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Selective inactivation of spores maintaining larvicidal activity in *Bt* serovar *israelensis* irradiated with gamma rays

Inativação seletiva de esporos mantendo atividade larvicida em *Bt* sorovar *israelensis* irradiada com raios gama

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Abstract

Bacillus thuringiensis is used to produce biopesticides against target-insects of importance in agroindustry and human health. However, *B. thuringiensis* can carry the same enterotoxin-encoding genes as *Bacillus cereus*, suggesting a potential risk of food poisoning. The objective of this work was to study the application of gamma radiation to eliminate spores in biopesticides based on *B. thuringiensis* serovar *israelensis* in order to avoid the liberation of these spores in the environment. The application of 20 kGy of radiation ensures total inactivation of the spores in the insecticide without changing the larvicidal activity. Results showed that it is possible to develop formulations containing *B. thuringiensis* serovar *israelensis* (IPS-82) eliminating viable spores with gamma radiation without affecting the larvicidal activity. Irradiated formulations would be safer for the environment and humans since the elimination of viable spores abolishes the pathogenic potential of *B. thuringiensis* strains.

Keywords: biopesticides, gamma radiation, larvicidal insecticides.

Resumo

Bacillus thuringiensis é usado na produção de biopesticidas contra insetos-alvo de importância na agroindústria e na saúde pública. Contudo, *B. thuringiensis* pode apresentar os mesmos genes codificadores de enterotoxinas presentes em *Bacillus cereus*, sugerindo a possível existência de risco para intoxicação alimentar. O objetivo deste trabalho foi estudar a aplicação de radiação gama para a eliminação de esporos em preparações de biopesticidas de *B. thuringiensis* sorovar *israelensis* de forma a se evitar a liberação de esporos viáveis no ambiente. Observou-se que a aplicação de 20 kGy garante a inativação total dos esporos no inseticida sem que haja mudança na atividade larvicida. Os resultados obtidos demonstram que é possível desenvolver formulações contendo *B. thuringiensis* sorovar *israelensis* (IPS-82) eliminando-se esporos viáveis sem alteração da atividade larvicida. As formulações irradiadas seriam mais seguras para o meio-ambiente e para seres humanos uma vez que a eliminação dos esporos viáveis neutraliza o potencial patogênico de cepas de *B. thuringiensis*.

Palavras-chave: biopesticidas, radiação gama, inseticidas larvicidas.

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Introduction

The use of chemical pesticides to control insects has revealed several different risks for man and the environment. The occurrence of chemical pesticides resistance in target insects and human health problems related to their use have been reported (Aranda *et al.,* 2000; van Rie and Ferrè, 2000; Cadavid-Restrepo *et al.,* 2012). These facts prompted the emergence of industrialized biopesticides based on strains of *Bacillus thuringiensis*, a member of *Bacillus cereus senso lato* group, that are capable of producing different crystal δ-endotoxins encoded by *cry* genes. These toxins are the active ingredients of a large number of preparations used against larvae of many insect orders, especially species of Coleoptera, Diptera and Lepidoptera (Aranda *et al.,* 2000; Libman and MacIntosh, 2000; Hansen and Salamitou, 2000).

In 1978, a previously described *B. thuringiensis* strain (Goldberg and Maragalit, 1977) was designated as the 14th *B. thuringiensis* serovar (*Bacillus thuringiensis* var *israelensis*, H-14) (de Barjac, 1978). This bacterium is highly toxic for mosquitos and blackflies (LC_{50} ranging from 10-13mg/ml against fourth instar larvae of many species) and presents three inclusions containing four main proteins, Cyt1A, Cry4A, Cry4B and Cry11A. These proteins have different molecular weights and act in synergy with other Cyt toxins (Federici *et al.* 2000; Crickmore *et al.* 2006; Cadavid-Restrepo *et al.,* 2012). This serovar became the active compound of industrialized biopesticides used all over the world in polycentric programs to control *Aedes spp.* larvae, especially those of *Aedes aegypti*, the yellow fever and dengue vector (Mittal, 2003). It is noteworthy that in Germany, *B. thuringiensis* serovar *israelensis* has been used since 1981 in a program that is conducted by the German Association for Mosquito Control (Kommunale Aktionsgemeinschaft zur Bekämpfung der Stechmückenplage - KABS) (Becker, 2002).

Despite the advantages of using biopesticides instead of chemical pesticides, there are some considerations to be made: *B. thuringiensis* shares a high degree of genetic similarity with *B. cereus*, and it can be distinguished from the last mainly by the production of parasporal bodies containing δ-endotoxins (Yuan *et al.,* 2002). It has been described that *B. thuringiensis* strains can carry the same enterotoxins as *B. cereus*, suggesting that this species may also pose potential risk of food poisoning (McIntyre *et al.,* 2008; Bartoszewicz *et al.,* 2008).

The aim of the present work was to evaluate the application of gamma radiation to reduce the presence of viable spores in biopesticides based on *B. thuringiensis* serovar *israelensis*, in order to decrease or prevent the release of these spores in the environments where the breeding sites of mosquito larvae and black flies are located. This inactivation methodology can be used as an alternative to create an effective, safer and more environment-friendly biopesticide.

Material and methods

Bacterial strains

The toxigenic strain *B. thuringiensis* serovar *israelensis* (H-14) IPS-82 (LFB-FIOCRUZ 584, deposited in the Coleção de Culturas do Gênero *Bacillus* e Gêneros Correlatos – CCGB since 1983) was used. This strain was preserved as lyophilized spores produced in AES medium according to Rabinovitch *et al*. (Rabinovitch *et al.,* 1975) and kept in refrigerator. Cytomorphological, biochemical and physiological characteristics were confirmed as previously described (Gordon *et al.,* 1973; Claus and Berkeley, 1986). The spores obtained from AES medium were conserved in distillated water under refrigeration as suspensions containing $8x10^6$ colony forming unit (CFU)/mL^{-1}.

Production of *B. thuringiensis* IPS-82 formulation

In order to prepare the primary inoculum, the spores contained into two tubes with AES medium (at least 90% of viable spores) were harvested and transferred to 2.0 mL of sterile distillated water and homogenized. One milliliter of this suspension (10^9-10^{10} CFU) was transferred to 100 mL of liquid medium and incubated at 35°C under 120 rpm for 4h. After that, 30 mL of this growth were inoculated in 6L of fermentation medium (Soy meal 60-70% of protein 20 g/L; glucose 3.0 g/L; Yeast extract - 1.0 g/L; NaCl - 2.0 g/L; $MgSO_4.7\ H_2O$ - 0.3 g/L; $MnSO_4.H_2O$ - 0.02 g/L; $ZnSO_4.7\ H_2O$ - 0.02 g/L; $FeSO_4.7H_2O$ - 0.02 g/L; $CaCl_2$ - 0.1 g/L; pH 7.0). Fermentation was carried out in a fermentator New Brunswick FS-314 (14L total capacity) under 500rpm promoted by two sets of submerged turbin-like blades (11cm apart from each other), with aeration of 3L of air/min^{-1} (0.5 vvm), temperature of 33°C and 20mL of antifoam SAG 471 (1:1 in water). Total fermentation time was 22h-24h. The end-point of the process was determined by observation under an optical microscope as the point when the sporogenesis reached at least 90% of free of spores and, in addition, by the pH measure (values between 8.5 and 9.0). After the fermentation, the pH of the fermented medium was adjusted to 4.5 with laboratory grade propionic acid. This medium was centrifuged in a continuous flow Alfa-Laval Gyro Tester centrifuge at 3450 rpm. The final formulation was obtained by the addition of thickener, preservatives, emulsifying, disintegrants and water to the centrifuged, not exceeding 2% (w/w). These additives were mechanically mixed at room temperature (25°C to 30°C) until reaching a uniform creamy texture. The biomass was kept in the refrigerator. The fermentation process generated around 170g of biomass with 70% water content.

Irradiation

Aliquots of formulated biomass were placed in neutral glass tubes with screw caps (75mm x 15mm) until filling them, and sent to IPEN-CNEN/SP, São Paulo to be exposed to an ionizing radiation source of Co-60 (Gammacell 220 Atomic Energy of Canada Ltd.) at an average dose of 4.86 h $^{-1}$ kGy and dose uniformity factor of 1.13. Doses ranged from 0 to 20 kGy.

Quantification of spores in the irradiated biomass

In order to evaluate the effects of different doses of irradiation on the viability of *B. thuringiensis* serovar *israelensis* IPS-82 spores, suspensions containing 25 mg or 50 mg of each irradiated and non-irradiated samples in 25 mL or 50 mL of sterile distillated water were prepared. Two milliliters of each suspension were heated in 100°C water bath for 15 min. After that, heat-treated suspensions were diluted in sterile distilled water at a ratio of 1:10 and 3 aliquots of 0.1 mL of the most diluted suspensions were spread in three Petri dishes containing nutrient agar culture medium (meat peptone 10 g/L; meat extract 3 g/L; NaCl 5g/L; agar-agar 20 g/L; pH 7.5). Colony counting was performed after incubation at 30°C for 24h-30h in order to determine the average number of colony forming units per mg (CFU mg^{-1}).

Biological assays and potency determination

The effect of the irradiation on the insecticidal activity was evaluated by assays with sensitive *Aedes aegypti* larvae. These assays were conducted according to the methodology recommended by the World Health Organization (WHO, 1981), with minor modifications, in order to determine the potency of the material expressed by International Toxic Units per mg (ITU mg^{-1}). For this purpose, 25 mg of each of the irradiated and non-irradiated samples were diluted in 50ml of sterile distilled water and, after homogenization, different aliquots were transferred into plastic cups containing 25 young L4 larvae of *A. aegypti* (Rockefeller strain) in 150 ml of dechlorinated water, comprising different concentrations, at least six. Four cups for each concentration and four cups for control samples, where no insecticide was added, were used. Mortality of the larvae was evaluated 24 hours after incubation at 27°C -28°C, and the lethal concentration to 50% of the larvae (LC_{50}) was determined by the analysis of the probits. The tests were repeated for each sample at least three times on different days and the mean value and the standard deviation were calculated. In addition to each test sample, the LC_{50} of the standard powder IPS-82 (batch #91519) was determined. This powder was provided by the Institut Pasteur (Paris, France) and preserved in refrigerator under vacuum. Its potency was arbitrated at 15,000 ITU per mg, and it allowed determining the average potency of the bioinsecticide samples exposed to different doses of radiation and also of those not irradiated.

Data analysis

The analysis was performed to evaluate the effect of irradiation on the larvicidal activity of formulations based on *B. thuringiensis* serovar *israelensis* IPS-82. The value of decimal reduction (D_{10}) for *B. thuringiensis* spores, representing the radioresistance of the spores, was calculated through linear regression. Larvicidal activity (expressed as potency) of samples of the insecticide that were exposed to different doses of gamma radiation was determined in *A. aegypti* and compared with that of the non-irradiated samples. The ANOVA test was applied in order to compare the potency of irradiated and non-irradiated samples.

Results

Effect of irradiation on the viability of B. thuringiensis serovar israelensis IPS-82 spores

Samples of formulated insecticides biomass containing an average of 3.5 x 10^6 viable spores per mg were subjected to different doses of irradiation. Table 1 shows the average number of viable spores (N) per mg for each dose of radiation (D) used for inactivation. A linear response between N and D logarithms was obtained (Figure 1). The value of decimal reduction (D_{10}) for *B. thuringiensis* spores was 2.89. According to the equation of the curve obtained by the correlation of the data showed in Table 1, only one spore, from 3.5x10^6 that were irradiated, survive after a dose of 19.83 kGy. The application of a dose of 20 kGy ensured total inactivation of the spores in the insecticide.

Table 1. Correlations among irradiation dose with Co-60, average potency and *Bacillus thuringiensis* sorovar *israelensis* IPS-82 residual viable spores (N) in formulated biomass.

Dose (kGy)	Average potency (ITU/mg) ±SD[1,2]	Average of viable spores (N) (CFU/mg)	Log N
0	833± 142	3.50 x 10^6	6.54
5	920 ± 27	4.10 x 10^5	5.61
7.5	838 ± 43	4.60 x 10^4	4.66
10	876 ± 218	2.25 x 10^3	3.35
12.5	1060 ± 277	6.00 x 10^1	1.78
15	1019±10	4.30x10^1	1.64
17.5	1033 ± 95	1.25 x 10^1	1.10
20	1146 ± 89	0	0

Notes: ([1]) Effect on *Aedes aegypti* young L4 larvae. ([2]) Average values for triplicate experiments.

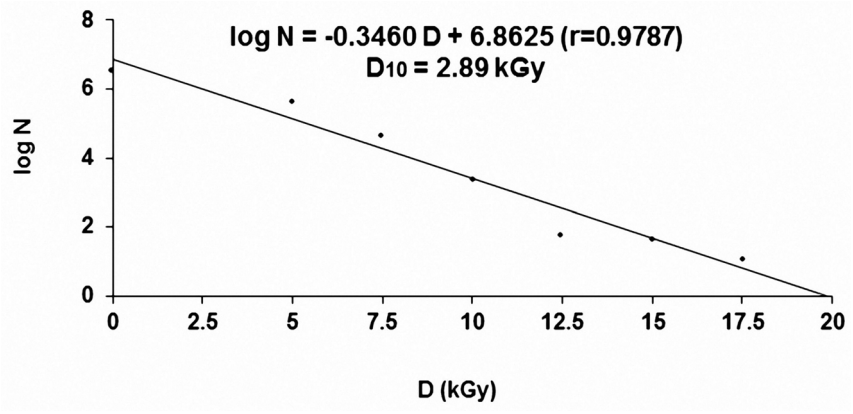

Figure 1. Radio-susceptibility of *Bacillus thuringiensis* serovar *israelensis* IPS-82 spores and decimal reduction value (D_{10}). D – radiation dose; N - residual viable spores in formulated biomass.

Effect of irradiation on the larvicidal activity of emulsifiable concentrate based on *B. thuringiensis* serovar israelensis IPS-82

The variance analysis of the larvicidal activity of samples of the insecticide (expressed as potency) revealed that there is no statistical significance between the mean values of different treatments (Table 1). These results indicate that there was no change in the effectiveness of insecticide after irradiation with Co-60 up to a maximum dose of 20 kGy (p=0.05, confidence interval 95%).

Discussion

This study showed that it is possible to develop formulations containing *B. thuringiensis* serovar *israelensis* (IPS-82) eliminating viable spores with gamma radiation without affecting the larvicidal activity. There are a few works in the literature reporting irradiation of *B. thuringiensis* serovar *israelensis* with Co-60. One of these works reported that gamma radiation doses of 20-25kGy produced a 20-30% reduction in the effectiveness of *B. thuringiensis* serovar *israelensis* powder against L4 Culicidae larvae (Becker, 2002). Melo-Santos *et al.* (2009) reported that two Bti IPS-82 preparations (tablet and technical powder) lost up to 83% of potency after irradiation. Asano *et al.* (2000) found that gamma irradiation of *B. thuringiensis* serovar *kurstaki* spores results in a dramatic reduction of their effect against the diamondback moth larvae, *Plutella xylostella*, indicating that live spores are necessary to achieve full insecticidal activity against these Lepidoptera larvae.

Our results showed that irradiation with Co-60 (ranging from 5 to 20 kGy) gradually decreases the amount of viable spores of *B. thuringiensis* serovar *israelensis* in the formulated biomass. Moreover, it was shown that the larvicidal activity of the preparation remains unchanged. Thus, the results suggest that the mosquitocidal activity of the *B. thuringiensis* serovar *israelensis* strain IPS-82 (LFB-FIOCRUZ 584) used in the formulation tested does not depend on the germination of the spore inside the *A. aegypti* larvae and thus it is not affected by radiation. Our results showed that irradiation with Co-60 (ranging from 5 to 20 kGy) gradually decreases the amount of viable spores of *B. thuringiensis* serovar *israelensis* in the formulated biomass, but the larvicidal activity of the preparation remains unchanged. The discrepancy between the results presented in this work and those described by Asano *et al.* (2000) and Becker (2002) regarding the insecticidal efficiency of irradiated spores may be, in part, due to the different *B. thuringiensis* strains and serovars that were used in each study. This is particularly more relevant when comparing our results with those of Asano *et al.* (2000) where the germination of the spores seems to play an important role on the pathogenicity of *B. thuringiensis* serovar *kurstaki* on *P. xylostella*.

In this work, an "in house" emulsifiable formulation was used instead of the commercial wettable powder used by Becker (2002) or the table and the technical powder used by Melo-Santos *et al.* (2009). In contrast with results reported in both studies, we did not observe any significant loss of potency after the irradiation. Moreover, it seems that the addition of proprionic acid to the fermentation product, creating an acidic environment, protects the crystalline structure of the protoxin. Our results suggest that the type of formulation may influence the conservation of the potency in irradiated products and this should be considered when developing these products.

The original, non-irradiated formulation described in the present study was once tested in the field in three streams with different flows located in Rio Grande do Sul, Brazil (Mardini *et al.,* 1999). The formulation tested against larvae of *Simulium* spp., in doses ranging from 20 to 40 ppm per point of application, was able to totally eliminate the larvae of this insect in a distance of 380m from the point of application (Mardini *et al.,* 1999). Nevertheless, further studies are necessary to evaluate the efficacy of the irradiated formulation in the field.

The present work demonstrates that it is possible to develop industrially formulated products containing *B. thuringiensis* serovar *israelensis* (IPS-82) eliminating viable spores with gamma radiation without affecting the larvicidal activity. Irradiated formulations would be safer for the environment and humans since the elimination of

viable spores abolishes the pathogenic potential of insecticidal *B. thuringiensis* strains. Further studies should evaluate the application of the methodology described here on different formulations using other *B. thuringiensis* serovars active against agricultural pests, in order to assess how the insecticidal activity of these strains is affected by radiation.

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References

ARANDA, E.; LORENCE, A.; TREJO, M.R. 2000. Rural production of Bacillus thuringiensis by solid state fermentation. *In:* J.F. CHARLES; A. DELÉCLUSE; C. NIELSEN-LEROUX (eds.), *Entomopathogenic Bacteria: from laboratory to field applications*. Dordrecht, Kluwer Academic Publishers, p. 317-332. http://dx.doi.org/10.1007/978-94-017-1429-7_17

ASANO, S.; OGIWARA, K.; INDRASITH, LS.; TAKAHASHI, M.; SUZUKI, N. and HORI, H. 2000. Synergism of the spore on insecticidal activity of δ-endotoxin of Bacillus thuringiensis against diamondback moth, Plutella xylostella (Lepidoptera: Yponomeutidae) is not observed at late stage in bioassay. *Applied Entomology and Zoology*, **35**(4):583-590.

BARTOSZEWICZ, M.; HANSEN, B.M.; SWIECICKA, I. 2008. The members of the Bacillus cereus group are commonly present contaminants of fresh and heat-treated milk. *Food Microbiology,* **25**(4):588-596. http://dx.doi.org/10.1016/j.fm.2008.02.001

BECKER, N. 2002. Sterilization of Bacillus thuringiensis israelensis products by gamma radiation. *Journal of American Mosquito Control Association*, **18**(1):57-62.

CADAVID-RESTREPO, G.; SAHAZA, J.; ORDUZ, S. 2012. Treatment of an Aedes aegypti colony with the Cry11Aa toxin for 54 generations results in the development of resistance. *Memórias do Instituto Oswaldo Cruz*, **107**(1):74-79. http://dx.doi.org/10.1590/S0074-02762012000100010

CLAUS, D.; BERKELEY, R.C.W. 1986. Genus Bacillus. *In:* P.H.A. SNEATH; N.S. MAIR; M.E. SHARPE; J.G. HOLT (eds.), *Berguey's Manual of Sytematic Bacteriology.* Baltimore, Williams & Wilkins, p. 1104-1139.

CRICKMORE, N.; BONE, E.J.; WILLIAMS, J.A.; ELLAR, D.J. 2006. Contribution of the individual components of the ä-endotoxin crystal of the mosquitocidal activity of Bacillus thuringiensis subsp. israelensis. *FEMS Microbiology Letters,* **3**(131):249-254.

DE BARJAC, H. 1978. Une nouvelle varieté de Bacillus thuringiensis très toxique pour les moustiques: B.thuringiensis var israelensis sérotype H14. Comptes Rendus de l'Académie des Sciences*,* **286**:797-800.

FEDERICI, B.; PARK, H.W.; BIDESHI, D.K. 2000. Genetic engeneering of bacterial insecticides for improved efficacy against medically important Diptera. *In:* J.F. CHARLES; A. DELÉCLUSE; C. NIELSEN-LEROUX (eds.), *Entomopathogenic Bacteria: from laboratory to field applications*. Dordrecht, Kluwer Academic Publishers, p. 461-484. http://dx.doi.org/10.1007/978-94-017-1429-7_25

GOLDBERG, L.J.; MARAGALIT, J. 1977. A bacterial spore demonstrating rapid larvicidal activity against Anopheles sargentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti and Culex pipiens. *Mosquito News*, **37**(3):355-358.

GORDON, R.E.; HAYNES, W.C.; PANG, C. 1973. Genus Bacillus. *USDA Handbook 427*, Washington DC, USDA, p. 97-98.

HANSEN, B.M.; SALAMITOU, S. 2000. Virulence of Bacillus thuringiensis. *In*: J.F. CHARLES; A. DELÉCLUSE; C. NIELSEN-LEROUX (eds.), *Entomopathogenic Bacteria: from laboratory to field applications*. Dordrecht, Kluwer Academic Publishers, p. 41-64. http://dx.doi.org/10.1007/978-94-017-1429-7_3

LIBMAN, G.N.; MACINTOSH, S.C. 2000. Registration of Biopesticides. *In*: J.F. CHARLES; A. DELÉCLUSE; C. NIELSEN-LEROUX (eds.), *Entomopathogenic Bacteria: from laboratory to field applications*. Dordrecht, Kluwer Academic Publishers, p. 333-353. http://dx.doi.org/10.1007/978-94-017-1429-7_18

MARDINI, L.B.; SOUZA, M.A.; RABINOVITCH, L.; ALVES, R.S.; SILVA, C.M. 1999. Field studies with the bacterial larvicide INPALBAC for Simulium spp. control in Rio Grande do Sul, Brazil. *Memórias do Instituto Oswaldo Cruz,* **94**(5):679-681. http://dx.doi.org/10.1590/S0074-02761999000500023

MCINTYRE, L.; BERNARD, K.; BENIAC, D.; ISAAC-RENTON, J.L.; NASEBY, D.C. 2008. Identification of Bacillus cereus group species associated with food poisoning outbreaks in British Columbia, Canada. *Applied and Environmental Microbiology,* **74**(23):7451-7453. http://dx.doi.org/10.1128/AEM.01284-08

MELO-SANTOS, M.A.V.; ARAÚJO, A.P.; RIOS, E.M.M.; REGIS, L. 2009. Long lasting persistence of Bacillus thuringiensis serovar israelensis larvicidal activity in Aedes aegypti (Diptera: Culicidae) breeding places is associated to bacteria recycling. *Biological Control*, **49**(2):186-191. http://dx.doi.org/10.1016/j.biocontrol.2009.01.011

MITTAL, P.K. 2003. Biolarvicides in vector control: challenges and prospects. *Journal of Vector Borne Diseases,* **40**(1-2):20-32.

RABINOVITCH, L.; PALMEIRA, M.L.; SILVA, S.M. 1975. A spontaneous Spo mutant of Bacillus licheniformis with increased respiratory metabolism. *Revista de Microbiologia,* **6**:47-49.

VAN RIE, J.; FERRÈ, J. 2000. Resistance to Bacillus thuringiensis Insecticidae crystal proteins. *In*: J.F. CHARLES; A. DELÉCLUSE; C. NIELSEN-LEROUX (eds.), *Entomopathogenic Bacteria: from laboratory to field applications*. The Netherlands, Kluwer Academic Publishers, p. 219-236. http://dx.doi.org/10.1007/978-94-017-1429-7_12

WORLD HEALTH ORGANIZATION (WHO). 1981. Guidelines. Report of informal consultation on standardization of Bacillus thuringiensis H-14 TDR/BCV/B.TH-14/811. WHO/VBC 81-828. Geneva, WHO, 15 p.

YUAN, Z.; HANSEN, B.M.; ANDRUP, L.; EILENBERG, J. 2002. Detection of enterotoxin genes in mosquito-larvicidal Bacillus species. *Current Microbiology,* **45**(3):221-225. http://dx.doi.org/10.1007/s00284-001-0105-6

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