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Effect of Domoic Acid on Growth and Heavy Metal Tolerance of Some Marine Bacteria Isolated from Samandag Shore and Iskenderun Bay

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In the current study, effects of domoic acid on some marine bacteria were studied. Domoic acid is a neurotoxin which affects human and animals and by some marine microalgea. For this study, heterotrophic marine bacteria were isolated from Iskenderun Bay and Samandag shore. 19 *Pseudomonas* sp. strains, 15 *Acinetobacter* sp. strains, 5 *Alteromonas* sp. strains and 9 *Vibrio* sp. strains were identified. For inhibition tests, varing concentrations of domoic acid (0.5μgr.ml^{-1} and 1 mgr.ml^{-1}) were tested. It was found that there is not any inhibition effect of domoic acid on the all bacteria with used concentrations. Stimulator effect was determined by getting growth curve from the bacteria cultures which was added varying concentrations of domoic acid. It was found that 2.5 μgr.ml^{-1} and 5 μgr.ml^{-1} domoic acid showed inducer effect on 1.6 *Pseudomonas* sp. strain and 4.4 *Vibrio* sp. strain growth. In this study, it was also investigated the effect of domoic acid on heavy metal tolerance of these bacteria. The results of these study will contribute novel information on the effect of domoic acid on marine bacteria.

Key words: Domoic acid; heavy metal; marine bacteria

Domoic acid is a neurotoxin which affects human and animals and produced by some marine microalgea. Domoic acid (DA) is the principle compound associated with the toxic syndrome Amnesic Shellfish Poisoning (ASP) and is produced by some species of the diatom genus *Pseudo-nitzschia*, including *P. multiseries*[1].

Domoic acid (DA), was originally isolated from the red algae *Chondria armata*, known in Japan as domoi, it being the antihelminthic agent in a long used traditional medicine[2].

It was later identified as the cause of an unusual shellfish poisoning syndrome, amnesic shellfish poisoning, that first occurred on Prince Edward Island in Canada[3]. The source of the toxin was the diatom *Pseudo-nitzschia* (previously *Nitzschia*) *pungens* forma *multiseries*[4]. Domoic acid (MW=311; $C_{15}H_{21}NO_6$) is a tricarboxylic acid similar to the glutamate receptor agonist kainic acid, being cyclised analogues of L-glutamate. It is only mildly toxic to mammals, with an LD_{50} to mice (intra-peritoneally) of 3.6 mg kg^{-1}. It is polar making it soluble in water and insoluble in organic solvents[5-6]. Structure of domoic acid resembles iron-complexing agents produced by terrestrial plants, such as mugineic acid. This similarity in chemical structure of domoic acid to other phytosiderophores suggest o role for domoic acid as a trace metal chelator[7].

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The interaction of bacteria with unicellular algae has long been recognized as playing an important role in the biology and ecology. The nature of this interaction encompasses a diverse array of both "loose" and "tight" associations which may be characterized as stimulatory or inhibitory to one or both participants[8]. Interactions between phytoplankton and bacteria have been studied form many years. These include competition for avaliable organic matter, provision of extracellular materials by the phytoplankton which are of benefit to the bacteria and vice versa[9]. Bates *et al*.,[10] found that *P. multiseries* axenic cultures produce substantially less DA than nonaxenic cultures.

Many species of marine microalgae produce substances that are highly toxic; because the algae are widespread and periodically from large concentarations in the form of population blooms, the amount of toxins produced are considerable[10]. These toxins constitute a significant environmental hazard as they can accumulate in molluscan shellfish, which rely on phytoplankton as food[11]. Many metal ions are essential as trace elements but at higher concentrations they became toxic. Bacteria and higher organisms have developed resistance mechanisms to toxic metals to make them innocuous. Organisms respond to heavy metal stress using different defence systems, such as making complexes and the synthesis of binding proteins[12]. Heavy metal resistant bacteria also play an important role in the biogeochemical cycling of those metal ions. The oxidation state of a heavy metals relates to the solubility and toxicity of the metal itself[13].

Interactions between bacteria and harmful algae have recently become the focus of much attention. The present study was aimed to determine the effects of domoic acid on growth and examine the relationship between domoic acid and heavy metal tolerance of some marine bacteria isolated from Samand gshore and Iskenderun Bay. This is the first study for this region.

MATERIALS AND METHODS

Chemicals and Medium

Marine Agar (2216) Difco, Marine Broth (2216) Difco, Nutrient Broth (CM1) Oxoid, Nutrient Agar (1.05450) Merck, Endo Agar (0006-17) Difco and EMB Agar (0076-17-0) were used in the tests. Domoic acid (purity 99 %) were purchased from Sigma. Minimal medim; Glucose 5 g, Na_2HPO_4 6 g, KH_2PO_4 3 g, NH_4Cl 1 g, NaCl 0.5 g, $MgSO_4$ 0.12 g and $CaCl_2$ 0.01 g and was supplemented with domoic acid (0,5 mg/ml).

Sampling site and isolation of bacteria

Marine broth and marine agar were used for isolation of heterotrophic marine bacteria. Water samples were collected from four different sites in Hatay; Samandag shore and Iskenderun bay. The samples were transported in sterilised jars and stored on ice until further treatment. Idetification of isolated bacteria were done according to the Bergey's Manuel of Determinative Bacteriology[14]. The bacterial isolates were maintained on marine agar slants for further study. Pure cultures were stored at –20 °C.

Domoic acid inhibition tests

The inoculum was grown in the marine broth for 24 h. 100 µl of a 24 h culture was spread evenly over the marine agar surface to form a bacterial lawn. Steril disks in which a range of domoic acid concentrations (0,5; 1,5; 2; 2,5; 20; 40; 60; $\mu g.ml^{-1}$ and 1 $mg.ml^{-1}$) had been absorbed were then pressed onto the agar surface. The plates were incubated at 25°C for 24-48 h. After the incubation, inhibition zones around the disks were determined.

Domoic acid stimulation tests

Selected bacterial isolates were inoculated (50 µl) in 2 ml marine broth to which varing concentrations of domoic acid and also in marine broth with no domoic acid. After samples were withdrawn after 3 hours intervals, those samples were spreaded onto marine agar and incubated at 24°C for 24 h. The bacterial growth was determined by plate count. Bacteria growth curve was determined.

Growth with domoic acid

Domoic acid were added to MM9 liquid media to final concentrations of 20µgr/ml. The inocula was standardised to 0,3 OD units at 600 nm. Isolates were inoculated (50 µl) in 2 ml MM9 liquid media and incubated at 24 °C for 24 h. After samples were withdrawn, bacterial growth was determined by spectrophotometer.

Determination of MTCs

For testing metal tolerance the metals Cu, Cd, Ni, Cr used as $CdCI_2.H_2O$, $CuSO_4.5H_2O$,

$NiCI_2.6H_2O$ and $K_2Cr_2O_7$ were added to sterilised marine agar medium at the following concentrations (mM); copper, 5 to 30; cadmium, 2 to 20; nickel, 2 to 20; chromium, 1 to 10. Plates were then spot inoculated and incubated at 25°C for two days[15]. The maxium tolerable concentrations (MTC) of heavy metal that allows growth after 2 days[16]. Metal-free control plates were used to evaluate the viability of the strains and culture media. The samples were kept away from metallic materials to avoid contamination.

Plasmid curing

To determine if the resistance gene encoded by a plasmid, ethidium bromide was used to eliminate the plasmids from the strains, and also heat treatment was applied as a second control. The strains were grown with ethidium bromide (100 µgr/ml) and then spread on marine agar plates, one containing metal salts and the other not containing. Replica plates were both media were incubated at 24 °C. Plasmids were considered to be eliminated from those colonies that grow on the metal- free medium only[17].

RESULTS AND DISCUSSION

All of the strains' physiological and morphological properties were determined. Bacteria isolates were identified in our laboratory following Bergey's Manuel of Determinative Bacteriology[14]. 19 *Pseudomonas* sp. strains, 15 *Acinetobacter* sp. strains, 5 *Alteromonas* sp. strains and 9 *Vibrio* sp. strains were identified. For inhibition tests, 0.5 $\mu gr.ml^{-1}$, 1.5 $\mu gr.ml^{-1}$, 2 $\mu gr.ml^{-1}$, 2.5 $\mu gr.ml^{-1}$, 20 $\mu gr.ml^{-1}$, 40 $\mu gr.ml^{-1}$, 60 $\mu gr.ml^{-1}$ and 1 $mgr.ml^{-1}$concentrations were tested. In the present study, all the strains were grown at these concentrations. Generally no harmfull influence of domoic acid

Table 1. MTC of the some strains with domoic acid and without

	with domoic acid (5 $\mu gr.ml^{-1}$)				without domoic acid			
	Cu	Ni	Cr	Cd	Cu	Ni	Cr	Cd
1.6Pseu	26	10	4	12	16	8	4	6
2.4Pseu	20	14	2	10	16	11	2	6
2.6Pseu	28	14	2	12	18	10	2	8
3.4Pseu	22	12	2	12	16	10	2	12
3.8Pseu	18	12	4	10	16	12	4	8
4.8Pseu	24	10	2	10	18	8	2	8
1.2Acin	16	8	1<	14	10	8	1<	12
2.8Acin	16	8	1<	12	10	6	1<	10
3.7Acin	10	8	1<	14	10	8	1<	12
1.10Vib	6	10	2	10	4	6	2	6
4.4Vib	6	12	4	10	4	8	2	8
2.3Alter	16	8	6	14	10	6	4	12
3.5Alter	16	12	6	14	10	12	6	12

concentrations was observed on the number of isolates. But other workers had shown that the domoic acid inhibition of the growth was concentration dependent[11]. Stewart *et al.*[11]showed inhibition for all bacteria were virtually the same, approximetely 9 mm for 5µmol DA, 8 mm for 2.5 µmol DA, 7 mm for 0.625 µmol DA and trace or none for 0.25 µmol DA. Maximum 1 $mgr.ml^{-1}$ concentaration was tested, because domoic acid had never been more than this concentration in nature. Doucette *et al.*[8] found maximum levels of 40 µg DA equiv. L-1 at 5m depth in Monterey Bay, CA during an August-September 2000 bloom of toxic *Pseudo-nitzschia australis*. But, toxin concentrations on molluscs and fish which consume toxic algae could be more than dissolved DA in seawater. Windust[17] found that 50. 10^{-6}g $.disk^{-1}$concentration of domoic acid was not showed inhibition effect on marine bacteria.

Stimulation test were carried out in a marine broth medium. The strains from each genus were randomly selected for stimulation tests. 1.6 *Pseudomonas*, 4.4 *Vibrio*, 2.3 *Alteromonas* and 2.9 *Acinetobacter* named strains were tested and

growth curve were determined with and without 5 µgr.ml^{-1} concentration of domoic acid. The bacterial isolates exihibited different growth patterns in the presence of domoic acid. Growth curves of the strains were shown Fig 1, Fig 2, Fig 3 and Fig 4.

When domoic acid was added to marine broth for the growth of 1.6 *Pseudomonas* sp. and 4.4. *Vibrio* sp., the effect was striking. Domoic acid was markedly stimulatory, increasing bacterial numbers 2-3 fold. Other isolates did not affected from domoic acid.

The growth of the isolates was determined using MM9 medium with domoic acid incorporated. All the strains were grown in MM9 plus domoic acid. It is important to note that the growth of bacteria presumably occurred as a direct result of the bacterial utilization of domoic acid. This shows that the strains could be use domoic acid for growth. Stewart *et al*.[11] (1998), suggest that bacteria could play a significant role in domoic acid elimination in certain molluscan species, e.g., *Mytilus edulis* and possibly *Mya arenaria,* but apperantly not in *Placopecten magellanicus*. Domoic acid is produced widely in nature, as it does not appear to accumulate in nature, mechanisms must exist for its degradation and disposal. As bacteria contained within the marine environment are prime choices to mediate this activity, a search for bacteria competent to utilize domoic acid was instituted[11].

Stewart *et al*.[11] proved that microbial metabolic capacities for the utilization of different compounds were products of the long-term availability of these compounds and the direct association of the microorganisms with the specific subsrates.

Domoic acid producing algae species were determined in mediterranean[18]. Polat *et al*.[19], investigated the distribution of potentially harmful phytoplankton species in Iskenderun bay. Potentially harmfull diatoms were *Pseudonitshia pungens*, *Leptocylindricus danicus* and *Cylindrotheca closterium* determined in the bay. But there is no data of the toxin analyses of the species in this region. It is also known that toxin production of species is depend on environmental conditions.

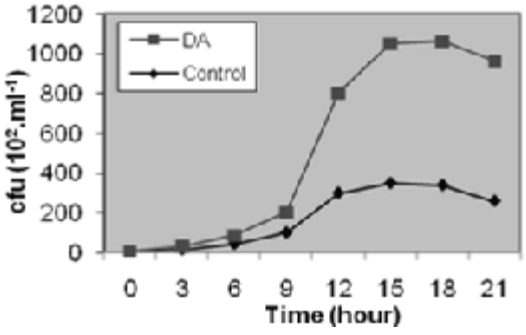

Fig. 1. Growth curve of 1.6 *Pseudomonas* sp.with DA

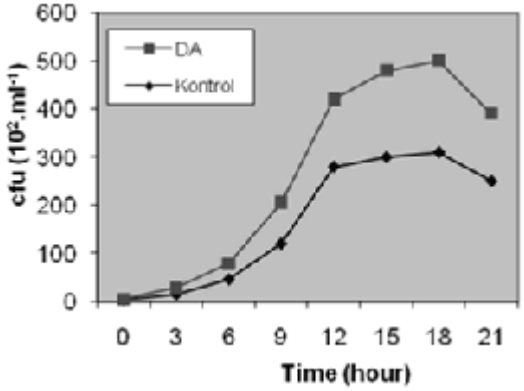

Fig. 2. Growth curve of 4.4 *Vibrio* sp with DA

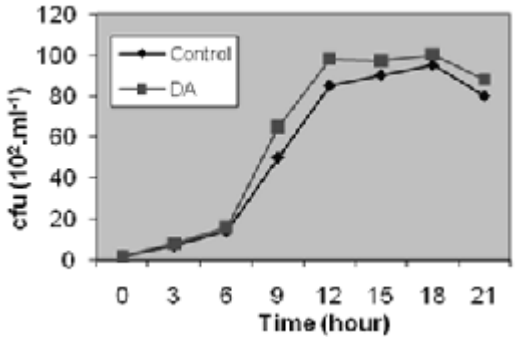

Fig. 3. Growth curve of 2.3 *Alteromonas* sp.with DA

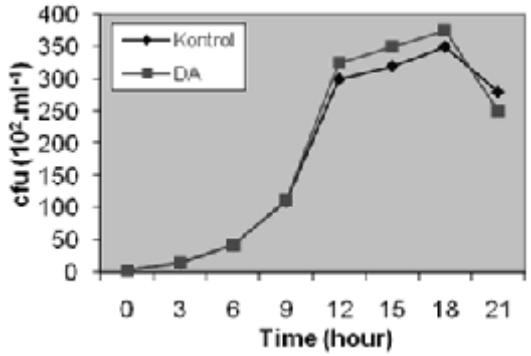

Fig. 4. Growth curve of 2.9 *Acinetobacter* sp.with DA

Several studies have examined the changes in bacterial flora over the development and decline of HABs[20]. Among the most interesting are the reports by Fukami *et al.*[21]on *Gymnodium nagasakiense* red showing that bacteria could either stimulate or inhibit dinoflagellate growth depending on the stage of the bloom. Bacteria are an inherent part of the physical environment of dinoflagellates, both in vitro and in vivo[22-23].

In this study, the effect of domoic acid on heavy metal tolerance of these bacteria was also investigated. All strains were tested if the resistance genes encoded by a plasmid. None of the strains had plasmid encoded heavy metal resistance. Table 1 shows MTC of the some strains with domoic acid and without.

The bacteria isolated from marine, growing in the presence of domoic acid stimulating growth concentration (5 µgr.ml^{-1}), were showing increased tolerance to copper, nickel and cadmium compared to without domoic acid.

The increased tolerance to heavy metals of the bacteria by domoic acid can be explained by: The interaction between heavy metals and domoic acid affecting the availability of the metals to bacteria cells. Production of DA could be a mechanism to bind free cupric ion, subsequently making it less available to the cell, rendering the cells more resistant to copper toxicity[7]. Since domoic acid can stimulate the growth of some marine bacteria species, domoic acid able to binding the metals may promote the development of heavy metal tolerance. Phytosiderophores are analogous to bacterial siderophores. They bind Cu sufficiently well to alleviate Cu toxicity in algal cultures[24].

The interaction of the bacteria with phytoplankton include competition for avaliable organic matter, uses or inhibition of bacteria by extracellular materials by the phytoplankton[25-26]. The interaction of bacteria and species of harmful algae has known as playing an important role in ecology of these organisms[20]. The results of these study will contribute novel information on the effect of domoic acid on marine bacteria.

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