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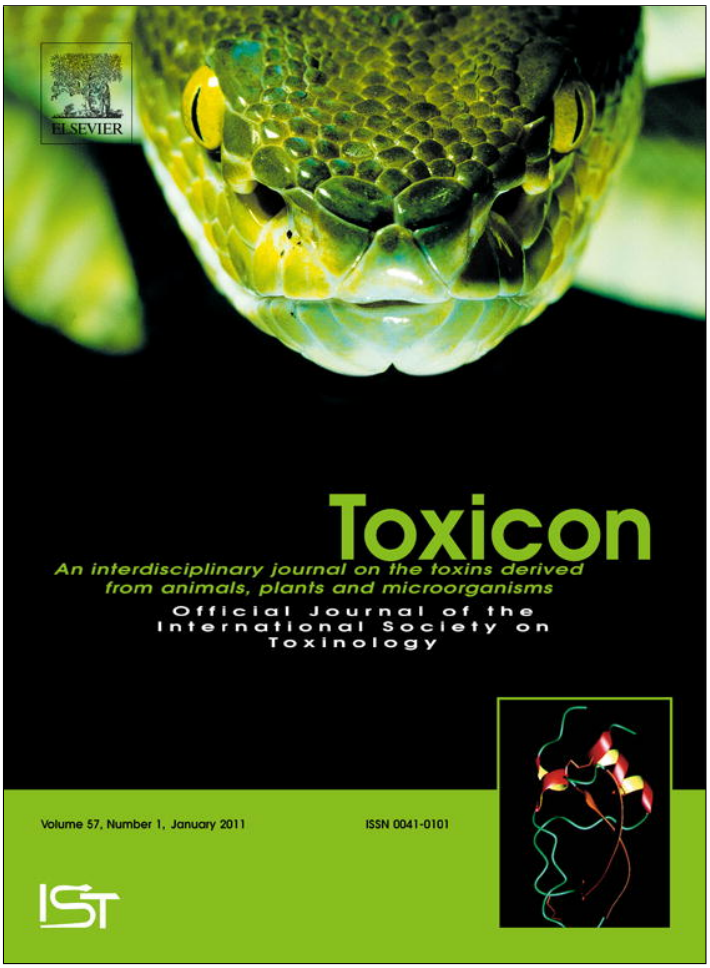

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Short communication

Evidence of *Methylobacterium* spp. and *Hyphomicrobium* sp. in azaspiracid toxin contaminated mussel tissues and assessment of the effect of azaspiracid on their growth

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ABSTRACT

A flagellar protein belonging to the genus *Methylobacterium* or *Agrobacterium* was previously observed by proteomics in azaspiracids (AZA) toxic mussels. Here, we report the isolation of two different *Methylobacterium* spp. (NTx1 and Tx1) from non-toxic and AZA toxic mussels, respectively, which when co-cultured with AZA exhibited significantly different growth responses - isolate Tx1 growth rate was enhanced, whereas growth of isolate NTx1 was adversely affected, compared to non-AZA supplemented control cultures. A *Hyphomicrobium* sp. (Tx2) also isolated from the toxic mussels achieved greater cell density in AZAs supplemented cultures.

The consumption of shellfish contaminated with algae-derived toxins is now of major global concern. Different classes of phycotoxins, including the fairly recently discovered azaspiracid toxins, have been reported and are known to cause human food poisoning. In the past decade, there has been an increasing distribution of azaspiracid toxin (AZA) outbreaks across European countries, but more recently in Morocco (Taleb et al., 2006), Japan (Ueoka et al., 2009) and Chile (Lopez-Rivera et al., 2010). The symptoms associated with AZA poisoning in humans (nausea, vomiting, severe diarrhoea, and stomach cramps) are very similar to those found following consumption of diarrhoeic shellfish poisons (DSPs), however AZAs were also found to induce neurotoxic symptoms (respiratory difficulties, spasm, paralysis of limbs and death) in mice (Ito et al., 2002). Amongst azaspiracid analogues, the most abundant in shellfish are AZA1, AZA2 and AZA3 (Figure S1). Despite considerable interest and importance, relatively little is yet known about the reasons for their sudden occurrence and the mechanisms underlying azaspiracid outbreaks.

Tillmann et al. (2009) recently identified an azaspiracids producer as a small (12–16 μm length and 7–11 μm width) photosynthetic dinoflagellate designated *Azadinium spinosum*. It was shown that this dinoflagellate produces the toxin in axenic conditions, seemingly excluding the possibility that bacteria influence the production of AZAs (Krock et al., 2009). However, employing a proteomic approach, we recently discovered an unexpected bacterial flagellar protein, with homology to *Methylobacterium* or *Agrobacterium* flagellar proteins, in highly AZA contaminated mussel protein profile (Nzoughet et al., 2009). This finding

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Table 1
Summary of PCR and culture results for non-toxic and toxic mussels.

Sample details		PCR result		Culture result	
		Methylobacterium genus-specific	Agrobacterium tumefaciens-specific	Pink/red isolates[a] on MMSM plates (count, CFU/g)	Sub-culture outcome[b] (MMSM broth)
Non-toxic mussels:					
HP	NTx1	+	–	–	–
	NTx2	+	–	–	–
	NTx3	+	–	–	–
RT	NTx1	+	–	$+(5.5 \times 10^2)$	+
	NTx2	+	–	$+(2.3 \times 10^2)$	–
	NTx3	+	–	$+(1.0 \times 10^2)$	–
Toxic mussels:					
HP	Tx1	+	–	$+(8.6 \times 10^2)$	+
	Tx2	+	–	$+(9.1 \times 10^2)$	+
	Tx3	+	–	$+(4.0 \times 10^2)$	–
RT	Tx1	+	–	$+(3.0 \times 10^2)$	–
	Tx2	+	–	$+(1.0 \times 10^2)$	–
	Tx3	+	–	$+(5.0 \times 10^2)$	–

[a] Suspected *Methylobacterium* spp.
[b] +, isolates showing good growth ($OD_{600\ nm}$ increase) on sub-culture; –, no appreciable growth on sub-culture from MMSM agar after 12 days.

raised questions about the implication of bacteria in azaspiracid outbreak since previous studies have highlighted the involvement of bacteria in the production of paralytic and amnesic shellfish toxins by dinoflagellate algae (Kodama et al., 1988). In light of the above facts, an investigation was carried out to determine which of the bacterial genera (*Methylobacterium* or *Agrobacterium*) was present in azaspiracid toxin contaminated mussels and then to examine the influence of AZAs on growth of the isolated bacteria.

Azaspiracid toxic mussels (*Mytilus edulis*) used for this study were harvested off the north coast of Ireland (McSwynes Bay) in 2005 during a toxin outbreak, and had been stored at −20 °C since that time. Mass spectrometric analysis revealed that these samples contained high levels of AZA1 (33230 μg/kg) and AZA2 (3630 μg/kg), well above the EU regulatory limit of 160 μg/kg. Non-toxic mussels (*M. edulis*) were collected from Rostreavor, Northern Ireland (sea water site, at the top end of Carlingford Lough) in 2009 and tested 'toxin negative' by the mouse bioassay. Toxic and non-toxic mussels were subjected to the same analytical procedures. A total of six individual mussels, three toxic mussels (referred to as Tx1, Tx2, Tx3) and three non-toxic mussels (referred to as NTx1, NTx2, NTx3), were aseptically removed from their shell. In each case the hepatopancreas (HP) was dissected from the remaining tissue (RT) and each part was equally divided into two portions. The first set of portions was used to extract DNA from each HP and RT sample. A tenfold dilution of each sample was prepared in TEN lysis buffer (2 mM EDTA, 400 mM NaCl, 10 mM tris–HCl, pH 8.0, 0.6% SDS). After cell lysis, by beadbeating (Lysing matrix B tubes, QBiogene, Cambridge) in FastPrep FP120 machine, DNA was extracted from all samples with phenol:chloroform:isoamylalcohol (25:24:1) (Sigma) (Dowhan, 2008). DNA purification was achieved using a Microclean kit (Microzone Ltd, Sussex, UK) and DNA samples were stored at −20 °C until required.

PCR was carried out on the HP and RT samples using *Methylobacterium* genus-specific primers (MB4 5′-CCG CGT GAG TGA TGA AGG-3′, MB 5′-AGC GCC GTC GGG TA AGA-3′, Podolich et al., 2009), and *Agrobacterium tumefaciens*-specific primers (Agro1r 5′-GTCTCCAATGCCCATACCC-3′ and α688f 5′-TATCTACGAATTTCACCT-3′, Süß et al., 2006) to test for the presence of *Methylobacterium* spp. or *Agrobacterium tumefaciens* DNA. PCR assays were carried out in 50 μl volumes containing 1X Platinum *Taq* polymerase buffer (Invitrogen), 10 pmol of each primer, 2.5 nmol dNTP mix, 1.5 mM $MgCl_2$, 2.5 U Platinum® *Taq* polymerase (Invitrogen), and ~50 ng template DNA. Cycling conditions were as described elsewhere (Podolich et al., 2009; Süß et al., 2006). The HP and RT of both toxic and non-toxic mussels exhibited the expected band at 1078 bp following *Methylobacterium* genus-specific PCR, whilst no PCR evidence of *A. tumefaciens* was obtained (Table 1).

The second portion of each toxic and non-toxic mussel sample was cultured on selective Methanol Mineral Salts Medium (MMSM) in an attempt to isolate *Methylobacterium* spp. (Green, 2007). Samples were serially diluted (10^{-1}, 10^{-2} and 10^{-3}) with Maximum Recovery Diluent (Oxoid, Basingstoke, UK) and 100 μl aliquot of each dilution was spread on MMSM plates followed by incubation at 30 °C. After 5 days incubation, suspected *Methylobacterium* spp. were observed as pink/red colonies on some plates (Table 1). The non-isolation of suspect *Methylobacterium* colonies from any of the non-toxic HP samples (Table 1) was an unexpected result as the *Methylobacterium* genus-specific PCR was positive for these samples. It might be that the *Methylobacterium* sp. present in non-toxic HP was in an unculturable state (Kelley et al., 2004), which provides a mechanism for the bacteria to survive harsh environments (Morris et al., 1997; Podolich et al., 2009). Phenotypic identification of suspected *Methylobacterium* spp. was then performed using standard tests. All isolates were Gram negative rods, oxidase-positive, catalase-positive, and motile. Growth was strictly aerobic. These characteristics are the same as those described in the literature for *Methylobacterium* spp. (Green and Bousfield, 1983), although we acknowledge that other bacterial genera may

exhibit the same results for the limited range of phenotypic tests applied. The motile nature of the bacterial isolates is in agreement with the detection of a *Methylobacterium* flagellar protein in the toxic mussels profile by proteomics, as reported by Nzoughet et al. (2009). Pink-pigmented bacterial colonies were subsequently sub-cultured into MMSM broth and the $OD_{600\ nm}$ of the cultures was monitored for a period of 12 days until growth was indicated by an increase in $OD_{600\ nm}$. Bacterial DNA was extracted from the three MMSM broth cultures which exhibited growth (namely Tx1, Tx2 and NTx1, Table 1) and *Methylobacterium* genus-specific PCR was performed on each. The expected PCR product (1078 bp) was obtained for all three isolates providing a preliminary identification of *Methylobacterium* sp. for all three isolates.

The isolates Tx1, Tx2 and NTx1 were then further identified by 16S rDNA sequencing as described elsewhere (Whiteley and Bailey, 2000). Sequences generated were assembled using ChromasPro 1.34 (www.technelysium.com.au), and subsequently imported into BioEdit (Hall, 1999) and aligned using CLUSTALW (Thompson et al., 1994). BLAST analysis indicated that bacterial isolates NTx1 (GU597367) and Tx1 (GU597368) shared 100% homology with GenBank sequences (Table S1) of mainly non-speciated *Methylobacterium* spp. *Methylobacterium populi*, as suggested by the original proteomics results (Nzoughet et al., 2009), did not feature amongst the BLAST matches. Isolate Tx2 (GU597369) was not identified as a *Methylobacterium* sp. but shared 99% sequence similarity to GenBank sequences for *Hyphomicrobium* sp. SAN-1 and *Hyphomicrobium facile* (Table S1). A Bayesian phylogenetic tree was generated using the 16s rDNA fragment sequencing results (Figure S2), which confirmed that Tx1 and NTx1 isolates were different *Methylobacterium* sp.

The impact of AZA on growth of the three mussel isolates (NTx1, Tx1 and Tx2) in MMSM broth was then assessed. Test tubes of MMSM broth (4 ml) were prepared in triplicate, supplemented with 300 ng/ml AZA1 and 33 ng/ml AZA2 (maximum AZA levels achievable due to limited stock of AZA), and inoculated with 100 μl stationary phase broth cultures of Tx1, Tx2 or NTx1. Control (No AZAs) cultures contained no added toxin just 100 μl broth cultures of Tx1, Tx2 or NTx1. Cultures were incubated at 30 °C and $OD_{600\ nm}$ was monitored over a period of 20 days. Growth curves were generated from the experimental data and fitted using the Gompertz equation (GraphPad Prism Version 3). Statistical analysis was performed using SPSS Statistics 17.0. Differences between control (No AZAs) and AZA supplemented cultures (+AZAs) were analysed by Student's (two tailed) *t* test, comparing the difference of the means to time zero OD value and also by comparison of daily OD values. Two different growth responses were observed in the presence of AZAs (Fig. 1). AZAs appeared to have some inhibitory effect on the growth of the *Methylobacterium* sp. NTx1 isolated from non-toxic mussels RT ($p < 0.001$), whilst they seemed to promote the growth of *Methylobacterium* sp. Tx1 ($p < 0.001$) and *Hyphomicrobium* sp. Tx2 ($p < 0.05$) isolated from toxic mussel HP. Both Tx1 and Tx2 achieved a significantly higher final OD in the AZA supplemented cultures compared to the control (No AZA) cultures.

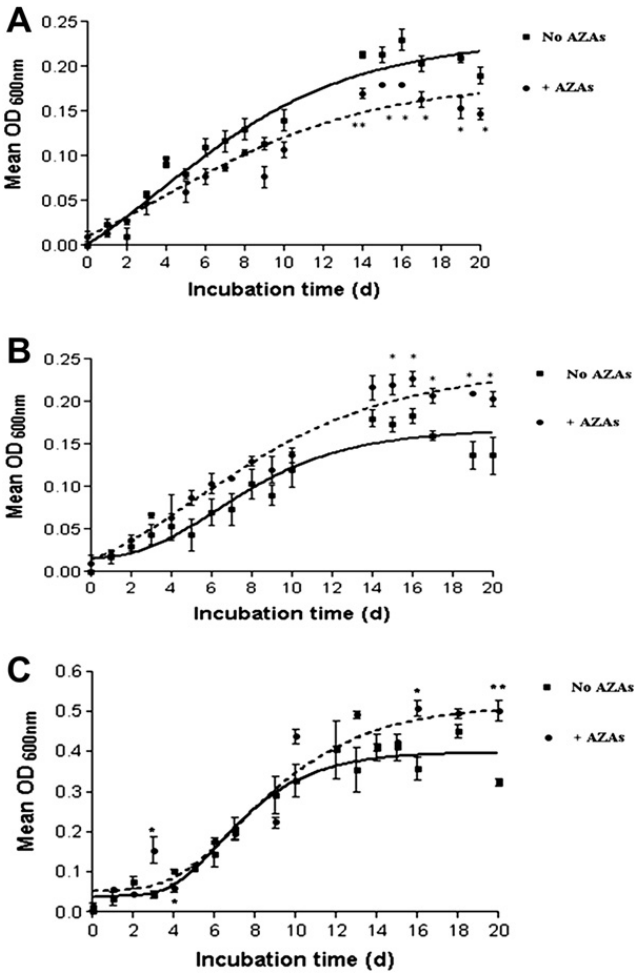

Fig. 1. Effect of presence of AZA1 and AZA2 (+AZAs) on the growth of (A) *Methylobacterium* sp. NTx1, (B) *Methylobacterium* sp. Tx1 and (C) *Hyphomicrobium* sp. Tx2 in MMSM broth. In the case of the two toxic mussel HP isolates (Tx1 and Tx2) the presence of AZA allowed cell density to reach a significantly higher level compared to control (No AZA) culture. The converse was the case for the non-toxic mussel RT isolate (NTx1). Error bars represent SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

Liquid chromatography coupled with tandem mass spectrometry was used to assess AZA concentrations at the end of the growth experiments. The HPLC and MS conditions (McNabb et al., 2005) employed here were a modified version of a method previously reported (Nzoughet et al., 2008). Up to ten AZA analogues were monitored in the related samples. Monitored transitions were: AZA1, 842.6 > 672.3; AZA2, 856.4 > 672.3; AZA2-OCH_3, 870.6 > 672.3; AZA3, 828.6 > 658.6; AZA4, 844.5 > 658.5; AZA5, 844.5 > 674.5; AZA6, 842.5 > 658.5; AZA17, 872.5 > 702.5; AZA19, 886.5 > 702.5; AZA21, 888.5 > 702.5. The results confirmed that the control cultures did not contain AZAs, and that at the end of the growth experiments AZA supplemented cultures contained approximately 1% of total AZA1 and AZA2 added at the beginning of the investigation; no other AZA analogue was detected.

In conclusion, this study revealed the presence of two different *Methylobacterium* spp. in non-toxic and toxic AZA mussels which exhibited different growth patterns when subsequently co-cultured with AZAs. We believe this to be

the first report of specific bacterial genera in association with AZA contamination of mussels. These findings are further evidence that relationships between algal-derived toxins and bacteria exist; in this case the newly discovered Azaspiracids and *Methylobacterium* spp. Tx1 and NTx1, or *Hyphomicrobium* sp. Tx2. Since the association of *Methylobacterium* spp. with plants seems to rely on a symbiotic relationship between the bacterium and the plant host, it is suggested that a similar scenario may exist between AZA algal producers and *Methylobacterium* spp. *or Hyphomicrobium* sp. Further research involving the bacterial isolates reported here and *A. spinosum* is underway to better understand the interactions between bacteria, dinoflagellate and AZA toxin production.

Acknowledgements

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Ethical Statement

None.

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.toxicon.2011.09.012.

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