A comprehensive comparative genomic approach for identifying saxitoxin synthesis genes

Jeremiah D. Hackett

Department of Ecology and Evolutionary Biology
University of Arizona
Saxitoxin genomics is a collaborative effort

PI: Gerry Plumley
Debashish Bhattacharya
Don Anderson
Jeremiah Hackett
Jeanette Loram
Ahmed Moustafa
Origin and assembly of the saxitoxin biosynthetic pathway in cyanobacteria
Paralytic shellfish poisoning (PSP)

- Potentially fatal syndrome
- Shellfish accumulate toxins
- Most wide-spread toxic syndrome associated with algae
- Affects humans, marine mammals and sea birds
Saxitoxin (STX)

- Most potent non-protein toxin
- LD50 ~8 ug/kg
- In vivo, produced from 3 arginine, 1 methionine, 1 acetate
- ~20 related compounds
- Potent and selective sodium channel blocker
- “Black capsule” poison

<table>
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<th>R₂</th>
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Saxitoxin

Saxitoxins, also known as Paralytic Shellfish Poisons (PSPs), are neurotoxic alkaloids that block the entrance of sodium ions into nerve cells and cause the paralysis characteristic of saxitoxin poisoning. Saxitoxins naturally gather in marine and freshwater microalgae, especially Anabaena circinalis in freshwater and Alexandrium catenella, Alexandrium minutum, Alexandrium ostenfeldii, Alexandrium tamarense, Gymnodinium catenatum and Pyrodinium bahamense var. compressum in saltwater. The Saxitoxins are passed on to marine life that ingests the microalgae. Saxitoxin was named after the mollusk Saxidomus giganteus in which it was first recognized. Due to its selective blockage of only the sodium channels but not affecting the potassium or calcium channels or the chloride ions count, saxitoxins have become biomedical tools in the study of nerve disorders.

Consisting of polar molecules, saxitoxins dissolve easily in water and low level alcohols. The toxins are stable in neutrals and acids even at high temperatures but are inactivated by strong alkalines. The threat of saxitoxin poisoning increases during red tide algae blooms, especially for shellfish eaters. Pufferfish poisoning results from saxitoxins. The most lethal non-protein toxin, 0.2 milligrams of saxitoxins can kill a human of average bodyweight.

Saxitoxins as a Biological Weapons Agent

1,000 times more toxic than sarin, saxitoxins have been explored by the United States biological and chemical weapons programs. Reportedly, the CIA used saxitoxins for suicide capsules and other covert uses in the 1950s. It was reported that Francis Gary Powers carried saxitoxins on the tip of a hidden drill bit inside a silver dollar. Named Agent TZ, saxitoxins were designated Schedule 1 chemical agent and stockpiled. In biochemical form, saxitoxins are easily dissolved into water and can be ingested or inhaled.
PSP producing organisms

Dinoflagellates

- *Alexandrium catenella*

Cyanobacteria

- *Cylindrospermopsis raciborskii*
- *Anabaena circinalis*

Pyrodinium bahamense
Identifying STX synthesis genes

- STX is a unique compound, novel genes.
- Dinoflagellates have the greatest PSP impact, but have large genomes.
- Analysis of STX+ cyanobacterial genomes could accelerate discovery of STX genes
Candidate saxitoxin synthesis cluster in cyanobacteria

Cylindrospermopsis raciborskii

Kellmann et al. 2008
Preliminary annotation of these genes suggests gene transfer had a role in gene cluster evolution

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<thead>
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<th>Gene</th>
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<th>Best BLAST Hit</th>
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Best BLAST hit:
- Cyanobacteria: 13
- Proteobacteria: 8
- Bacteroidetes: 2
- Firmicutes: 1
- Actinobacteria: 1

*Kellmann et al. 2008*
Comparative genomics STX+ organisms

Cyanobacteria

Dinoflagellates

*Alexandrium catenella*
*Alexandrium tamarense*

*Pyrodinium bahamense*

*Aphanizomenon issatschenkoi*
Evolutionary history of Stx genes

- Combined genomic and phylogenomic approach

- Sequenced genomes of:
  - Stx+ *Anabaena circinalis* ACBU02
  - Stx - *Anabaena circinalis* ACFR02
  - Stx+ *Aphanozomenon flos-aquae*

- Address 3 questions:
  - Are the *Cylindrospermopsis* candidate saxitoxin genes other saxitoxin producing cyanobacteria?
  - What is the structure of the Sxt cluster in other cyanobacteria?
  - What are the phylogenetic origins of these genes?
Cyanobacterial genomes

- **Anabaena circinalis ACBU02**
  - Saxitoxin producer
  - 417 contigs
  - 25 scaffolds
  - 4.46 Mb
  - 5,188 predicted genes
  - 87.5% annotated

- **Anabaena circinalis ACFR02**
  - No saxitoxin
  - 913 contigs
  - 18 scaffolds
  - 4.60 Mb
  - 5,203 predicted genes

Moustafa et al. in review
SSU rDNA tree of cyanobacteria

- **Chroococcales**
  - *Synechococcus elongatus* PCC7942
  - *Synechococcus elongatus* PCC6301
  - *Cyanotoche sp* 51142
  - *C. sp* PCC8801
  - *T. elongatus* BP1

- **Gloeobacterales**
  - *Gloeobacter violaceus* PCC8105
  - *Gloeobacter violaceus* PCC7421

- **Oscillatoriales**
  - *Trichodesmium contortum* IMS101

- **Nostocales**
  - *Nostoc sp* PCC7120
  - *Anabaena variabilis* ATCC29413
  - *Nostoc punctiforme* PCC73102
  - *Nostoc sp* 8963
  - *Anabaena circinalis* ACBU02
  - *Anabaena flos-aquae* NRC441
  - *Aphanizomenon flos-aquae* NH5

0.05 substitutions/site
Phylogenomic analysis of toxic ACBU02

- Determined the phylogenetic origin of all genes in the genome.
- Searched a database of >500 genomes.
- All prokaryotes, representative viral and eukaryotic genomes.
- Similarity search, build alignments, construct ML trees.
- Trees are sorted on topology criteria with >50% bootstrap support.

*Anabaena circinalis*
PhyloSort: automated tree sorting.
Moustafa and Bhattacharya 2008 BMC Evolutionary Biology.
Phylogenomic analysis of toxic ACBU02

- Trees inferred for 3,865 (75%) genes
- 35% (1,612) are cyanobacteria-specific
- 30.5% (1,401) shared by cyanobacteria and other bacteria
- 17% (779) shared by bacteria and Archaea
- 1.8% (73) are of viral origin

Moustafa et al. in review

LGT candidates
### Predicted toxin genes in *A. circinalis*

25 of 26 predicted STX genes found in toxic *A. circinalis*.

Distributed across ~10 contigs

- *sxtX* not found.
- Only found in cyanos that make neosaxitoxin

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<th>Gene</th>
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<th>Contig</th>
<th>Description</th>
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<td><em>sxtW</em></td>
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<td><em>sxtY</em></td>
<td>ABI75117</td>
<td>contig267</td>
<td>Phosphate uptake regulator</td>
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<td><em>sxtZ</em></td>
<td>ABI75118</td>
<td>contig267</td>
<td>Two-component sensor histidine kinase</td>
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<td>ABI75114</td>
<td>contig129</td>
<td>Saxitoxin-binding protein</td>
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<td><em>sxtU</em></td>
<td>ABI75108</td>
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<td>Short-chain alcohol dehydrogenase</td>
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<td><em>sxtF</em></td>
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Saxitoxin gene cluster rearrangements

Kellmann et al. 2008

Cylindrospermopsis raciborskii T3 saxitoxin gene cluster (~35Kb)

Anabaena circinalis ACBU02 saxitoxin genes, contig129 (~16Kb)

Moustafa et al. in review
Origins of the saxitoxin genes

Examined trees from all 26 predicted saxitoxin genes

Tree topologies fell into three categories:

1. Genes common to Stx +, Stx -, and other cyanobacteria
   • Show pattern of vertical inheritance, may not be Stx specific

2. Genes of cyanobacterial origin in only Stx + cyanos
   • Not found in stx - strains, show lateral transfer from other cyanos

3. Genes that originated by LGT in Stx + taxa from non-cyanobacterial sources
   • Not found in stx -, show LGT from other bacteria
Type I - cyanobacterial, not STX+ specific

- Shared by Stx+ and Stx- cyanobacteria
- Show vertical inheritance
- 5 genes ($sxtO$, $sxtV$, $sxtW$, $sxtY$, $sxtZ$)
- Trees show sister relationship of Stx+ and Stx- strains
- Stx+ strains not monophyletic
- Generally agree with SSU rDNA tree
- Probably not specific to saxitoxin production (e.g. $sxtW =$ ferredoxin)
sxtY - phosphate uptake regulator
sxtZ - two-component sensor histidine kinase
Type II - STX specific, cyanobacterial

- Saxitoxin producing cyanos form a clade within the cyanobacteria, excluding STX- strains. (10 genes)
  - 2 genes \((sxtP\) and \(sxtU\)) appear vertically inherited from Nostocaceae ancestor
  - 2 genes \((sxtM\) and \(sxtF\)) transferred from Chlorococcales
  - 6 genes \((sxtD, L, N, X, J,\) and \(K\)) transferred from (or to) toxin-producing Oscillatoriales
    - *Lyngbya* sp. (STX) and *Trichodesmium erythraeum* (unknown neurotoxin)
sxtN - sulfotransferase

Shared by toxin producing cyanobacteria

0.6 substitutions/site
Type III - STX specific, non-cyanobacterial

- 11 genes, 7 have a proteobacterial source.
- Encode key enzymes in saxitoxin synthesis
  - sxtA (PKS), sxtB (cytidine deaminase), sxtG (aminotransferase), sxtI (O-carbamoyltransferase)
**sxtH** and **sxtT**

Phenylpropionate dehydrogenase

Verrucomicrobial origin

2 copies in *M. infernorum*

1 transferred
sxtA

- Key first enzyme in saxitoxin synthesis
- Multidomain polyketide synthase
  - SAM-dependent methyl transferase
  - Acetyl-transferase
  - Acyl carrier protein
  - Class II aminotransferase
sxtA

N-terminus
Proteobacteria

C-terminus
Actinobacteria

Burkholderia ambifaria AMMD 115360667
Burkholderia ubonensis Bu 167588068
Myxococcus xanthus DK 1622 108760503
Alexandrium catenella 186964724 sxtA1(+)
Anabaena circinalis ACBU02 sxtA1(+)
Anabaena circinalis AWQC131C 114462387 sxtA1(+)
Cylindrosporomepsis raciborskii T3 114462352 sxtA1(+)
Geobacter uraniireducens Rf4 148265129
Candidatus Endobugula sertula 121624848
Flavobacteriales bacterium ALC-1 163788868
Flavobacterium johnsoniae UW101 146299776
Psychrophilus torquis ATCC 700755 91218228
Dokdonia donghaensis MED138 86131935
Kordia algicida OT-1 163754777
Nostoc punctiforme PCC 73102 186683360(-)
Frankia sp Cc13 86742422
Frankia sp EAN1pec 158312847
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Anabaena circinalis ACBU02 sxtA2(+)
Cylindrosporomepsis raciborskii T3 114462352 sxtA2(+)
Pyrococcus furiosus DSM 3638 18976637
Pyrococcus abyssi GE5 14521874
Thermus thermophilus HB27 46199521
Thermus thermophilus HB8 55981551
Acidobacteria bacterium Ellin345 94970945
Solibacter usitatus Ellin6076 116624248
Porphyromonas gingivalis ATCC 33277 188995585
Porphyromonas gingivalis W83 34541403
Pseudomonas syringae pv phaseolicola 71736009
Pseudomonas fluorescens PfO-1 77461396

1.0 substitutions/site
Proteobacterium (e.g., *Myxococcus xanthus*)

Acyl-CoA N-acyltransferase

PP-binding

Actinobacterium (e.g., *Frankia alni*)

Aminotransferase class I and II

HGT & fusion

Ancestor of saxitoxin-producing cyanobacteria

Polyketide synthase (*sxtA*)

~1200 amino acids
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<th>A. circinalis ACBU02</th>
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**Phylogenomics of Stx summary**

- **Not Stx specific**
  - Cyanobacterial
  - LGT from non-cyanobios

- **Cyanobacterial, Stx specific**
- **LGT from non-cyanobios Stx specific**
Methyltransferase domain of sxtA

N-terminus of sxtA shows very weak similarity with SAM-dependent methyltransferases

Kellmann et al. 2008

Proteobacterium (e.g., *Myxococcus xanthus*)

Acyl-CoA N-acyltransferase

PP-binding

HGT & fusion

Ancestor of saxitoxin-producing cyanobacteria

Polyketide synthase (sxtA)

~1200 amino acids

Actinobacterium (e.g., *Frankia alni*)

Aminotransferase class I and II

Mustafa et al. in review
The missing methyl transferase

- `sxtA` MTF region poorly matches methyltransferases
- We identified 2 SAM-dependent methyltransferases in ACBU02
- 1 is shared with non-toxic ACFR02
- Other is similar (66% identity) to a firmicute protein, FkbM SAM-dependent methyltransferase.
- This protein also matches an EST from toxic dinoflagellate *Alexandrium tamarense*.
Stx+ specific SAM-dependent methyl transferase
Conclusions

• Phylogenomic analysis has revealed the extent of gene transfer in A. *circinalis*.
• 25 of 26 predicted STX genes were found.
• 5 are not STX+ specific.
• 10 are of cyanobacterial origin.
• 11 are of non-cyanobacterial origin.
• *sxtA* is a fusion of transferred genes from different sources.
• May have discovered a methyltransferase that performs the initial reaction in STX synthesis.
Search for Stx genes in dinoflagellates

- *Alexandrium tamarense*, saxitoxin producing dinoflagellate
- 100 Gb genome, 25-35,000 genes.

- Sanger 3’ EST database of ~13,000 EST unigenes
- 10MB of 454 transcriptome data
- ~5,500 contigs
Search for Stx genes in dinoflagellates

• We have identified candidate saxitoxin genes \( sxtA, sxtG, sxtN, sxtZ, \) new FkbM methyltransferase

• More \( A. \) tamarense and \( A. \) catenella, \( P. \) bahamense transcriptome data soon.

• Testing 3 hypotheses:
  1. STX in dinoflagellates arose by LGT from cyanobacteria.
     • Many or just a few genes
  2. STX in dinoflagellates arose independently
  3. STX in dinoflagellates is caused by symbiotic bacteria
“Desert lab of marine genomics”

Jennifer Hughes

Chris Schvarcz

The University of Arizona
Ecology & Evolutionary Biology
Proposed STX synthesis pathway

FIG. 3. Revised pathway for STX biosynthesis and the putative functions of sxt genes (see text for description).
Key enzymes in pathway arose through LGT

Transferred
Non-cyano

Transferred
Toxic cyano

Vertical
Shared with
Non-toxic