Bull Environ Contam Toxicol (2007) 78:239–244
DOI 10.1007/s00128-007-9148-0

Bio-safety Assessment of Validamycin Formulation on Bacterial and Fungal Biomass in Soil Monitored by Real-Time PCR

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Received: 15 October 2006 / Accepted: 19 March 2007 / Published online: 3 May 2007
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Validamycin is a biological fungicide used in the agriculture sector. It can control sheath blight caused by *Rhizoctonia solani* in rice, potatoes, vegetables, as well as damping off diseases in vegetable seedlings, cotton, sugar beets and other plants on a large scale (NIOSH, 1993). It is absorbed by cells of *Rhizoctonia solani* and hydrolyzed to a potent inhibitor of trehalase-validoxylamine A. Validamycin has been considered as a low-toxicity material that can be degraded easily by some bacterium, such as *Flavobacterium saccharophilum*, *Pseudomonas* sp. HZ519 and *Stenotrophomonas maltrophilia* CCTCC M 204024 under lab conditions (Asano et al., 1984; Zheng et al., 2005; zhang et al., 2005). To the best of our knowledge, few studies have focused on the effects of validamycin formulation on the agro-ecosystem. Crystal proteins of *Bacillus thuringiensis* (Bt) also serve as a biological pesticide, also regarded as non-toxic several decades ago. However, following the emergence of Bt-transgenic plants, some reports showed that Bt toxins could accumulate in the soil, resulting in potential risks to soil biological processes including microorganism biomass and soil enzyme activity (Castaldini et al., 2005; Donegan et al., 1995; Wang et al., 2005; Wu et al., 2004a, b). In essence, a biological pesticide is a chemical substance with a definite molecular weight, and can also cause environmental disturbance, just like chemical pesticides (Shen, 1997). Bearing in mind these concerns, it is necessary to assess the effects of validamycin formulation on agro-ecosystem and the corresponding risks.

The widespread and nonjudicious agricultural use of pesticides results in their entry into the soil and water ecosystems. Soil is the most diverse terrestrial habitat, and soil microbial communities play important roles in decomposition, nutrient cycling, and energy flow (Waldrop and Firestone, 2006; Wardle and Giller, 1997). Much work has been directed towards understanding the complexity of pesticide–microbial interactions in soil, indicating that in general soil microbial populations are affected by pesticide (Griffiths et al., 2006; Lu et al., 2006). Conventional microbial methods that rely on microbial cultivation can only identify a small fraction (0.01–10%) of the total microbial biomass (Ferguson et al., 1984), because they are unable to culture the majority of microorganisms from soil samples. This was a main obstacle for the better understanding of microbial ecology and diversity. Recently, soil microbiologists have developed several molecular methods to overcome this limitation by allowing particular genes to be monitored directly in the environment. Such methods include the polymerase chain reaction (PCR), and microarray-based genomic technology. Real-time PCR is a method based on the use of fluorescent probes or dyes to quantify the copy number of target DNA in a sample. This technology, which is not limited by the cultivability of soil microorganisms, has been successfully applied in the medical and environmental fields for the quantification of bacteria (Kolb et al., 2003), viruses (Schaade et al., 2000) and fungi (Lees et al., 2002). In this investigation, we developed a real-time PCR assay that can quantify soil bacteria and fungi by using specific primers based on 16S

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ribosomal DNA (16S rDNA) and 18S ribosomal DNA (18S rDNA) with the fluorescent Sybr Green I dye. From the environmental bio-safety point of view, we further assessed the effects of the validamycin formulation on the biomass of soil microorganisms.

Materials and Methods

A yellow loamy soil collected from the 0–20 cm layer from the botanical gardens of Zhejiang University, Hua-jia-chi Campus, Hangzhou, China was used in this investigation. Soil samples were air-dried at room temperature, sieved through 2 mm mesh soil sieve to remove plant materials, soil macrofauna and stones, and then homogenized in a rotary cylinder. The chemical composition of soil samples included 1.41% total organic C content, and 115.8 mg kg^{-1} available N, 25.2 mg kg^{-1} available P, 58.5 mg kg^{-1} available K with a pH 7.10 (H_2O), 0.4% total soluble salts and a pH in water (1:2.5 w/v) of 7.2.

Soils were placed in cylindrical plastic pots, with 2 kg in each pot. Then, 500 ml of sterile deionized water was added to submerge the soils to a depth of 2 cm. The soils were incubated in the dark at 28 ± 1°C for two weeks to recover the soil microorganisms. The validamycin formulation was produced by Qianjiang Biochemical Ltd., China. Zero (control), 0.375,0.75,1.5,3,6,12 (g kg^{-1} dry soil) validamycin effective ingredient was added in treatments lablled 0–6, respectively, and incubated at 28°C for four weeks. Loss of water by evaporation was compensated everyday to avoid dryness. Soil subsamples were taken after 3, 7, 14, 21 and 28 days of incubation following pesticide application to assay the copy numbers of 16S rDNA and 18S rDNA.

DNA extraction from the soil samples followed the protocol described by Qian et al. (2005). In order to investigate the presence of bacterial and fungal strain, universal primers specific for 16S rDNA of eubacteria 338f (5′- ACT CCT ACG GGA GGC AGC AG–3′) and 518r (5′- ATT ACC GCG GCT GCT GG –3′) (Liu et al., 1997); and for 18S rDNA of Fungal EF4f (GGAAGGG[G/A]TGTATTTATTAG) and Fung5r (GTAAAAGTCCT GGTTCCC) (vanElsas et al., 2000; He et al., 2005) were used to amplify and generate about 220-bp and 530-bp DNA fragments. Primers were then used for real-time PCR amplification in the soil samples. For real-time PCR, 1 µl of 50-fold diluted sample was added to 5 µl of a PCR mixture prepared from 2× Sybr Green PCR Master Mix (TaKaRa), with each primer at a concentration of 400 nM. The cycle parameters were as follows: 10 s at 95°C and 40 cycles of 5 s at 95°C and 31 s at 60°C according to the instructions of the manufacturer. The template DNA was amplified and monitored using an ABI Prism SDS 7300 instrument (PE Applied Biosystems, Foster City, CA, USA).

One copy of 16S rDNA or 18S rDNA fragment was cloned in a pGEM-T Easy Vector System (Sangon, China). A standard curve was also generated by using recombinant plasmid containing 16S rDNA or 18S rDNA fragments. *Escherichia coli* strain JM109 (TaKaRa) was transformed with the cloning vector, and transformants were selected by blue–white selection on Luria-Bertani agar plates containing ampicillin (100µl ml^{-1}), X-Gal (5-bromo-4- chloro-3-indolyl-beta-D-galactoside; 1 mg/plate), and isopropyl-beta-D-thiogalactopyranoside (IPTG; 2.38 mg/plate). High-purity plasmid was obtained by extracting 500 µl of an *Escherichia coli* culture with a Plasmid Maxi kit (Qiagen). DNA was quantified by using an Ultrospec-4300 Pro (Amersham Biosciences). Serial dilutions of DNA were prepared by using sterile water and PCR was performed as described below. Standard curves based on threshold cycles (Ct) for the 10-fold dilution series of the corresponding plasmid DNA (10^3–10^{10} copy number per µl) were constructed for bacterium and fungus. The standard curve was obtained by plotting the Ct value, which is defined by the crossing cycle number or crossing point, against the logarithm of the concentration of each 10-fold dilution series of plasmid DNA. As Ct values may vary slightly between experiments, parallel sets of the three dilution series of pure standard DNA were run in all experiments.

The relationship between Ct and the target gene copy number is: $\log T_0 = -\log E \times Ct + \log K$, where E is the PCR efficiency and was calculated for one cycle in the exponential phase according: $E = 10[-1/\text{slope}]$ (Rasmussen, 2001), T_0 is the initial amount of DNA and K is the calculated initial amount of DNA for a Ct value of 0. The copy numbers of the real-time PCR standards were calculated by assuming average molecular masses of 660 Da for 1 bp of double-stranded DNA (PCR applications manual, 2nd ed., Roche Diagnostics GmbH, Mannheim, Germany, 1999). This can be calculated using the following equation: copies per nanogram = $(n \times mw)/(NL \times 10^{-9})$, where n is the length of the standard in base pairs, mw is the molecular weight per bp, and NL is Avogadro's constant (6.02×10^{23} molecules per mole). Because the total numbers of microorganisms in the control also varied during the experimental process, the stimulated intensity (SI) was also used as an evaluated index, defined as the ratio of the total number of microorganisms in the treated sample with that in the control. One-way analysis of variance (ANOVA) was performed in each environment to determine differences in 16S rDNA and 18S rDNA copy numbers. Methods were compared using the least significant difference (LSD) test at a 0.05 significance level.

Results and Discussions

Serially diluted DNA originating from two recombinant plasmid DNA segments including 16S rDNA and 18S rDNA fragments showed the single expected amplicons of 220 bp and 530 bp, respectively, as determined using agarose gel electrophoresis (Fig. 1). The use of standard curves based on a known copy of the target genes makes it possible to quantify DNA from any source. In this study, standard curves generated for 16S rDNA [log (16S rDNA) = log 0.340×Ct + 14.314, $R^2 = 0.978$] and 18S rDNA [log (18S rDNA) = log 0.352×Ct +14.450, $R^2 = 0.977$] target molecules were linear from 10^3 to 10^{10} (10^9 for 18S rDNA) copies.

16S rDNA target molecules, ranging from 1.70×10^{10} to 536.53×10^{10} copies per gram of dry weight of environmental sample, were significantly different among some of the treatments (Fig. 2a). The copy numbers of 16S rDNA of all the treatments fluctuated during the experiment; the amount of fluctuation in the seven treatments was different but showed a similar trend in each. When treatment lasted longer than three days, the copy numbers of 16S rDNA began to change, but only the copy number in treatment 4 (where the concentrations of validamycin formulation was 3 $g.kg^{-1}$ dry soil), which rose up to 3.36-fold, was significantly higher than that of the control (Table 1). During the seventh treatment day, the copy number of 16S rDNA of treatments 2, 3, 4, 5, and 6 (with concentrations of validamycin formulation of 0.75, 1.5, 3, 6, 12 $g.kg^{-1}$ dry soil, respectively) increased by varying amounts. The intensity of the stimulation was dose dependent, but not in a linear relationship with the concentration of validamycin formulation. The higher the concentration of validamycin formulation from 0–3 $g.kg^{-1}$ dry soil, the higher the copy numbers of 16S rDNA . The highest copy number was 536.53×10^{10} g^{-1} dry soil, over 25 times higher than that of the control in treatment 4. During treatment days 14 to 28, the copy numbers of 16S rDNA dropped quickly, but were still higher than that of the control in the treatments with high concentrations of the validamycin formulation; the stimulated intensity also dropped.

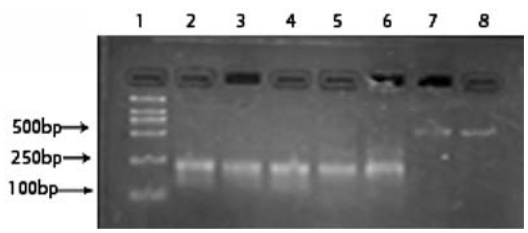

Fig. 1 Agarose gel (1.5%) electrophoresis for real-time PCR amplification of 16S rDNA and 18S rDNA. Line 1: molecular weight marker; line 2–6: PCR amplification of 16S rDNA (about 220bp); line 7–8: PCR amplification of 18S rDNA (about 530bp)

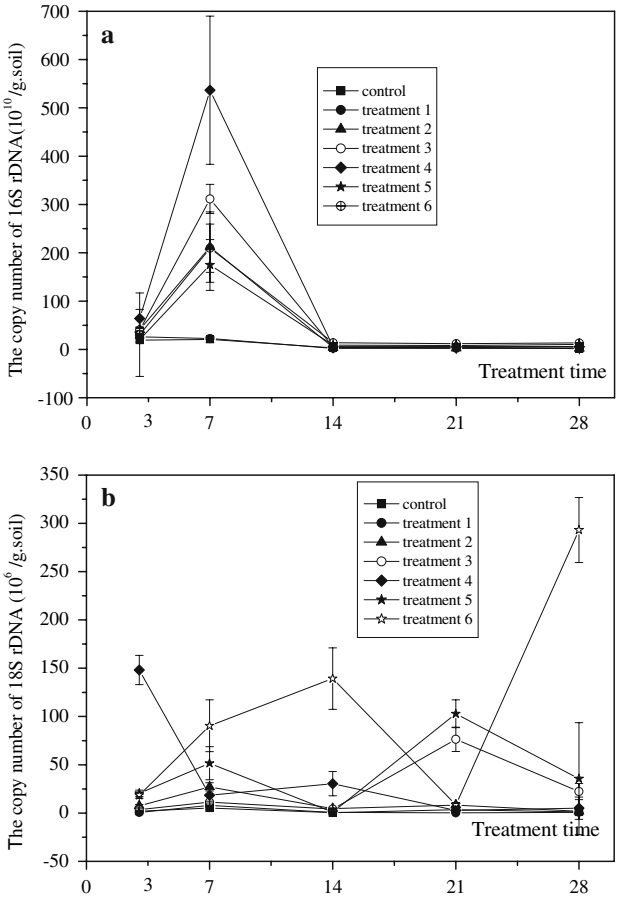

Fig. 2 a. Effect of validamycin on the copy numbers of 16S rDNA b. The effect of validamycin on the copy numbers of 18S rDNA

The copy numbers of 18S rDNA in soil treated at the six dosages, ranging from 0.71×10^6 to 293×10^6 copies per gram of dry weight of environmental sample, showed different responses (Fig. 2b). The validamycin formulation at low concentrations had no significant effect on the copy numbers of 18S rDNA during most of the incubation periods; only validamycin formulation at high concentration (as in treatments 4–6) could increase the copy numbers of 18S rDNA. The promotion effect recorded from day 3 to 28 in the sample subjected to treatment 6 was especially notable; the highest stimulated intensity (about 284-fold) appeared after day 14 of treatment, and the highest copy number appeared after day 28. The copy numbers in the other treatments recovered to the level of the control after 28 days of treatment.

Rapid real-time PCR can result in the development of appropriate control measures and/or eradication procedures more rapidly and accurately than conventional methods for microorganisms isolation and quantification (Bart et al., 2006; Gonod et al., 2006). In this study, two standard curves with R^2 values above 0.97 for both 16S rDNA and 18S rDNA were constructed after real-time PCR

Table 1 The effect of validamycin on 16S rDNA copy numbers

Treatment/day	16S rDNA copy numbers per gram dry weight ($\times 10^{10}$)									
	3d		7d		14d		21d		28d	
	SI		SI		SI		SI		SI	
Control 0 g. kg^{-1}dry soil	1.00	a	1.00	a	1.00	a	1.00	b	1.00	a
Treatment 1 0.375g. kg^{-1}dry soil	1.37	a	1.09	a	0.72	a	0.50	a	1.57	a
Treatment 2 0.75 g. kg^{-1}dry soil	2.14	ab	10.16	a	1.24	b	0.58	b	1.21	a
Treatment 3 1.5 g. kg^{-1}dry soil	2.10	ab	14.95	b	1.71	c	1.19	b	3.48	b
Treatment 4 3 g. kg^{-1}dry soil	3.36	bc	25.75	c	1.81	c	1.20	b	3.27	b
Treatment 5 6 g. kg^{-1}dry soil	1.07	a	8.39	a	2.76	d	1.76	c	6.12	c
Treatment 6 12 g. kg^{-1}dry soil	1.59	a	10.06	a	4.40	e	2.57	d	7.84	c

Means with the same letter are not significantly different at $P < 0.05$. SI: stimulated intensity, the ratio of the copy number in the treatment and the control

Table 2 The effect of validamycin on 18S rDNA copy numbers

Treatment/day	18S rDNA copy numbers per gram dry weight ($\times 10^{6}$)									
	3d		7d		14d		21d		28d	
	SI		SI		SI		SI		SI	
Control 0 g. kg^{-1}dry soil	1.00	a	1.00	a	1.00	a	1.00	a	1.00	a
Treatment 1 0.375g. kg^{-1}dry soil	0.35	a	1.50	a	1.27	a	0.08	a	0.67	a
Treatment 2 0.75 g. kg^{-1}dry soil	3.70	a	5.13	ab	9.44	a	2.41	a	1.66	a
Treatment 3 1.5 g. kg^{-1}dry soil	1.79	a	2.15	a	8.48	a	21.99	b	19.40	a
Treatment 4 3 g. kg^{-1}dry soil	73.13	b	3.51	ab	62.33	a	0.80	a	4.44	a
Treatment 5 6 g. kg^{-1}dry soil	1.07	a	9.76	bc	2.17	a	29.62	c	30.94	a
Treatment 6 12 g. kg^{-1}dry soil	9.09	a	17.09	c	284.75	b	2.45	a	254.89	b

Means with the same letter are not significantly different at $P < 0.05$. SI: stimulated intensity, the ratio of copy number in the treatment and control

amplification of two pure recombinant plasmids, confirming the linearity of the quantification process between exponential increases of DNA concentrations and real-time PCR threshold cycles.

The quantity of 16S rDNA may vary among bacterial species depending on the number of copies per genome and the growth phase of the cell. However, only 1–12 ribosomal operons per genome are present in bacterial cells (Fogel et al., 1999), not several thousand, so quantification of 16S rDNA through real-time quantification can serve as an indicator of the bacterial biomass. Because most bacteria contain several operons of the rRNA genes, the

measured target numbers are higher than actual cell numbers. However, DNA extraction efficiencies of less than 100% will underestimate the cell number in any DNA-based method. Overall, rather close correlation of the measured target numbers to the added cells show that real-time PCR can indeed measure the target numbers from environmental samples and that these are close to the actual cell numbers (Stubner, 2004). Several studies have compared real-time PCR measurements both with rRNA genes and functional markers with data from other enumeration methods and also found good correlation (Stubner, 2004). Molecular identification of fungi to the species level has generally utilized 18S rDNA. Although the taxonomic resolution of 18S rDNA may not be sufficient always to identify fungal species and strains, this gene is conserved enough to allow comparison across a wide range of fungal taxa and is therefore potentially useful for an initial assessment of the total fungal communities. Hunt et al. (2004) showed the EF4/Fung5 and EF4/EF3 PCR primers to be highly specific for fungal 18S rDNA sequences. The copy number of the rRNA operon is known to vary among different fungal species (Hibbett, 1992). However, the exact number of copies contained by the genome of any single species remains unknown in most species at present. This variation among species complicates the quantification of different fungal species in a mixed DNA sample, but the 18S rRNA gene is still the most widely used. In this context, we used eubacteria 16S rDNA primers (338f and 518r) and fungal 18S rDNA primers (EF4f and Fung5r) to quantify the copy numbers of 16S rDNA and 18S rDNA, and we also used the copy number of rDNA as an indicator of the biomass of soil bacteria and fungi. Furthermore, the numbers for eubacterial 16S rDNA targets in soil samples (approximately 10^{10} per gram dry weight) are similar to the cell counts from previous studies (Reichardt et al., 1997).

The major problems emerging from the use of pesticides in agriculture are their persistence in the soil, toxicity to non-target organisms and the selection of resistant pest species. As microorganisms, mainly bacteria and fungi, are responsible for the biological transformations that make nutrients available for plants, it is particularly interesting to relate pesticide-induced soil microorganisms changes to soil fertility (Crecchio et al., 2001). Validamycin formulation is a biological pesticide. It is a misconception that, because they are natural, biological pesticides are inherently safe. Naturally produced molecules can also be extremely toxic. Nicotine or *Clostridium botulinum* toxins are of similar or greater toxicity than the most acutely toxic synthetic pesticides. Organisms such as *Plasmodium falciparum, Escherichia coli 0157* or *Listeria* spp. have resulted in significant numbers of deaths or cases of disease in humans. Therefore, the potential of biological pesticides must be evaluated for their adverse affect on the ecosystem (Dewhurst, 2001). In our previous study, the activities of catalyse, urease, acid phosphatase changed significantly in soil samples treated with validamycin formulation, and the variation between samples was related to specific soil enzymes and the treatment concentration of the validamycin formulation and treated time (Qian et al., 2007). In this study, the copy numbers of 16S rDNA increased for almost all the concentrations of validamycin formulation, corresponding to an increase of bacterial quantity. The highest copy numbers of 16S rDNA were 25 times higher than the control in the 3 g. kg^{-1}dry soil validamycin formulation after the seventh treatment. The increase during the seven-day treatments increased according to the concentration change of the validamycin formulation from 0 to 3 g. kg^{-1}dry soil, and then dropped for higher concentrations (3–6 g. kg^{-1}dry soil), although still higher than that of the control. The stimulating intensity of the following treatment time slowly became weak. Only validamycin formulation treatment at a high dosage (treatments 5 and 6) maintained some degree of stimulation until day 28. The validamycin formulation could also affect the number of fungal species; the effect is dosage dependent. During the whole treatment, the numbers of fungi were not affected by low-dose validamycin formulations, but were stimulated strongly by high-concentration formulations. The highest stimulation effect was about 284 fold, far higher than the highest stimulating intensity to bacteria (about 25-fold). Validamycin formulation is a nonsystemic antibiotic with fungicide attributes, but it did not decrease the total numbers of soil fungi in our experiment. Some possible reasons for this include: (1) the validamycin formulation may be easily degraded in the soil environment, as has been seen in vitro (Qian et al., 2007). The population trend of bacteria, which increases sharply in a short time and then drops to the control level, shows that the validamycin formulation could be utilized by bacterium quickly in the beginning, when the bacteria counts are higher than the fungi counts. This may largely prevent its inhibition effect on the fungi biomass, while the degradation products may actually promote the growth of fungi. (2) Real-time PCR focuses on DNA analysis but not the activity of microorganisms. PCR can also amplify DNA from dead or inactive organisms. However, DNA from dead cells in soils would be degraded rapidly (England et al., 1998).

Our results clearly demonstrate that real-time PCR by Sybr green detection with corresponding primers is suitable for the quantification of bacteria and fungi in complex habitats. To our knowledge, this is the first PCR-based approach that enables absolute quantification of bacteria and fungi populations in soil treated with a validamycin formulation. The results indicate that the validamycin formulation significantly affected the numbers of bacteria

and fungi in the soil, like other pesticides. While the biomass of soil bacteria and fungi could recover to a normal level in low-dose validamycin formulation treatment in about one month, high doses of validamycin formulation may decrease the quantity of soil microorganisms, and require relatively long times to recover.

Acknowledgements This work was financially supported by the Natural Science Foundation of China (no. 20607020), the Natural Science Foundation of Zhejiang Province (no. Y504076) and the Ministry of Education Key Laboratory of Environment Remediation and Ecological Health of China (no. 050402).

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