ROLE OF DINOFLAGELLATE-ASSOCIATED BACTERIA IN TOXIN PRODUCTION : APPLICATION TO *DINOPHYSIS SPP* AND *PROROCENTRUM LIMA*

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Résumé : *des travaux récents ont été réalisés sur le rôle des bactéries associées aux dinoflagellés. Ils tendent à montrer que les bactéries seraient impliquées dans la production de toxine, voire qu'elles en seraient les productrices. Aucun travail n'ayant à ce jour été réalisé sur les bactéries associées aux dinoflagellés responsables d'intoxication DSP, nous avons extrapolé ces données à ce type de dinoflagellé afin d'évaluer le rôle des bactéries associées à deux dinoflagellés producteurs de DSP (*Prorocentrum lima *et* Dinophysis spp.*) dans la production de toxines. Une étude ultrastructurale de ces deux dinoflagellés au MEB a permis de visualiser les très nombreuses bactéries de forme hélicoïdale fixées aux cellules de* P.lima*, ainsi que le faible nombre de bactéries, de forme classique, fixées sur les cellules de* Dinophysis*. Une recherche d'acide okadaïque par dosage HPLC dans les bactéries libres (non fixées aux cellules phytoplanctoniques) a été entreprise dans les cultures de* P.lima*. Les premiers résultats montrent que les bactéries libres associées à* P.lima *contiendraient une faible quantité d'acide okadaïque; et que la stimulation de la croissance de ces bactéries n'augmente pas la teneur en toxine dans les bactéries libres.*

Abstract : *recent studies tend to show that bacteria associated with dinoflagellates are involved in toxin production or are themselves toxin producers. As bacterial association with DSP-producing dinoflagellates has not been investigated, we studied this relationship and the possible role of bacteria in toxin production in two dinoflagellates,* Prorocentrum lima *and* Dinophysis spp.*. An ultrastructural study in scanning electron microscopy showed a large number of helicoid bacteria bound to* P. lima *cells and a small number of classical bacteria bound to* Dinophysis spp. *cells. Screening for okadaic acid by high pressure liquid chromatography assay in free-living bacteria (not bound to phytoplankton cells) was performed in* P. lima *cultures. Initial results indicate that free-living bacteria associated with* P. lima *contain a low quantity of okadaic acid and that bacterial growth stimulation does not increase toxin concentration.*

Introduction

There has been increasing interest in recent years in the role of bacteria in toxin production by microalgae (essentially toxic dinoflagellates). Certain abnormalities and observations suggest that bacteria associated with dinoflagellates may either be implicated in toxin production or actually produce toxin themselves (Rausch de Traubenberg and Lassus, 1991). Although very few studies have been performed, more and more specialists are intrigued by this possibility. No data exist to date on the bacteria associated with dinoflagellates producing okadaic acid and its derivatives. The organisms concerned, *Prorocentrum lima* and various species of the genus *Dinophysis*, have been implicated in diarrheic shellfish poisoning (DSP) after human consumption of mollusks in Europe, the U.S.A., India and Japan. This paper provides a brief review of the most recent studies on bacteria associated with toxic dinoflagellates and presents our initial results relative to *P. lima* and *Dinophysis*.

Recent studies of dinoflagellate-associated bacteria

The dinoflagellate *Gymnodinium breve* is known to produce quite extensive toxic

blooms off the Florida coasts which have caused massive mortality in fish. Buck and Pierce (1989) succeeded in isolating a toxic bacterium from dinoflagellate cultures as well as from seawater in the presence and absence of blooms. Unfortunately, no chemical assay of the toxin from this bacterium has confirmed these results.

Two dinoflagellates, *Ostreopsis lenticularis* and *Gambierdiscus toxicus*, have been implicated in ciguatera, a type of poisoning well known in tropical regions. Tosteson et al. (1989) showed a correlation between *O. lenticularis* toxicity and the percent total bacteria directly associated with these cells. Moreover, the composition of bacterial microflora was found to be different in clonal cultures of *O. lenticularis* and *G. toxicus* when there were great toxicity variations.

The most important studies have been performed on *Alexandrium tamarense* and other dinoflagellates which cause paralytic shellfish poisoning (PSP) in various parts of the world. Kodama *et al.* (1988) extracted one of the PSP toxins (saxitoxin) from a bacterium of the *Moraxella genus* considered to be intracellular with *A. tamarense*. Subsequently, PSP toxins were detected in 0.45 to 5 µm particles (the size of an *A. tamarense* cell is between 30 and 35 µm) during a toxic episode in the absence of any dinoflagellate (Kodama, 1989). These particles contained bacteria of the *Moraxella genus* (Kodama, 1989). Ogata *et al.* (1990) isolated 10 bacterial strains producing these same toxins from 4 species of dinoflagellates known to produce them. Finally, Kodama *et al.* (1990) showed that PSP toxin production by *Moraxella* is more elevated in a poor than a rich environment.

Thus, toxin production by these associated bacteria has been confirmed only for species implicated in PSP. Existing data on bacteria associated with species implicated in other types of poisoning are still inadequate to prove this hypothesis for other toxic dinoflagellates.

Initial results for bacteria associated with *Prorocentrum lima* and *Dinophysis* spp.

Ultrastructural study : scanning electron microscopy (SEM) visualized a few bacteria bound to Dinophysis cells which proved to be bacilla of classical shape. However, *P. lima* was found to have a large number of bacteria, most of which had an unusual morphology, i.e., large (1 to 2 µm) helicoid bacilla (Plate 1). These bacteria were bound directly to the theca (Plate 1b) or agglomerated in the mucus (Plate 1a).

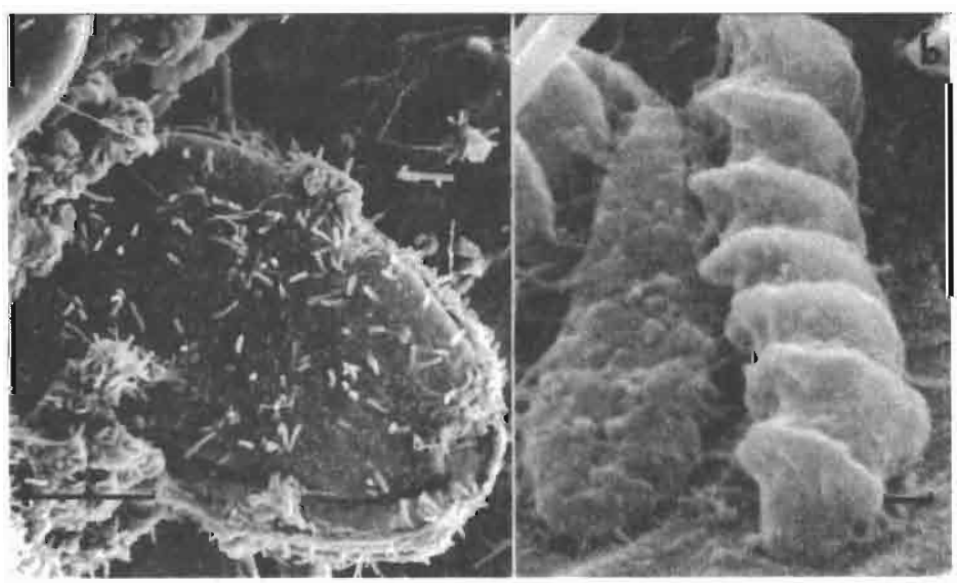

Plate 1 : Bacteria bound to *P. lima* (external SEM view).
(a) Bacteria bound to the theca and agglomerated in the mucus (bar = 10 µm).
(b) Detail of (a) showing bacteria on the theca (bar = 0,1 µm).

Detection of okadaic acid in free-living bacteria associated with *P. lima*

In addition to bound bacteria, endosymbiotic as well as free-living bacteria may be present. The latter are not bound to the cell wall but always present in the environment around the microalgae. In fact, all algae release metabolites of photosynthesis beneficial to bacteria which proliferate in their vicinity.

Two successive filtrations performed from a *P. lima* culture enabled us to obtain two fractions differing in particle size. One contained particles larger than 5 µm, i.e., *P. lima* cells, and bound bacteria ; and the other particles between 0.2 and 5 µm, i.e., free-living bacteria. The okadaic acid content in each of these fractions was determined by high pressure liquid chromatography (HPLC) after extraction, derivation and clean-up according to the method of (Lee *et al.*, 1987) as adapted by Masselin *et al.*, 1991.

Toxicity of 20 "free-living bacteria" fractions from P. lima *cultures*

A HPLC assay was performed from 20 free-living bacteria fractions taken from the *P.lima* culture at different growth stages (Table 1). The results show that 7 fractions (30 % of the extracts) contained a low quantity of detectable okadaic acid. Concentrations in the "alga + bound bacteria" fractions were usually 100 to 1000 times as high as in the free-living bacteria fractions for the same volume of initial culture. There was no apparent correlation between fraction toxicity and the number of bacteria nor between toxicity and culture age.

Serie a			Serie b	
Culture age (days)	**Toxicity ng/100 ml**	**Bacteria/ml**	**Toxicity ng/100 ml**	**Bacteria/ml**
0	17,6	5.10^3	ND	5.10^3
9	ND	2.10^5	9,56	$2,4.10^6$
15	ND	$4,95.10^6$	22,8	—
23	ND	—	8,5	$4,6.10^6$
30	ND	$1,9.10^7$	7,08	$2,55.10^6$
37	ND	$1,14.10^6$	ND	$4,5.10^6$
43	ND	$1,20.10^6$	ND	$3,6.10^6$
51	ND	$5,5.10^6$	ND	$5,45.10^6$
58	31.9	$1,75.10^6$	ND	—
64	25.2	$2,5.10^5$	ND	$1,25.10^6$

Table 1 : toxicity of 20, 0,2 - 5 µm fractions (ng/100 ml of culture) from *P. lima* cultures (ND = not detectable)

Toxicity of the two fractions after bacterial growth stimulation in P. lima *culture*

Bacterial growth was stimulated in *P. lima* culture by addition of marine broth during 12 h in darkness. The number of bacteria increased tenfold. A HPLC assay was performed on both fractions according to the same method. The results (Table 2) show that there were no significant differences between the control and broth-enriched cultures. Bacterial stimulation with marine broth failed to increase okadaic acid concentration in the free-living bacteria.

	Controle cultures	**Marine broth enriched cultures**
> 5 µm fractions	$3,28.10^3 \pm 0,71.10^3$	$3,27.10^3 \pm 0,5.10^3$
0,2 µm- 5 µm fractions	35,13 ± 3,67	39,33 ± 3,21

Table 2 : Toxicity of > 5 µm and 0,2 µm - 5 µm fractions after bacteria growth stimulation (ng/100 ml of culture, n = 3)

Conclusion

In view of the low quantity of toxin detected in free-living bacteria, these initial results must be interpreted with due caution. Our DSP results for *P. lima* can be compared to those obtained by Kodama and co-workers for *A. tamarense* and other PSP toxin-producing organisms with respect to the possibility of detecting toxin particles smaller than the dinoflagellate and the failure of marine broth-enriched cultures to increase the quantity of toxin detected in bacteria. This last observation may be attributed either to the fact that toxin production is not related to bacterial production or that the medium employed had no effect on toxin production.

In the present state of our knowledge, it cannot be concluded that bacteria cultured with *P. lima* produce okadaic acid since there is a possibility that toxin is transferred from the dinoflagellate to the bacteria. These initial results suggest other approaches, e.g., screening for toxin-producing bacteria after isolation and monospecific culture, which could confirm whether okadaic acid is produced by bacteria associated with *P. lima*.

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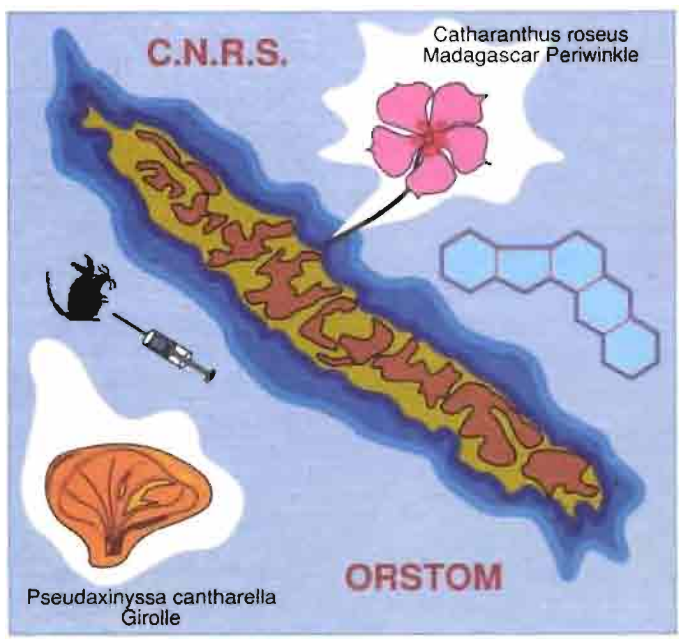

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