



ACADEMIC
PRESS

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Environmental Research 93 (2003) 88–91

Environmental
Research

<http://www.elsevier.com/locate/envres>

Identical ribosomal DNA sequence data from *Pfiesteria piscicida* (Dinophyceae) isolates with different toxicity phenotypes

Torstein Tengs,^a Holly A. Bowers,^a Howard B. Glasgow Jr.,^b JoAnn M. Burkholder,^b and David W. Oldach^{a,b,*}

^a*Institute of Human Virology, University of Maryland at Baltimore, 725 West Lombard Street, Baltimore, MD 21201, USA*

^b*Center for Applied Aquatic Ecology, North Carolina State University, 620 Hutton Street, Suite 104, Raleigh, NC 27606, USA*

Received 9 July 2002; received in revised form 30 October 2002; accepted 22 November 2002

Abstract

Complete small subunit ribosomal RNA, internal transcribed spacer 1 and 2, 5.8S, and partial large subunit ribosomal RNA gene sequences were generated from multiple isolates of *Pfiesteria piscicida*. Sequences were derived from isolates that have been shown to be ichthyotoxic as well as isolates that have no history of toxic behavior. All of the sequences generated were identical for the different cultures, and we therefore conclude that differences in toxicity seen between isolates of *P. piscicida* are linked to factors other than genetic strain variation detectable by ribosomal gene sequence analyses.

© 2003 Elsevier Science (USA). All rights reserved.

1. Introduction

The heterotrophic dinoflagellate *Pfiesteria piscicida* has been linked to fish kills and human health problems repeatedly in both the Albemarle–Pamlico Estuarine System and Chesapeake Bay in recent years (Glasgow et al., 1995; Burkholder and Glasgow, 1997; Grattan et al., 1998; Steidinger et al., 1996). The species has been shown to be ichthyotoxic in laboratory settings (Burkholder et al., 1992, 1995; Burkholder and Glasgow, 1997; Fairey et al., 1999), but the mechanism(s) of toxicity are not well established because the toxin(s) are not yet fully characterized (Fairey et al., 1999; Levin et al., 2000), as is true for certain other dinoflagellate species (Falconer, 1993).

Like many other toxic algal species (e.g., Gentien and Arzul, 1990; Anderson, 1991; Skulberg et al., 1993; Bates et al., 1998; Edvardsen and Paasche, 1998), *P. piscicida* has both toxic and noninducible (NON-IND) strains. Burkholder has defined strains that do not produce detectable toxin and that are incapable of causing fish death or disease, even in the context of appropriate stimulation in fish bioassays, as noninducible (NON-IND functional type, Woods Hole Oceanographic

Institute; WHOI, 2000; Burkholder et al., 2001). In contrast, strains defined as actively toxic (TOX-A functional type) are triggered to produce toxin when they detect secretions and excretions from live fish (Burkholder et al., 1992; Burkholder and Glasgow, 1997). In the absence of live fish, these strains become temporarily nontoxic (Burkholder et al., 1995; Burkholder and Glasgow, 1997) and are referred to as the nontoxic or the TOX-B functional type. When cultured with live fish, toxic strains demonstrate potent ichthyotoxicity at similar cell concentrations as occur in estuarine fish kills that have been linked to toxic *P. piscicida* (Burkholder et al., 1995; Glasgow et al., 1995; Burkholder and Glasgow, 1997).

One hypothesis to explain the differences in observed toxicity among isolates of *P. piscicida* is that these isolates represent genetically distinct strains. To test this hypothesis, we generated DNA sequence data from both evolutionarily conserved (small and large subunit ribosomal RNA genes [SSU and LSU rRNA] and 5.8(S) and more variable regions of the ribosomal gene region (internal transcribed spacer 1 and 2 [ITS 1 and 2]) from TOX-A, temporarily nontoxic (TOX-B), and NON-IND isolates of *P. piscicida*. Various isolates of different geographical origins (toxicity not tested) were also sequenced to see if genetic differences could be found in any of the loci examined.

*Corresponding author. Fax: +1-410-706-1992.

E-mail address: oldach@umbi.umd.edu (D.W. Oldach).

2. Materials and methods

DNA was extracted from 21 different cultures of *P. piscicida* (Table 1) as described elsewhere (Oldach et al., 2000). Cultures were acquired from NCSU (North Carolina State University, Center for Applied Aquatic Ecology), CCMP (Provasoli-Guillard National Center for Culture of Marine Phytoplankton), and Florida DEP (Department of Environmental Protection). PCR amplifications were performed using two primer sets; in order to avoid amplification of prey organisms (i.e., cryptomonad species), selective primers were used in combination with general primers designed from more conserved regions of the genes. A general 5' SSU rRNA primer (primer "A", Tengs et al., 2000) was used in combination with a *P. piscicida* specific 3' SSU rRNA primer (primer "Chic", Oldach et al., 2000). An overlapping fragment spanning the 3' end of SSU rRNA gene, ITS 1 and 2, 5.8S and a part of the LSU rRNA gene was generated using a dinoflagellate-selective primer (primer "Dino", Oldach et al., 2000), and a general 5' LSU rRNA primer designed from an alignment comprised of 44 dinoflagellate LSU rRNA sequences downloaded from GenBank (Primer "LSU380"-0.5'TTTCATCTTTCCTCACGGTACTT3'). Amplifications were done using 2 mM [Mg²⁺] and *Taq* DNA polymerase (GIBCO). PCR products were sequenced directly using an ABI 377 automatic sequencing machine (Perkin–Elmer) and a DYEnamic terminator cycle sequencing kit (Amersham Pharmacia Biotech). All of the fragments were control-sequenced, and both strands were read for >90% of the amplicons.

Overlapping fragments were assembled and compared using the software SeqPup (Gilbert, 1996).

At times approximate to DNA extraction, fish bioassays were performed as described elsewhere (Burkholder et al., 1995; Burkholder and Glasgow, 1997; Glasgow, 2000; WHOI, 2000; Marshall et al., 2000).

3. Results

The rRNA gene region sequenced had a total length of 2779 base pairs (GenBank accession numbers AF330600-AF330620), and was 100% identical for all of the isolates, regardless of toxicity phenotype (see Table 1 for details).

4. Discussion

Recent findings have indicated that some *Pfiesteria* species can have harmful effects on fish as a function of micropredation rather than excretion of an exotoxin (Vogelbein et al., 2002). Other studies have argued that certain potentially toxin-associated genes (i.e., *PKS* and *NRPS* genes) do not reside in the genomes of these organisms (Berry et al., 2002), but none of these studies have in any way excluded the possibility that *Pfiesteria* is capable of producing a toxic compound. As of yet, no genes directly involved in toxin production have been described from any dinoflagellate species (Plumley,

Table 1
Isolates of *P. piscicida* with identical 18S, ITS1 & 2, 5.8S, and partial 23S sequences

Isolate ID	Functional type	Date and place of collection
NCSU B-125-4 (12/9)	TOX-A	12/96, Beaufort Pt., North Carolina, USA
NCSU B-125-PAC	TOX-A	12/96, Beaufort Pt., North Carolina, USA
NCSU 125-4	TOX-A ^a	9/97, Beaufort Pt., North Carolina, USA
NCSU 125-4 (12/2)	TOX-A ^a	12/96, Beaufort Pt., North Carolina, USA
NCSU 125-4 1997	TOX-A ^a	12/96, Beaufort Pt., North Carolina, USA
NCSU B-113-3	TOX-B	9/97, Neuse River, North Carolina, USA
NCSU B-125-4	TOX-B	12/96, Beaufort Pt., North Carolina, USA
NCSU B-89B	TOX-B	7/98, Neuse River, North Carolina, USA
NCSU 102-1	NON-IND	8/97, Pocomoke River, Maryland, USA
NCSU B-98T	NON-IND	9/97, Chicamacomico River, Maryland, USA
NCSU 113-2	Not tested	9/97, Neuse River, North Carolina, USA
NCSU 114-1-5	Not tested	9/97, Neuse River, North Carolina, USA
NCSU 97-2 1997	Not tested	9/97, Chicamacomico River, Maryland, USA
NCSU 97-1	Not tested	9/97, Chicamacomico River, Maryland, USA
NCSU 98-3 1997	Not tested	9/97, Chicamacomico River, Maryland, USA
NCSU 98-A	Not tested	9/97, Chicamacomico River, Maryland, USA
NCSU 113-4	Not tested	9/97, Neuse River, North Carolina, USA
NCSU 113-3-B	Not tested	9/97, Neuse River, North Carolina, USA
Florida DEP ^b	Not tested	9/97, Chicamacomico River, Maryland, USA
CCMP 1830	Not tested	1/98, Chicamacomico River, Maryland, USA
CCMP 1831	Not tested	1/98, Chicamacomico River, Maryland, USA

^aCultures inoculated into an algal culture and never inoculated back into fish bioassay to confirm toxicity again.

1997), and no genetic markers unique for pathogenic strains of any dinoflagellate species have been reported.

Our data address in part the question of genetic strain variation among *P. piscicida* isolates with different toxicity phenotypes. While genetic sequence distinction between toxic, temporarily nontoxic, and NON-IND isolates of *P. piscicida* remains possible, our study demonstrates that such a locus is highly unlikely to reside in the ribosomal gene region. Although we find it implausible that any ribosomal loci are directly involved in toxin production, we do not rule out the possibility that “pathogenicity genes” might exist elsewhere in the genome (as seen in bacteria; see Hacker et al., 2000), and that these genes (when described) will be candidates for use as markers for toxic strains.

The fact that identical sequences were derived from isolates from different localities is not too surprising, since all cultures were established from the Chesapeake Bay and North Carolina. In other species of algae, isolates from large geographical ranges have been shown to have identical ITS sequences (for example, see Connell, 2000).

Differences in nutrient levels, salinity, water temperature, and other environmental variables are important factors that can affect *P. piscicida* toxicity (e.g., Burkholder and Glasgow, 1997). *P. piscicida* is common in multiple rivers in the Albemarle–Pamlico and Chesapeake Bay (Burkholder et al., 1992, 1995; Burkholder and Glasgow, 1997; Rublee et al., 1999) and can often be found in high concentrations without having any apparent negative effects on the environment or on fish populations (Burkholder et al., 1995, 1999; Burkholder and Glasgow, 1997). How can it be that some isolates of *P. piscicida* can not be induced to produce toxins even if grown to high densities, exposed to live fish, and otherwise maintained under growth conditions identical to those for isolates that show ichthyotoxicity? In some species of harmful algae, bacteria associated with the eukaryote cells have been shown to produce toxic compounds. For instance, bacteria associated with *Alexandrium* species can produce saxitoxins (Galacher et al., 1997; for review see Doucette, 1995). We hypothesize that an interaction between specific (most likely epi- or intra-cellular) bacteria and *P. piscicida* may be necessary for toxin production by toxic strains, and that the bacterial flora associated with *P. piscicida* in a natural setting are dependent upon complex interactions between the species and multiple environmental variables. This premise would offer an explanation for the observation that in *P. piscicida* cultures, toxicity seems to be a phenotype that can change over time depending not only on the conditions the culture has been kept under before it is tested in toxicity assay, but also on whether it was isolated from a fish kill event (Burkholder and Glasgow, 1997; WHOI, 2000; Glasgow, 2000). More than 99% of *Pfiesteria* TOX-B strains will

lose their ability to kill fish (even when exposed to live fish) within 1.5–2 months if they are fed only algae (Burkholder et al., 2001). The TOX-B strains used in our study had been cultured on algal prey for 1–5 weeks, whereas the NON-IND strains had not been stimulated by fish excreta for >1 y. All of our strains have previously been shown to be ichthyotoxic in laboratory settings before this study. It is likely that the associated bacterial flora of these cultures changed over time, and we hypothesize that such changes may be reflected in observed toxicity functional types (i.e., from TOX-A to TOX-B to NON-IND). This is an area of ongoing investigation in our laboratories.

Alternative hypotheses could be evoked, involving mechanisms such as epigenetic inheritance and gene methylation, viral transfection, presence or absence of key metabolic substrates, or combinations of the above. Until purified toxin standards are available and the biosynthetic pathways of these compounds have been described in some detail, the actual mechanisms underlying *P. piscicida*'s ability to kill fish and affect human health will remain unclear. Recent progress in ongoing efforts to isolate and characterize *Pfiesteria* associated toxins (Moeller et al., 2002) are very encouraging in this regard.

References

- Anderson, D.M., 1991. Toxin variability in *Alexandrium*. In: Granéli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), *Toxic Marine Phytoplankton—Proceedings of the Fourth International Conference on Toxic Marine Phytoplankton*. Elsevier, New York.
- Bates, S.S., Garrison, D.L., Horner, R.A., 1998. Bloom dynamics and physiology of domoic-acid-producing *pseudo-Nitzschia* species. In: Anderson, D.M., Cembella, A., Hallegraeff, G.M. (Eds.), *Physiological Ecology of Harmful Algae*, NATO ASI Series G: Ecological Sciences, Vol. 41. Springer, Berlin.
- Berry, J.P., Reece, K.S., Rein, K.S., Baden, D.G., Haas, L.W., Ribeiro, W.L., Shields, J.D., Snyder, R.V., Vogelbein, W.K., Gawley, R.E., 2002. Are *Pfiesteria* species toxicogenic? evidence against production of ichthyotoxins by *Pfiesteria shumwayae*. *Proc. Natl. Acad. Sci. USA* 99 (17), 10970–10975.
- Burkholder, J.M., Noga, E.J., Hobbs, C.W., Glasgow Jr., H.B., Smith, S.A., 1992. New phantom dinoflagellate is the causative agent of major estuarine fish kills. *Nature* 358 (6385), 407–410.
- Burkholder, J.M., Glasgow Jr., H.B., Hobbs, C.W., 1995. Distribution and environmental conditions for fish kills linked to a toxic ambush-predator dinoflagellate. *Mar. Ecol. Prog. Ser.* 124, 43–61.
- Burkholder, J.M., Glasgow Jr., H.B., 1997. *Pfiesteria piscicida* and other toxic *Pfiesteria*-like dinoflagellates: behavior, impacts, and environmental controls. *Limnol. Oceanogr.* 42, 1052–1075.
- Burkholder, J.M., Mallin, M.A., Glasgow Jr., H.B., 1999. Fish kills, bottom-water hypoxia, and the toxic *Pfiesteria* complex in the Neuse River and Estuary. *Mar. Ecol. Prog. Ser.* 179, 301–310.
- Burkholder, J.M., Glasgow Jr., H.B., Deamer-Melia, N.J., 2001. Overview and status of the toxic *Pfiesteria* complex (dinophyceae). *Phycologia* 49, 186–214.
- Connell, L.B., 2000. Nuclear ITS region of the alga *Heterosigma akashiwo* (Chromophyta: Raphidophyceae) is identical in isolates from Atlantic and Pacific basins. *Mar. Biol.* 136 (6), 953–960.

- Doucette, G.J., 1995. Assessment of the interaction of prokaryotic cells with harmful algal species. in: Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Technique et Documentation, Lavoisier, Intercept Ltd., Paris, France.
- Edwardsen, B., Paasche, E., 1998. Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. In: Anderson, D.M., Cembella, A., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algae, NATO ASI Series G: Ecological Sciences, Vol. 41. Springer, Berlin.
- Fairey, E.R., Edmunds, J.S., Deamer-Melia, N.J., Glasgow Jr., H.B., Johnson, F.M., Moeller, P.R., Burkholder, J.M., Ramsdell, J.S., 1999. Reporter gene assay for fish-killing activity produced by *Pfiesteria piscicida*. Environ. Health Perspect. 107, 711–714.
- Falconer, I.R. (Ed.), 1993. Algal Toxins in Seafood and Drinking Water. Academic Press, New York.
- Gallacher, S., Flynn, K.J., Franco, J.M., Brueggemann, E.E., Hines, H.B., 1997. Evidence for production of paralytic shellfish toxins by bacteria associated with *Alexandrium* spp. (Dinophyta) in culture. Appl. Environ. Microbiol. 63, 239–245.
- Gentien, P., Arzul, G., 1990. Exotoxin production by *Gyrodinium* cf. *aureolum* (Dinophyceae). J. Mar. Biol. Assoc. UK 70, 571–581.
- Gilbert, D., 1996. SeqPup, Version 0.6, a biological sequence editor and analysis program.
- Glasgow, H.G., 2000. The biology of the toxic *Pfiesteria* complex in the Neuse Estuary, North Carolina. Ph.D. Dissertation, Department of Marine, Earth and Atmospheric Sciences, North Carolina State University, Raleigh.
- Glasgow Jr., H.B., Burkholder, J.M., Schmechel, D.E., Fester, P.A., Rublee, P.A., 1995. Insidious effects of a toxic dinoflagellate on fish survival and human health. J. Toxicol. Environ. Health 46, 501–522.
- Grattan, L.M., Oldach, D.W., Perl, T.M., Lowitt, M.H., Matuszak, D.L., Dickson, C., Parrott, C., Shoemaker, R.C., Kauffman, C.L., Wasserman, M.P., Hebel, J.R., Charache, P., Morris, J.G., 1998. Learning and memory difficulties after environmental exposure to waterways containing toxin-producing *Pfiesteria* or *Pfiesteria*-like dinoflagellates. Lancet 352, 532–539.
- Hacker, J., Kaper, J.B., 2000. Pathogenicity islands and the evolution of microbes. Annu. Rev. Microbiol. 54, 641–679.
- Levin, E.D., Rezvani, A.H., Christopher, N.C., Glasgow Jr., H.B., Deamer-Melia, N.J., Burkholder, J.M., Moser, V.C., Jensen, K., 2000. Rapid neurobehavioral analysis of *Pfiesteria piscicida* effects in juvenile and adult rats. Neurotoxicol. Teratol. 22 (4), 533–540.
- Marshall, H.M., Gordon, A.S., Seaborn, D.W., Dyer, B., Dunstan, W.M., Seaborn, M., 2000. Comparative culture and toxicity studies between the toxic dinoflagellate, *Pfiesteria piscicida* and a morphologically similar cryptoperidinioid dinoflagellate. J. Exp. Mar. Biol. Ecol. 255, 65–74.
- Moeller, P.D.R., Ramsdell, J.S., Morton, S.L., Mitchell, B.A., Eaker, S., Burkholder, J.M., Glasgow, H.B., Deamer-Melia, N.J., 2002. Isolation, characterization and current chemical structural information on a water soluble toxin derived from *Pfiesteria piscicida*. Presented at The Xth International Conference on Harmful Algae, October 21–25, 2002, St. Pete Beach, FL, USA.
- Oldach, D.W., Delwiche, C.F., Jakobsen, K.S., Tengs, T., Brown, E.G., Kempton, J.W., Schaefer, E.F., Bowers, H.A., Glasgow, H.B., Burkholder, J.M., Steidinger, K.A., Rublee, P.A., 2000. Heteroduplex mobility assay-guided sequence discovery: elucidation of the small subunit (18S) rDNA sequences of *Pfiesteria piscicida* and related dinoflagellates from complex algal culture and environmental sample DNA pools. Proc. Natl. Acad. Sci. USA 97, 4303–4308.
- Plumley, F.G., 1997. Marine algal toxins: biochemistry, genetics and molecular biology. Limnol. Oceanogr. 42, 1252–1264.
- Rublee, P.A., Kempton, J.W., Schaefer, E.F., Burkholder, J.A., Glasgow Jr., H.B., Oldach, D.W., 1999. PCR and fluorescent in-situ hybridization detection extends the range of *Pfiesteria piscicida* in estuarine waters. Va. J. Sci. 50 (4), 325–335.
- Skulberg, O.M., Carmichael, W.W., Codd, G.A., Skulberg, R., 1993. Taxonomy of toxic Cyanophyceae (cyanobacteria). In: Falconer, I.R. (Ed.), Algal Toxins in Seafood and Drinking Water. Academic Press, New York.
- Steidinger, K.A., Burkholder, J.A., Glasgow, H.B., Truby, E.W., Garrett, J.K., Noga, E.J., Smith, S.A., 1996. *Pfiesteria piscicida* gen et sp nov (Pfiesteriaceae, fam nov), a new toxic dinoflagellate with a complex life cycle and behavior. J. Phycol. 32, 157–164.
- Vogelbein, W.K., Lovko, V.J., Shields, J.D., Reece, K.S., Mason, P.L., Haas, L.W., Walker, C.C., 2002. *Pfiesteria shumwayae* kills fish by micropredation not exotoxin secretion. Nature 418 (6901), 967–970.
- Woods Hole Oceanographic Institute. 2000. Glossary of *Pfiesteria*-related terms. Consensus document by the *Pfiesteria* Interagency Coordination Working Group, chaired by J. Macknis, US EPA, Baltimore, MD and including representatives from the US E.P.A., the CDC, NOAA, various state agencies and academic institutions, 2000; available at <http://www.redtide.whoi.edu/pfiesteria/documents/glossary.html>.