Vol. 381: 157–165, 2009
doi: 10.3354/meps07933

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published April 17

OPEN ACCESS

Bacillus insecticides are not acutely harmful to corals and sponges

Andrew P. Negri*, Rochelle M. Soo, Florita Flores, Nicole S. Webster

Australian Institute of Marine Science, PMB 3 MC, Townsville, Queensland 4810, Australia

ABSTRACT: *Bacillus thuringiensis* is a Gram-positive bacterium that produces crystalline endotoxins and is widely considered an environmentally safe insecticide to control mosquitoes and a number of agriculture pests. Bacteria closely related to *B. thuringiensis* have recently been discovered in association with diseased sponges, which has raised concerns that *Bacillus* insecticides may be harmful to tropical marine invertebrates. We exposed coral larvae and juvenile corals to the insecticides VectoBac® G (containing *B. thuringiensis israelensis*) and VectoLex® G (containing *B. sphaericus*). VectoBac G and VectoLex G had no effect on the survival and metamorphosis of *Acropora millepora* and *A. tenuis* larvae at very high concentrations (5000 µg l^{-1}). The juvenile corals of the same species were also unaffected after 4 sequential 48 h exposures to *B. thuringiensis israelensis* and *B. sphaericus* at different stages of development. Adult corals (*A. millepora*) and sponges (*Ianthella basta*) were exposed to a single 6 h pulse of 1000 µg l^{-1} VectoBac G. Although *B. thuringiensis israelensis* was detected in the seawater using denaturing gradient gel electrophoresis, it was not detected in association with the corals or sponges. No evidence of coral or sponge disease was observed during the following 2 wk. These results indicate that insecticides containing endotoxin-producing *Bacillus* spp. are unlikely to be acutely pathogenic to corals and sponges. However, the effect on most tropical marine invertebrates remain untested and the risks of seed populations of alien *Bacillus* becoming established on reefs and horizontal transfer of toxin genes to native bacteria also need to be addressed.

KEY WORDS: Insecticide · *Bacillus* · Coral · Sponge · Larvae · Pesticide · Pollution · Pathogen

INTRODUCTION

Bacillus thuringiensis is an endospore-forming Gram-positive bacterium capable of producing crystalline protein δ-endotoxins (Höfte & Whiteley 1989). These proteins are toxic to several groups of insects and cause necrosis of the gut epithelium that in turn results in starvation and eventual mortality. The use of *B. thuringiensis* is widespread in the tropics, where it is used to control mosquitoes (Lacey & Undeen 1986) and pests that affect plantations of palm, sugar cane and cotton (Hoong & Hoh-Christopher 1992, Walker et al. 2003). Commercial *B. thuringiensis* is generally considered environmentally benign due to its high specificity (Höfte & Whiteley 1989); however, its effects on tropical marine organisms have not been explored.

Although *Bacillus* spp. are a common component of marine microbial populations (Ivanova et al. 1999), toxic spore-forming *B. thuringiensis* comprise only a small subset of *Bacillus* reported in the marine environment (Maeda et al. 2001). Two *Bacillus* strains were recently isolated from diseased sponges, *Ianthella basta*, from reefs off Papua New Guinea (Cervino et al. 2006). One strain was most closely related to the group containing *B. cereus*, *B. thuringiensis* and *B. anthracis*, while the other was most closely related to *B. pumilus* (which can be used as a fungicide) (Jacobsen et al. 2004). Simultaneous re-infection of *I. basta* with 5 isolates (both *Bacillus* strains and 3 additional *Pseudomonas* strains) resulted in the onset of disease in this sponge species (Cervino et al. 2006). This result raised concerns that accidental overspray or runoff contain-

*Email: a.negri@aims.gov.au

ing commercial *Bacillus* spp. may contribute to unexplained disease in coral reef invertebrates (Cervino et al. 2006).

To test the potential pathogenicity of commercial *Bacillus* spp. to important tropical marine species we sequentially exposed larvae and juveniles of the corals *Acropora millepora* and *A. tenuis* to the mosquitocides VectoBac G containing *B. thuringiensis israelensis* and VectoLex G containing the closely related *B. sphaericus*. We measured coral larval survival and metamorphosis, and the survival and development of juvenile corals. We also assessed the potential for commercial preparations of VectoBac G and VectoLex G to infect adult *A. millepora* colonies and the sponge *I. basta*. The coral and sponge exposures mimicked potential exposures by direct accidental overspray or recent runoff with freshly prepared insecticide.

MATERIALS AND METHODS

Insecticide preparation. Suspensions of bacterial insecticides were prepared in 0.2 µm filtered seawater (FSW) from VectoBac G (*Bacillus thuringiensis israelensis*, Strain H-14 and VectoLex G (*Bacillus sphaericus* Serotype H5a5b, Strain 2362 (both from Valent BioSciences). Fresh suspensions were prepared 2 h before each inoculation, maintained at 28°C and resuspended by periodic shaking. Subsequent inoculations mimicked events where corals or sponges might be exposed via recent accidental overspray. Exposures were expressed as µg insecticide (VectoBac G or VectoLex G) l^{-1} and were 10- to 100-fold higher than concentrations that affect target mosquitoes (Brown et al. 2000, Russell et al. 2003).

Coral and sponge collection. Reproductively mature colonies of the 2 broadcast spawning coral species, *Acropora millepora* (Ehrenberg) and *A. tenuis* (Dana), were collected from 3 to 5 m depths at Magnetic Island (19° 10′ S, 146° 52′ E). These corals were maintained outdoors in 27 to 29°C flowing seawater in 80 % shaded aquaria at the Australian Institute of Marine Science, Townsville. Gametes were collected and larvae cultured following the methods described in Negri & Heyward (2000). Following coral spawning, 3 mature colonies of *A. millepora* were fragmented into replicate 8 × 8 cm pieces and allowed to acclimate for 1 wk. The sponge *Ianthella basta* (Pallas) was collected from a 15 m depth at Davies Reef (18° 50′ S, 147° 38′ E).

Inoculation of larval and juvenile corals and mosquito larvae with *Bacillus* spp. Coral larval exposures were conducted in sterile 6-well, 12 ml, polystyrene cell culture plates (Nunc) at 28°C and 70 µmol quanta m^{-2} s^{-1} using compact fluorescent tubes (55 W) emitting strongly between 420 and 440 nm under a 12 h light:12 h dark cycle. These intensity and spectral conditions mimic sheltered reef habitats of ~8 m depth where these coral larvae would normally settle. Individual wells contained 10 to 15 *Acropora millepora* or *A. tenuis* 6 d old larvae in 10 ml FSW. Wells from each plate hosted all 4 treatments. To test the effects of *Bacillus* spp. on a variety of developmental stages, repeated 48 h exposures were performed (1) on pre-competent (to metamorphose) larvae, (2) when the same larvae became competent, (3) following metamorphosis as azooxanthellate juveniles and (4) once the juveniles had taken up symbiotic zooxanthellae (Table 1). The FSW was changed daily and larvae of both species were exposed to 0, 100, 1000 or 5000 µg l^{-1} VectoBac G or VectoLex G for each of the stages. Larvae were assessed for survival (still mobile) at 5 d and survival and metamorphosis at 9 d respectively (Markey et al. 2007). Larval metamorphosis was induced by the addition of 5 µl of crustose coralline algae (CCA) extract (Markey et al. 2007). Juveniles were inoculated with symbiotic dinoflagellates (*Symbiodinium* spp.) prepared from their parental colonies

Table 1. *Acropora millepora* and *A. tenuis*. Dosing regime and coral health assessments at different larval and juvenile stages. Both species were repeatedly dosed throughout their development with 0, 100, 1000 or 5000 µg l^{-1} VectoBac G (VB) (*Bacillus thuringiensis israelensis*) for each larval stage. In addition, *A. millepora* larvae were dosed with the same concentrations of VectoLex G (VL) (*B. sphaericus*). Seawater was changed in each well on a daily basis. Symbol Y indicates when inoculations occurred. Assessment abbreviations: srv = survival; mm = metamorphosis; sym = symbiont uptake; pol = polyp numbers assessed

Life stage	Age (d)	VB or VL inoculation	Assessment
Pre-competent larvae	0		
	2		
	3	Y	
	4	Y	
	5		srv
Competent larvae	6		
	7	Y	
	8	Y	
	9[a]		srv
Azooxanthellate juveniles	1		mm
	2	Y	
	3	Y	
	4		
	5		
	6		
Zooxanthellate juveniles	7[b]	Y	
	8	Y	
	28		srv, sym, pol

[a]Metamorphosis induced with crustose coralline algae extract
[b]Inoculation with *Symbiodinium* spp. (10^5 cells per 10 ml well)

as per the methods described by Owen et al. (2003). Juveniles were assessed for survival and symbiont uptake (*Symbiodinium* spp. visible in polyps) and the number of polyps per colony counted under a dissecting microscope at 28 d. The efficacy of both VectoBac G and VectoLex G were tested against target salt-marsh mosquito larvae (third instar *Ochlerotatus vigilax* obtained from The Queensland Institute of Medical Research) using assays as described by Russell et al. (2003). Ten *O. vigilax* larvae were transferred into 4 replicate 150 ml beakers and inoculated with 0 to 1000 µg l^{-1} VectoBac G or VectoLex G (under the same light and temperature conditions as for the coral larvae). Survival after 24 h was indicated by movement of larvae following gentle prodding with a glass pipette.

Inoculation and sampling of sponges and adult corals with VectoBac G. *Acropora millepora* (n = 9) and *Ianthella basta* (n = 7) were placed in partially shaded (80%) outdoor, 1000 l flow-through aquaria (1 µm filtered) at 28°C. Three specimens of *A. millepora* and a single *I. basta* were removed at time $t = 0$ as controls. The water flow was stopped and the remaining corals and sponges were inoculated to a final concentration of 1000 µg l^{-1} VectoBac G. Water flow was maintained with a submerged pump and aeration. Flow-through water was resumed after 6 h for all treatments. The seawater (1 l) was sampled before inoculation, at 6 h and again at 14 d for detection of *Bacillus thuringiensis israelensis*. The sponges (n = 3) and adult corals (n = 3) were sampled for microbes at 1 and 14 d and visually assessed for disease at 14 d.

DNA extraction of corals and sponges. Sample tissues (approx. 250 mg) were aseptically transferred to sterile 2 ml microfuge tubes and 500 µl of grinding buffer (2 ml 1 M Tris, 4 ml 0.5 M EDTA, 2 ml 10% sodium dodecyl sulphate [SDS], 400 µl 5 M NaCl and 11.6 ml distilled water) were added to each replicate sample. Tubes were immersed in liquid nitrogen and ground with plastic pestles. Samples were incubated at 65°C for 60 min before the addition of 187 µl of 5 M potassium acetate. Samples were then incubated on ice for 30 min and centrifuged at 8000 × *g* for 15 min. The supernatants were transferred to fresh tubes and DNA was precipitated with a 0.8‰ volume of isopropanol, washed with 70% ethanol and resuspended in distilled water.

DNA extraction of seawater. To determine whether *Bacillus thuringiensis israelensis* was retained in the seawater, 1 l samples were filtered through 0.2 µm Sterivex filters (Millipore), filled with 1.8 ml lysis buffer (40 mM EDTA, 50 mM Tris and 0.75 M sucrose) and frozen at –20°C. Sterivex filter units were then filled with 200 µl of lysozyme (10 mg ml^{-1}) and incubated at 37°C for 45 min. A 200 µl aliquot of Proteinase K (0.2 µg ml^{-1}) in 1% SDS was added to each sample and the filters were incubated at 55°C with rolling for 1 h. The lysates were then recovered from each filter bell into 2 × 2.5 ml microfuge tubes. The DNA was extracted from all tubes using an equal volume of phenol:chloroform:indoleacetic acid (IAA) (25:24:1, pH 8.0) and a further extraction with an equal volume of chloroform:IAA (24:1). DNA was precipitated with a 0.7‰ volume of isopropanol, washed with 70% ethanol and resuspended in distilled water.

DGGE. To assess bacterial changes in adult corals and sponges following the addition of VectoBac® G, all samples were processed for denaturing gel gradient electrophoresis (DGGE). The 16S rRNA gene from each sample was amplified by PCR with universal bacterial primers 1055f: 5′-ATG GCT GTC GTC AGC T-3′ and 1406r: 5′-ACG GGC GGT GTG TAC-3′ (Ferris et al. 1996). Products from duplicate PCR reactions were combined and applied to a 40% wt/vol polyacrylamide (37–5:1) gel containing a 50 to 70% denaturing gradient of formamide and urea. The gel was electrophoresed at 60°C for 17 h in 1 × Tris-acetate-EDTA (TAE) buffer at 50 V using the Ingeny D-Code system. The gel was stained with 1 × Sybr Gold for 30 min, visualised under UV illumination and photographed. Representative bands were excised, re-amplified by PCR with the original primers and checked for correct mobility on another 50 to 70% DGGE gel. PCR products were sequenced using the forward primer and the PRISM Ready Reaction Kit (PE Applied Biosystems) and ABI 310 and 373 automated sequencers.

Survival of *Bacillus* insecticides in seawater. Sterile FSW and sterile filtered ultrapure (Milli-Q) water (0.2 µm) were inoculated with 1000 µg l^{-1} VectoBac G or VectoLex G in 3 replicate 1 l flasks. These suspensions were incubated at 28°C for 1 wk and subsampled at 2 and 7 d. Serial dilutions of the incubations were spread-plated in triplicate on Marine Agar 2216 (Difco Laboratories) for detection and enumeration of bacterial cells as previously described (Webster & Hill 2001).

Data analysis. Statistical analyses were performed using STATISTICA 6.0 (Statsoft). Data from larval survival and metamorphosis, and all juvenile experiments were arcsine transformed to meet the assumptions of normality and homogeneity of variance. Differences between means were tested using 1-factor ANOVA. Significant differences between treatment means were assigned at $p < 0.05$. If significantly different, post hoc comparisons of the means for significant factors in the ANOVA were carried out using Tukey's HSD multiple comparisons tests. Untransformed bacterial counts (CFU) taken from different water types at different times were tested using 2-way ANOVA.

RESULTS

Effects of *Bacillus* spp. insecticides

Coral larvae

The sequential exposure of *Acropora millepora* and *A. tenuis* to VectoBac G (containing *Bacillus thuringiensis israelensis*) and VectoLex G (containing *B. sphaericus*) at concentrations up to 5000 µg l^{-1} had no observable effect on coral larvae. Mean larval survival was 93 to 98% for both species after 9 d in control treatments (0 µg l^{-1} VectoBac G and VectoLex G) and this did not change following any of the *Bacillus* treatments (Table 2). Mean metamorphosis for *A. millepora* larvae was 87 ± 5% (mean ± SE) for the VectoBacG controls and 88 ± 4% for the VectoLex G controls, while metamorphosis success was slightly lower for *A. tenuis* VectoBac G controls (78 ± 6%) (Table 2). No significant effect on metamorphosis was observed at VectoBac G or VectoLex G concentrations up to 5000 µg l^{-1} (Fig. 1, Table 2).

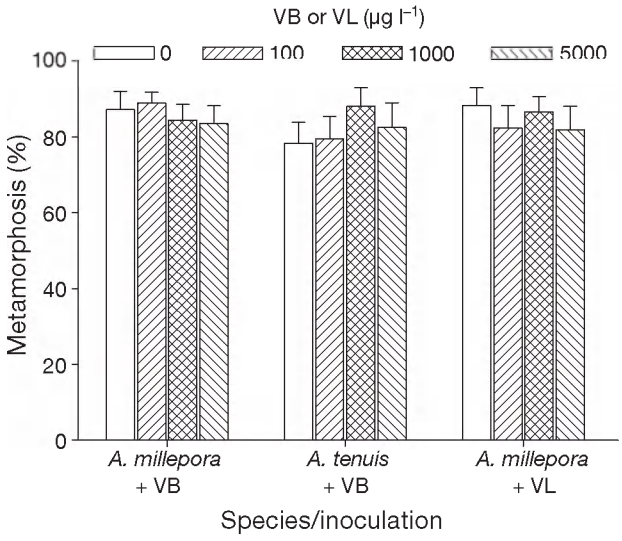

Fig. 1. *Acropora millepora* and *A. tenuis*. Metamorphosis of 9 d old juvenile corals following sequential exposure to 0, 100, 1000 or 5000 µg l^{-1} VectoBac G (VB) or VectoLex G (VL) during the pre-competent and competent phases. Bars represent mean percent metamorphosis (+SE), n = 6 replicate wells containing 10 to 15 larvae each

Table 2. Coral larval and juvenile and mosquito larval health statistics. Mean values (±SE) for each of the control treatments (0 µg l^{-1}) are provided along with ANOVA results ($p < 0.05$). Larvae and juveniles (n = 10 to 15) were assessed from each of 6 replicate wells for corals and 4 replicate jars for mosquitoes. Six juveniles were randomly assessed for polyp numbers (pol, mean ± SE) in each treatment. VB = VectoBac G (containing *Bacillus thuringiensis israelensis*), VL = VectoLex G (containing *B. sphaericus*). Assessment definitions: See Table 1

Species	Insecticide	Stage	Assessment	Control (%); Polyps (n)	*F*	df, SS	p
Corals							
Acropora millepora	VB	Pre-competent larvae (5 d old)	srv	98 ± 2	0.0847	3, 0.0047	0.97
		Post-competent larvae (9 d old)	srv	95 ± 3	0.130	3, 0.0159	0.94
		Post-competent larvae (9 d old)	mm	87 ± 5	0.172	3, 0.0206	0.92
		Juveniles (28 d old)	srv	87 ± 5	0.616	3, 0.277	0.62
			sym	76 ± 17	0.376	3, 0.791	0.91
			pol	3.2 ± 0.6	0.259	3, 0.00155	0.85
	VL	Pre-competent larvae (5 d old)	srv	98 ± 2	0.151	3, 0.0102	0.93
		Post-competent larvae (9 d old)	mm	88 ± 4	0.287	3, 0.0461	0.83
		Post-competent larvae (9 d old)	srv	93 ± 5	0.123	3, 0.0166	0.95
		Juveniles (28 d old)	srv	87 ± 10	0.198	3, 0.0681	0.90
			sym	85 ± 11	0.0984	3, 0.0851	0.96
			pol	2.8 ± 0.6	0.363	3, 0.00255	0.78
A. tenuis	VB	Pre-competent larvae (5 d old)	srv	98 ± 2	0.199	3, 0.0102	0.90
		Post-competent larvae (9 d old)	srv	95 ± 3	0.423	3, 0.0395	0.74
		Post-competent larvae (9 d old)	mm	78 ± 6	0.608	3, 0.0959	0.62
		Juveniles (28 d old)	srv	86 ± 7	0.292	3, 0.0777	0.83
			sym	92 ± 8	0.179	3, 0.146	0.91
			pol	2.0 ± 0.5	0.648	3, 0.00275	0.59
Mosquitoes							
Ochlerotatus vigilax	VB	3rd instar larvae (7 d old)	srv	100 ± 0	257	5, 12.0	<0.001
	VL	3rd instar larvae (7 d old)	srv	100 ± 0	25.3,	5, 5.28	<0.001

Mosquito larvae

All third instar *Ochlerotatus vigilax* larvae survived for 24 h in control treatments (Figs. 2 & 3A). VectoBac G was highly pathogenic to mosquito larvae, with only 5 ± 3% surviving after 24 h exposure to 30 µg l^{-1} (Fig. 2). VectoLex G was far less pathogenic to the mosquito larvae with a significant reduction in survival (to 66 ± 7%) observed following exposure to 300 µg l^{-1}. These results indicate that both of these *Bacillus* spp. insecticides were actively pathogenic to their target species.

Juvenile corals

Juvenile *Acropora millepora* and *A. tenuis* survived longer than 28 d (86 to 87%) in the absence of VectoBac G or VectoLex G (Table 2). Symbiont uptake by the juveniles was high in control treatments, ranging from 76 ± 17% of *A. millepora* colonies to 92 ± 8% of *A. tenuis* colonies in VectoBac G controls (Table 2). The juveniles of both species also developed between 2.0 and 3.2 polyps per colony after 28 d of development (Fig. 3B, Table 2). There was no effect of VectoBac G or VectoLex G on juvenile survival (Fig. 4), symbiont uptake (Fig. 5) or juvenile development as measured by polyp number at 28 d (Fig. 6).

Viability of *Bacillus* insecticides in seawater

The mean colony forming units (CFU) of *Bacillus thuringiensis israelensis* isolated from sterile seawater and sterile fresh water inoculated with 1000 µg l^{-1} VectoBac G were 70 000 ± 10 000 and 42 000 ± 5000 CFU ml^{-1},

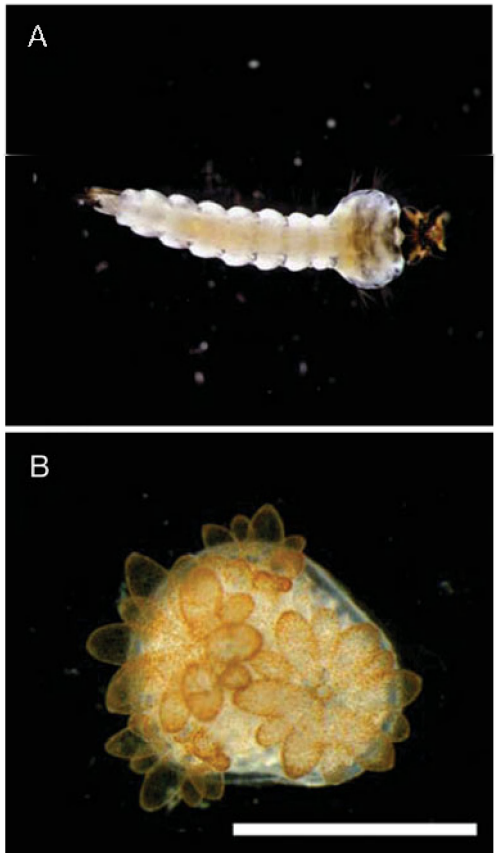

Fig. 3. Species used in assays. (A) Third instar mosquito *Ochlerotatus vigilax* larvae, a target species of the insecticide VectoBac G (*Bacillus thuringiensis israelensis*). (B) Non-target *Acropora millepora* juvenile coral after 28 d of development harbouring *Symbiodinium* sp. This colony had been exposed to 4 sequential 5000 µg l^{-1} exposures of VectoBac G. Scale bar = 500 µm

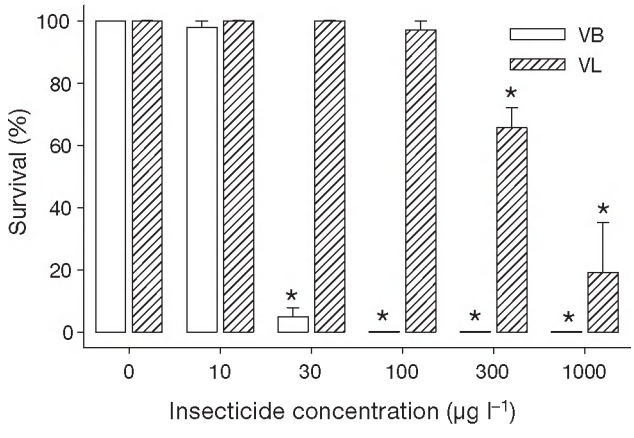

Fig. 2. *Ochlerotatus vigilax*. Survival of mosquitoes following 24 h exposures to VectoBac G (VB) or VectoLex G (VL). Bars represent mean percent survival (+SE), n = 4 replicate wells containing 10 larvae each. Asterisk (*) indicates a significantly reduced survival ($p < 0.05$, Table 2)

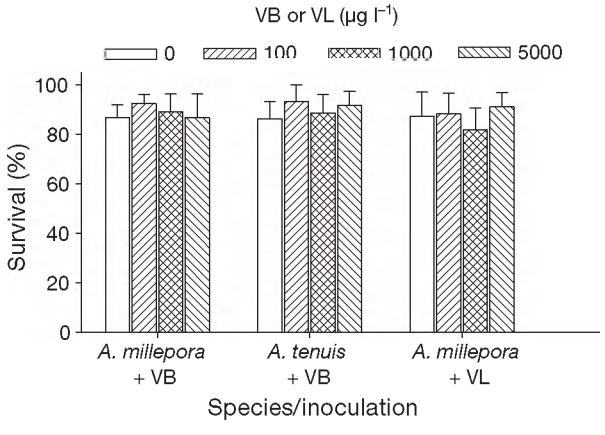

Fig. 4. *Acropora millepora* and *A. tenuis*. Survival of 28 d old juvenile corals following sequential exposure to 0, 100, 1000 or 5000 µg l^{-1} VectoBac G (VB) or VectoLex G (VL) throughout the larval and juvenile phases. Bars represent mean percent survival (+SE), n = 6 replicate wells containing 3 to 8 larvae each

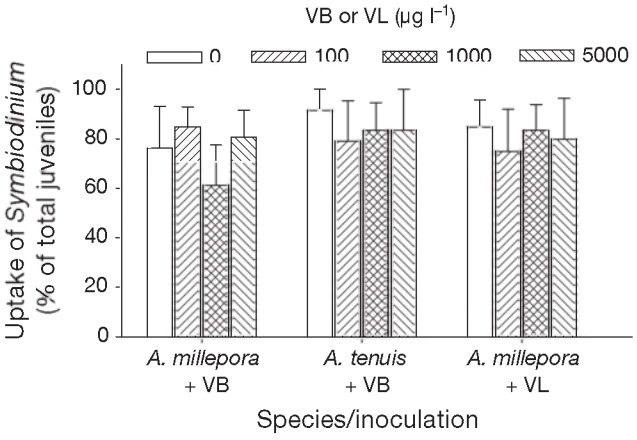

Fig. 5. *Acropora millepora* and *A. tenuis*. Uptake of *Symbiodinium* spp. (% of juveniles with visible symbionts) in 28 d old juvenile corals following sequential exposure to 0, 100, 1000, or 5000 µg l^{-1} VectoBac G (VB) or VectoLex G (VL) throughout the larval and juvenile phases. Bars represent mean percent uptake of *Symbiodinium* sp. (+SE), n = 6 replicate wells containing 3 to 8 juveniles each

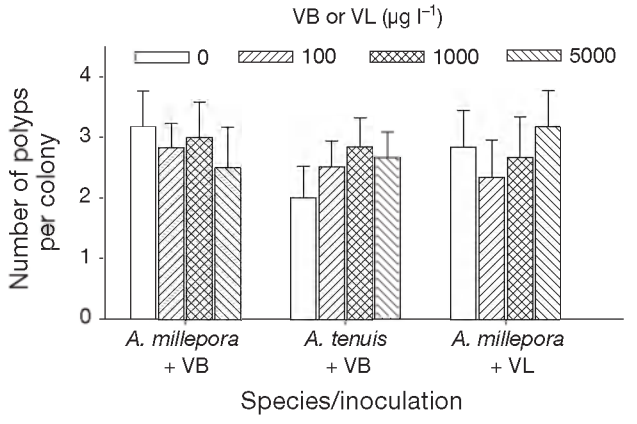

Fig. 6. *Acropora millepora* and *A. tenuis*. Number of polyps per juvenile in 28 d old coral colonies following sequential exposure to 0, 100, 1000 or 5000 µg l^{-1} VectoBac G (VB) or VectoLex G (VL) throughout the larval and juvenile phases. Bars represent mean no. polyps per colony (+SE), n = 6 replicate juveniles each

respectively, and this did not change significantly after 2 and 7 d (Fig. 7, Table 3). Similar colony numbers of *B. sphaericus* (44 000 ± 6000 CFU ml^{-1}) were successfully cultured from sterile seawater inoculated with 1000 µg l^{-1} VectoLex G.

Effects of *Bacillus thuringiensis israelensis* on adult corals and sponges

The commercial VectoBac G used in the adult exposure contained numerous bacteria as evidenced by

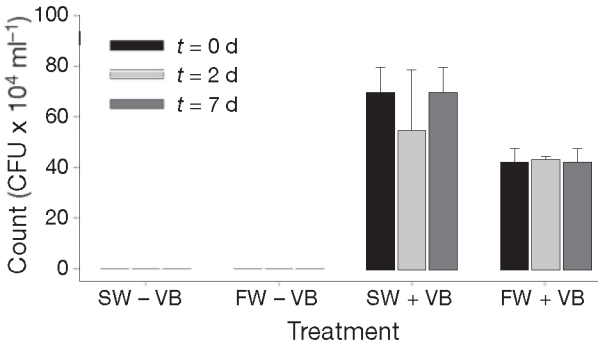

Fig. 7. *Bacillus thuringiensis*. Counts of *B. thuringiensis* in control sterile seawater without VectoBac G (SW – VB) and control sterile ultrapure (Milli-Q) water without VectoBac G (FW – VB), and sterile SW and FW after addition of 1000 µg l^{-1} VectoBac G (SW + VB and FW + VB, respectively). Counts are expressed as colony forming units (CFU) × 10^4 ml^{-1} (+SE), n = 3

the multiple bands present in the crude preparation. The 2 main DGGE bands detected in the VectoBac G preparation had 99% sequence homology to *Bacillus thuringiensis* strain SRDD (EF063149) (Band a, EU-784823) and 99% sequence homology to *B. cereus* strain NBRAJATH9 (EU661712) (Band b, EU784824) (Table 4). Both of these bands were identified by DGGE in seawater from the infection tanks immediately after addition of VectoBac G and after a further 6 h (Fig. 8). However, they could not be detected in seawater after 14 d and were not detected in any samples of *Acropora millepora* or *Ianthella basta* at any of the sampling times (Fig. 8). No adult *I. basta* or *A. millepora* exposed to a single 6 h pulse of 1000 µg l^{-1} VectoBac G exhibited observable onset of disease in the present study. The normal symbiotic bacterial community (DGGE bands c–h, Fig. 8) associated with *I. basta* was not disrupted by the addition of VectoBac G (Table 4).

DISCUSSION

The *Bacillus* insecticides VectoBac G (containing *B. thuringiensis israelensis*) and VectoLex G (containing *B. sphaericus*) were not acutely harmful to non-

Table 3. Statistical results for *Bacillus thuringiensis israelensis* viability from 2-way ANOVA for colony forming units (CFU) × 10^4 ml^{-1} in sterile seawater (SW) and sterile ultrapure (Milli-Q) water (FW) at times *t* = 0, 2 and 7 d after inoculation with 1000 µg l^{-1} VectoBac G

	SS	df	MS	*F*	p
Water type (SW or FW)	21.8	1	21.8	5.38	0.0389
Time (0, 2, 7 d)	1.96	2	0.981	0.242	0.788
Water type × Time	2.56	2	1.28	0.316	0.735
Residuals	48.6	12	4.06		

Table 4. Sequence results of bands from DGGE analysis of *Ianthella basta* following exposure to 1000 µg l^{-1} VectoBac G

Band ID	Nearest relative	% similarity	Accession no.
A (EU784823)	*Bacillus thuringiensis* strain SRDD	99	EF063149
B (EU784824)	*Bacillus cereus* strain NBRAJATH9	99	EU661712
C (EU784825)	*Gammaproetobacteria* from the sponge *Halichondria okadai*	98	AB054161
D (EU784826)	*Gammaproetobacteria* from the sponge *Microciona prolifera*	99	EF414032
E (EU784827)	*Gammaproetobacteria* from the sponge *Halichondria okadai*	98	AB054136
F (EU784828)	Unidentified bacterium from oceanic crust	95	EU491250
G (EU784829)	*Alphaproteobacteria* from the sponge *Rhopaloeides odorabile*	88	EU183877
H (EU784830)	*Alphaproteobacteria* from the sponge *Rhopaloeides odorabile*	87	EU183808

target coral larvae and juveniles at high concentrations (5000 µg l^{-1}). Conversely, both *Bacillus* insecticides were pathogenic to target mosquito larvae at concentrations at least 166-fold and 16-fold lower (for VectoBac G and VectoLex G, respectively). *B. thuringiensis israelensis* and *B. sphaericus* germination and survival was confirmed in seawater, yet corals and sponges exposed to high concentrations of VectoBac G did not exhibit any signs of disease after 14 d. It is likely that experimental doses (500 to 5000 µg l^{-1} VectoBac G or VectoLex G) were high compared with concentrations near coral reefs. Concentrations of *B. thuringiensis* have not been reported from seawater in the vicinity of coral reefs, but if VectoBac G was accidentally sprayed onto a reef in 1 m of seawater at the recommended application rate of 1.5 kg ha^{-1}, this would expose corals and sponges to 150 µg l^{-1} VectoBac G (if evenly distributed). These results indicate that the commercial *Bacillus* insecticides tested are not likely to be acutely harmful to the species tested.

There may be several reasons why VectoBac G and VectoLex G did not affect the corals and sponges in the present study. (1) The protein δ-endotoxins of *Bacillus thuringiensis* (VectoBac G) and *B. sphaericus* (VectoLex G) are highly specific to a limited number of insect types because solubilisation of the δ-endotoxins is required for toxicity, and this only occurs under highly alkaline conditions (e.g. within mosquito gut) (Höfte & Whiteley 1989). (2) The high selectivity of these toxins is also dependent on organisms possessing specific toxin binding sites (Gill et al. 1992) that may not be present in the corals or sponges. (3) The ingestion of viable *Bacillus* spp. from VectoBac G and VectoLex G may not have occurred under the expe-

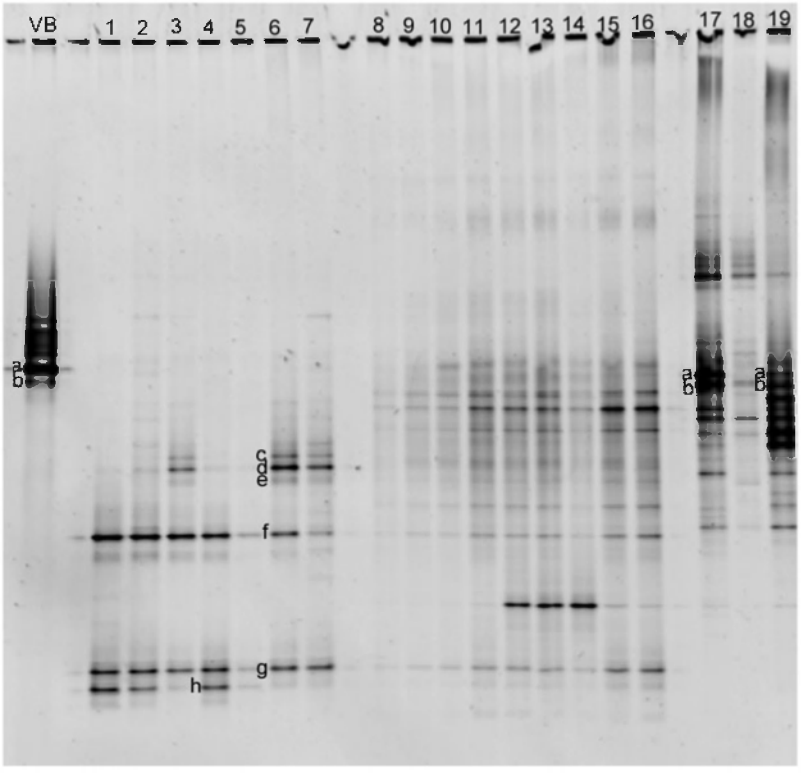

Fig. 8. Denaturing gel gradient electrophoresis (DGGE) of sponges and corals. DGGE profile of 16S rRNA-defined bacterial populations from replicate sponge and coral samples exposed to 1000 µg l^{-1} VectoBac G (VB). Lane 7 is sponge at time t = 0 d, Lanes 4 to 6 are sponges immediately after VB addition and Lanes 1 to 3 are sponges at t = 14 d after VB addition. Lanes 14 to 16 are corals at t = 0 d, Lanes 11 to 13 are corals immediately after VB addition and Lanes 8 to 9 are corals at t = 14 d after VB addition. Lane 17 is seawater immediately after VB addition, Lane 19 is seawater at t = 6 h after VB addition and Lane 18 is seawater at t = 14 d after VB addition. Bands a to h have been submitted to GenBank under the accession numbers EU784823 to EU784830

rimental conditions. (4) The *Bacillus* strains may have expressed lower δ-endotoxins in full salinity seawater (Nayar et al. 1999). The δ-endotoxins were not analysed during the exposures so the reasons for inactivity are yet to be determined.

No adult *Ianthella basta* or *Acropora millepora* exposed to a single 6 h pulse of 1000 µg l^{-1} VectoBac G exhibited an observable onset of disease. This result contrasts with the findings from a previous study where paling and necrosis of *I. basta* were observed following experimental infection using a bacterial consortium consisting of 2 *Bacillus* spp. and 3 *Pseudomonas* spp. isolated from diseased sponges (Cervino et al. 2006). There are several reasons why this may be the case. The *Bacillus* strains isolated in Cervino et al. (2006) may have been different from the commercial strains used in the present study. The spore-forming *Bacillus* species, including *B. thuringiensis*, *B. anthracis* (the causative agent of anthrax) and *B. cereus* (a causative agent of gastroenteritis), are closely related and difficult to separate on the basis of 16S rRNA gene sequences (Helgason et al. 2000). The DGGE sequence for *B. thuringiensis israelensis* (EU784823) and the sequence used in the *I. basta* disease study are not in completely overlapping regions of the 16S rRNA gene. However, where there was overlapping sequence for direct comparison (145 bp) there was 100% similarity between *B. thuringiensis israelensis* from VectoBac G and strain SDB21A from diseased *I. basta* sponges. The methods of infection also differed between the studies. The present study used a 6 h passive exposure to suspensions of VectoBac G. This method was used to successfully infect another species of sponge with a natural pathogen (Webster et al. 2002). In contrast, Cervino et al. (2006) attached infected media patches directly to the surface of the sponges for 72 h. Furthermore, Cervino et al. (2006) found that only a combination of 5 bacterial isolates (*Bacillus* spp. and *Pseudomonas* spp.) successfully infected the sponges and that the *Bacillus* spp. isolates alone were not infective.

It is likely that some coral reefs and other sensitive marine environments are periodically exposed to viable *Bacillus* strains via runoff from agriculture or overspray of mosquito control. Contamination by insecticides containing *B. thuringiensis israelensis* and *B. sphaericus* is also likely to be greatest on reefs already affected by other human impacts such as elevated nutrients and turbidity. Unlike contamination by other insecticides such as chlorpyrifos or endosulfan, *Bacillus* insecticides are living organisms that may not disappear from ecosystems over years or decades. *B. thuringiensis israelensis* from the VectoBac G preparation was able to survive well in both sterile seawater and sterile fresh water following 7 d of incubation (Fig. 7), confirming that accidental exposure of reef organisms to viable VectoBac G from mosquito control overspray or runoff from agriculture is possible (Cervino et al. 2006). If *Bacillus* insecticide strains remain viable in marine environments for significant periods of time, they may be triggered into activity and rapid growth under high nutrient conditions or within the gut of non-target species (Hendriksen & Hansen 2002). Although the overwhelming evidence suggests that *Bacillus* insecticides are environmentally benign, their effects on most marine invertebrates remain untested.

Acknowledgements. This research was conducted with the support of funding from the Australian Government's Marine and Tropical Sciences Research Facility and the Australian Institute of Marine Science. We thank Darren Alsemgeest from the Townsville City Council for providing the VectoBac® G and VectoLex® G samples and Kay Marshall from the Queensland Institute of Medical Research for providing advice on raising *Ochlerotatus vigilax* larvae. We also thank Eneour Puill-Stephan and Adrian Lutz for their help in the field, Rose Cobb for laboratory assistance and Craig Humphrey for editing advice.

LITERATURE CITED

Brown MD, Watson TM, Green S, Greenwood JG, Purdie D, Kay BH (2000) Toxicity of insecticides for control of freshwater *Culex annulirostris* (Diptera: Culicidae) to the nontarget shrimp, *Caradina indistincta* (Decapoda: Atyidae). J Econ Entomol 93:667–672

Cervino JM, Winiarski-Cervino K, Polson SW, Goreau TJ, Smith GW (2006) Identification of bacteria associated with a disease affecting the sponge *Ianthella basta*, in New Britain, Papua New Guinea. Mar Ecol Prog Ser 324: 139–150

Ferris MJ, Muyzer G, Ward DM (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62:340–346

Gill SS, Cowles EA, Pietrantonio PV (1992) The mode of action of *Bacillus thuringiensis* endotoxins. Annu Rev Entomol 37:615–634

Helgason E, Okstad OA, Caugant DA, Johansen HA and others (2000) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl Environ Microbiol 66:2627–2630

Hendriksen NB, Hansen BM (2002) Long-term survival and germination of *Bacillus thuringiensis* var. *kurstaki* in a field trial. Can J Microbiol 48:256–261

Höfte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 53:242–255

Hoong HW, Hoh-Christopher KY (1992) Major pests of oil palm in Sabah. The Planter (Malaysia) 68:193–210

Ivanova EP, Vysotskii MV, Svetashev VI, Nedashkovskaya OI and others (1999) Characterization of *Bacillus* strains of marine origin. Int Microbiol 2:267–271

Jacobsen BJ, Zidack NK, Larson BJ (2004) The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases. Phytopathology 94: 1272–1275

Lacey LA, Undeen AH (1986) Microbial control of black flies and mosquitoes. Annu Rev Entomol 31:265–296

Maeda M, Mizuki E, Hara M, Tanaka R, Akao T, Yamashita S, Ohba M (2001) Isolation of *Bacillus thuringiensis* from intertidal brackish sediments in mangroves. Microbiol Res 156:195–198

Markey KL, Baird AH, Humphrey C, Negri AP (2007) Insecticides and a fungicide affect multiple coral life stages. Mar Ecol Prog Ser 330:127–137

Nayar JK, Knight JW, Ali A, Carlson DB, O'Bryan PD (1999) Laboratory evaluation of biotic and abiotic factors that may influence larvicidal activity of *Bacillus thuringiensis serovar. israelensis* against two Florida mosquito species. J Am Mosq Control Assoc 15:32–42

Negri AP, Heyward AJ (2000) Inhibition of fertilization and larval metamorphosis of the coral *Acropora millepora* (Ehrenberg, 1834) by petroleum products. Mar Pollut Bull 41:420–427

Owen R, Knap A, Ostrander N, Carbery K (2003) Comparative acute toxicity of herbicides to photosynthesis of coral zooxanthellae. Bull Environ Contam Toxicol 70:541–548

Russell TL, Brown MD, Purdie DM, Ryan PA, Kay BH (2003) Efficacy of VectoBac (*Bacillus thuringiensis* variety *israelensis*) formulations for mosquito control in Australia. J Econ Entomol 96:1786–1791

Walker K, Mendelsohn M, Matten S, Alphin M, Ave D (2003) The role of microbial BT products in US crop protection. In: Medz M (ed) *Bacillus thuringiensis*: a cornerstone of modern agriculture. Food Products Press, Binghampton, p 31–52

Webster NS, Hill RT (2001) The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium. Mar Biol 138: 843–851

Webster NS, Negri AP, Webb RI, Hill RT (2002) A spongin-boring alpha proteobacterium is the etiological agent of disease in the Great Barrier Reef sponge, *Rhopaloeides odorabile*. Mar Ecol Prog Ser 232:305–309

Editorial responsibility: Inna Sokolova, Charlotte, North Carolina, USA

Submitted: June 11, 2008; Accepted: January 16, 2009
Proofs received from author(s): March 26, 2009