revealed only 39.6% of identical sites and 51.5% of identity. The comparison between the *B. subtilis* amylase gene and the more compact

form of *B. cereus* resulted in a lower percentage of identical sites (19,5%). As follows, we hypothesized that this smaller amylase could be nonfunctional since it is unlikable that homologous recombination has occurred in both endogenous amylase genes. Considering that BC25GM expresses GFPmu1 (Figure 2B) and the absence of the amylase activity (Figure 2D), the homology was sufficient for recombination between the plasmid and the endogenous gene. In the same way, *B. mojavensis* was also transformed using the same pSG1154 plasmid (Olubajo and Bacon 2008).

BC25GM was evaluated on its colonization ability in the intestinal tract of *L. vannamei*. GFPmut1 expression was showed using histological analyses (Figure 3). The relative fluorescence was also detected using the protein extract of the shrimp intestinal tract (Figure 4), showing that this strategy allows the identification of the probiotic. A basal fluorescence was observed in the midgut of the shrimps fed only with the regular feed. As described (Jamme et al. 2013) is possible to identify an autofluorescence signal from ex-cited biologic material, such as cell or tissue. BC25GM was detected until 17 days after the previous administration (data not presented). Similar to our findings was reported that a *B. cereus* strain (used for humans) also colonized for 18 days the intestinal tract of the mice after restriction of supply (Duc et al. 2004). In that report, the authors did not recommend the use of *B. cereus* as a probiotic for humans due to the presence of toxin-encoding genes. Regarding this question, we performed an investigation to identify those genes in the *B. cereus* #25 genome. The results pointed out 12 toxin genes related to intestinal infections and health risks in humans (Duc et al. 2004; Zhu et al. 2016). Among them are genes coding for cytotoxin K (*cytK*), non-hemolytic enterotoxin (*NheI*), and hemolysin BL (*Hbl*).

Although the BC25GM presented genes encoding for enterotoxins, its administration did not show toxic effects for *L. vannamei*, since 100% of the survivals were observed in the experimental groups (Table 2). The growth and water quality results revealed no differences compared to the control group. Therefore, the native strain has potential application in carciniculture, once it was obtained from a crustacean intestinal tract. This fact indicates that the microorganism is adapted to the Pacoti River environment, where strong salinity variations daily are commons. Maybe other *Bacillus* species could not be able to colonize crustaceans living there. However, the viability of the *B. cereus* #25 as a probiotic depends on the deletions of the enterotoxin genes. In this case, recent technologies for genome editing such as CRISPR/Cas9 could be employed. Thus, native strains could be implemented as bioreactors for the production and delivery of benefits molecules. One example is the production and delivery of dsRNA working for the activation of the RNA interference system, promoting advantages for the immunology of crustaceans against viruses (Feijó et al. 2015). Another substantial way could employ for molecules, such as phytases, promoting better use of the phosphorus in the feed, helping the nutrition and reducing environmental impacts by fecal excretion (Cheng et al. 2013).

Conclusion

Our study revealed that a manipulated native *Bacillus* strain can colonize the intestinal tract of shrimps. The administration of the strain in the feed did not show toxic effects for *L. vannamei*. In order to increase production in aquaculture systems, we suggest the possibility to implement native strains as a biotechnology tool applied to aquaculture, especially due to the special role to deliver beneficial molecules.

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Disclosure statement

The authors declare that they have no conflict of interest.

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