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Isolation and verification of anatoxin-a producing clones of *Anabaena flos-aquae* (Lyngb.) de Breb. from a eutrophic lake

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Abstract: We present a technique to isolate and confirm anatoxin-a producing clones (single trichome-isolates) of *Anabaena flos-aquae* (Lyngb.) de Breb. from blooms of this cyanobacterium. A single trichome is isolated from a field sample and grown in ASM medium. Single trichomes are then isolated from this culture and grown in ASM medium to produce single clone cultures. Mouse bioassay, and thin-layer chromatography (TLC) using purified anatoxin-a as reference is then used to confirm the anatoxin-a producing clones. Using this methodology, *Anabaena flos-aquae* samples collected during July 1991 from Hebgen Lake, Montana, were found to contain only 8.7% anatoxin-a producing clones. This minor proportion of anatoxin-a producing clones apparently accounts for the anatoxin-a produced by the entire population of *A. flos-aquae*. Our technique is simple and reproducible. A selected clone of *A. flos-aquae* that produces anatoxin-a and one that does not produce anatoxin-a were deposited in the UTEX culture collection, University of Texas at Austin.

Key words: Anatoxin-a; Toxic cyanobacteria; Cyanobacterial toxin; Cyanobacterial clone; Cyanobacterial strain; Intra-species variants of cyanobacteria; *Anabaena*

Introduction

Cyanobacteria that produce toxins are found in various parts of the world including The United States and Canada (Carmichael et al., 1985). Most cyanobacterial poisonings in North America are associated with blooms of *Anabaena flos-aquae* (Moore 1977; Juday et al., 1981; Carmichael, 1981). *Anabaena flos-aquae* produces the potent nicotinic cholinergic alkaloid, anatoxin-a, a bicyclic secondary amine (2-acetyl-9-azobicyclo [4.2.1]non-2-ene) (Devlin et al., 1977; Moore, 1977; Stevens and Krieger, 1988). However, all strains of *A. flos-aquae* do not produce anatoxin-a (Carmichael and Gorham, 1978; Stevens and Krieger, 1988; Moore, 1977). Hence it is important to formulate techniques that are simple, efficient and reliable to isolate and characterize anatoxin-a producing clones of *A. flos-aquae* so that sound water quality judgements can be made.

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Because *A. flos-aquae* produces at least four different kinds of anatoxin (anatoxin a, b, c and d) anatoxin-a being the most potent (Carmichael and Gorham, 1978), it is important that the technique should be able to accurately confirm the presence of anatoxin-a.

Materials and Methods

Unialgal populations of *A. flos-aquae* (Lyngb.) de Breb. collected during July 1991 from four random locations in the Greyling Arm of Hebgen Lake, Montana were combined and maintained for up to 7 days at a temperature of 25 ± 1°C and a photon flux density of 150 μmol quanta m^{-2} s^{-1} supplied by cool white fluorescent lamps under a 12 h L/D cycle.

Five random aliquots of 5 ml each were removed from the stock culture while stirring, and combined for use in the isolation procedure. The 25 ml aliquot thus obtained was gently passed twice through 22-gauge hypodermic needle fitted on a 30 ml syringe to disperse aggregates of the cyanobacterium. The dispersed cyanobacterial sample was then treated with 0.1 ml Tween-80 to prevent the trichomes from reaggregating. The dispersed aliquot was then diluted with ASM medium (Allen, 1968) with 10 μM $NaNO_3$ to obtain approximately 15–25 trichomes ml^{-1}. While stirring, 0.15–0.20 ml aliquots of the cyanobacterial suspension were transferred to 9 cm diameter plastic petri dishes and viewed under a dissecting microscope at a magnification of 25–30 ×. A 23-gauge hypodermic needle fitted on a 1 ml syringe was then used to remove a single trichome from the 0.15–0.20 ml aliquot under the dissecting microscope. The syringe content was transferred to a new petri dish to view under the dissecting microscope to ensure that a single trichome only was removed. After adding a few more drops of ASM medium to the culture sample containing the single trichome, it was transferred using a fresh 1 ml syringe to a test tube containing 5–6 ml ASM medium with 20 μM $NaNO_3$. This isolate was incubated under the same conditions as the stock culture with gentle agitation every 2 days for up to 10 days. After 10 days, samples from each culture tube were viewed under the dissecting microscope. Those cultures that contained growing *A. flos-aquae* were subjected one more time to the same procedure of dispersion, dilution and single trichome isolation in order to be sure the final culture samples were derrived from a single trichome. The *A. flos-aquae* clones thus obtained were allowed to grow for about 2 weeks in the test tubes before transferring to ASM medium of the same composition in 250 ml conical flasks. We produced a total of 23 clone-cultures (C-1 to C-23) of *A. flos-aquae*.

Each one of the 23 single trichome clones were tested for anatoxin-a production using (1) mouse bioassay, and (2) thin-layer chromatography (TLC) combined with mouse bioassay. 75 μl packed cell volume obtained by centrifuging acidified (using 0.2 mM HCl to pH 3.0) culture was disrupted thoroughly in 1 ml ice-cold ASM medium at pH 8.2 using an ultrasonic probe-disruptor (Braun, at a power setting of 5 on a 50/50 duty cycle for 3–5 min). The 0.5–0.6 ml extract (pH adjusted to 7.0 using 0.1 N NaOH) obtained after centrifugation was intra-peritoneally (IP) injected into a male Balb/cBy mouse with an average body weight of 26 g. A 0.55 ml portion of 0.9% (w/v) saline was injected into a control mouse. The injected mice were closely observed for at least 30 min.

For TLC, 125 μl packed cell volume of each clone of *A. flos-aquae* was extracted in a 2:1:2 (v/v/v) mixture of water/absolute ethanol/acetone according to the sonication procedure described for the mouse bioassay. The supernatant was concentrated to about half its original volume using a N_2-jet from a hypodermic needle. The concentrated supernatant was then used to spotdry polyester TLC plates (Sigma Chemical Co.) coated with 250 μm thick layer of silica gel with a mean particle size of 2–25 μm, and mean pore diameter of 60 Å. A control spot (0.02 μg) containing pure anatoxin-a ((+)-isomer obtained from Biometric Systems Inc., Eden Prairie, MN) was used as a control on every TLC plate. The extract from each clone was sufficient for about 5–6 spots. The silica gel had a fluorescent indicator at a wave length of 254 nm. The plates were

run at 4 °C for over 2 h using a solvent mixture containing 2 : 1 : 2 (v/v/v) water/ absolute ethanol/acetone in a closed TLC jar pre-equilibrated with the solvent mixture. The plates were dried using a blow-dryer, viewed under a broad spectrum UV source and the UV sensitive spots along the elution path of each spot including that corresponding to the authentic anatoxin-a spot was demarcated with a pencil. The spots having the same R_f value (relative to the solvent front, i.e., ratio of distance of the spot to the distance of the final solvent front from the original sample spot) in each clone were accumulated (excluding the control elution path having the authentic anatoxin-a) after carefully scraping individual spots. The silica gel samples from spots accumulated were then separately suspended in 1.5 ml of 0.3% (w/v) saline, vortexed to ensure dissolution of the soluble compounds from the samples, and centrifuged to eliminate silica particles. The 0.6 ± 0.05 ml supernatant obtained, was tested by mouse bioassay as described above. Both experiments were repeated to yield two replicate observations.

Results

Out of 23 clones of *A. flos-aquae* isolated, 2 clones (C-2 and C-18) killed test mice within 5 min of injection. Death was preceeded by acute convulsions, gasps and tremors, all of which are characteristic symptoms of anatoxin-a poisoning (Devlin et al., 1977; Moore, 1977; Stevens and Krieger, 1988). Three clones (C-5, C-8 and C-13) produced laziness and slight ataxia in the injected mice but they recovered after about 30 min. All other clones had no noticable influence on the test mice. These results were consistant in both experiments.

Anabaena flos-aquae clones C-2 and C-18 showed prominent UV sensitive spots (R_f value of 0.30–0.35) corresponding to the reference anatoxin-a spot on the TLC plates. Of these, clone C-2 consistantly showed the most intense spot. The rest of the clones of *A. flos-aquae* showed no visible UV sensitive spots corresponding to the authentic anatoxin-a. In the mouse bioassays when spots were eluted from the TLC plates and injected into test mice the spot with an R_f value 0.30–0.35 from clone C-2 killed the mouse in 5–6 min whereas that from C-18 killed the mouse after about 10 min. Symptoms were characteristic of anatoxin-a poisoning. None of the other clones or other spots from clones C-2 and C-18 produced significant toxic symptoms. These results were consistant in both experiments.

The most toxic clone C-2, and clone C-3 that produced no symptoms or UV sensitive spot corresponding to the authentic anatoxin-a in any of our experiments were selected for our further studies on anatoxin-a. These two selected clones were deposited in the UTEX culture collection (Department of Botany, University of Texas at Austin) under reference numbers LB 2557 (C-2) and LB 2558 (C-3).

Discussion

Our studies show that less than 9% of the *A. flos-aquae* clones from our study area produce anatoxin-a; only 8.7% (2 out of 23 clones) were able to produce anatoxin-a. This minor proportion of anatoxin-a producing clones of *A. flos-aquae* is apparently enough to kill cattle and wildlife in Hebgen Lake, Montana, a phenomenon which has been documented on numerous occasions since the mid 1970's (Juday et al., 1981; Priscu, unpublished data). The symptoms of laziness and slight ataxia caused by the clones C-5, C-8, and C-13 may have been caused by other kinds of toxic substances that may be prevalent in those clones.

The technique we used to isolate the anatoxin-a producing clones of *A. flos-aquae* is simple and reliable due to the stringent steps used to isolate and culture a single trichome from which the entire clone-culture is produced. Even though we lost about 8–9% of the isolated single trichomes during prolonged culturing, our culture technique to induce a single trichome to produce a population was very successful. The 2-step isolation of a single trichome used in our technique adds to the

reliability of purity and genetic homogeneity of the clones we isolated. Toxic strains of *A. flos-aquae* isolated by others (e.g., Gorham et al., 1964), apparently did not originate from a single trichome. Some colonies they produced may have originated from a micro-aggregate of trichomes. Hence, the degree of genetic purity of those strains is questionable. Further, the confirmation of anatoxin-a production by those strains was not performed using reference anatoxin-a. Even though this is not the first time a technique to isolate clones of *A. flos-aquae* is reported, our technique has several advantages and important merits. One of the important advantages of our isolation technique is that it will allow quantitative data on anatoxin-a production by a genetically uniform culture. Because it is difficult to separate viable single cells from trichomes, a single trichome may be the alternative to produce clones. Based on the fact that theoretically short trichomes from fresh cultures would very rarely have cells that are genetically different, the single trichome clones we have produced should be genetically homogeneous.

Our TLC technique even though combined with mouse bioassay was a significant step in the identification of anatoxin-a in the isolated clones of *A. flos-aquae*. Importantly, by converting the anatoxin-a into its hydrochloride form by acidification in the initial extraction process we protected the secondary amine N of this toxin from oxidation (see also Stevens and Krieger, 1988). The authentic anatoxin-a sample we used in our TLC was a hydrochloride form too, and a (+)-isomer because it is in this form anatoxin-a is produced naturally by *A. flos-aquae* (Spivak et al., 1982).

In summary, we have developed a simple technique for isolating and verifying anatoxin-a producing clones of *A. flos-aquae*. Using this method we showed that less than 9% of the trichomes of *A. flos-aquae* from a eutrophic lake contained anatoxin-a. Selected anatoxin-a producing and non-anatoxin-a producing clones isolated and identified by this procedure have been deposited with the UTEX culture collection (LB 2557-toxic, LB 2558-non toxic) and are readily available to scientists.

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