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TOXIC ALGAE
IV. ISOLATION OF TOXIC BACTERIAL CONTAMINANTS

by

W.K. THOMSON, A.C. LAING, and G.A. GRANT

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TOXIC ALGAE
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ABSTRACT

1. Bacteria, toxic to mice, have been isolated from four strains of blue-green algae.
2. These four strains have been grown successfully on solid and in liquid media.
3. *Microcystis aeruginosa* (Western strain) has been purified and found non-toxic in the purified state under all the conditions of growth investigated.
4. Two theories have been advanced which might ascribe a role to the algal cells in growth and toxin production.

INTRODUCTION

Blue-green algae (Cyanophyta) have been reported (3) to produce a toxic material that sometimes causes death of cattle drinking from pond waters in which algae have accumulated.

The literature on toxic algae has been reviewed in some detail in an earlier paper (1) in which it was concluded that the toxic material, not present in active form in fresh algae, develops or is released by partial putrefaction of the algal material. This was demonstrated experimentally using two different strains of blue-green algae. It has also been shown (2) that *Anacystis montana f minor* was toxic for mice at doses of 21 mg./kgm. after incubation of the material for 36 hr. at 37° C.

After an investigation (2) of the toxicity of *Microcystis aeruginosa* (Western strain), it was reported that 100 per cent mortality occurred with mice at doses of 22 mg./kgm. body weight. These deaths occurred after intraperitoneal injection of resuspended freeze-dried algae after the material had been incubated.

Both of these strains, the latter before and after purification, have been investigated in this laboratory (DRKL). Two strains of Nostoc have been studied to a lesser degree.

The main purposes of the study were:

(1) To produce greater growth or toxicity by varying culturing conditions, and

(2) to culture algae free from mould and bacterial contamination and use such purified strains for toxicity studies.

EXPERIMENTAL

Materials and Methods

The following strains of blue-green algae were studied:

Anacystis montana f minor, Microcystis aeruginosa (Western strain)*, *Nostoc ellipsosporum* and *Nostoc sp.* The first two strains have been grown in some quantity and the toxicity estimated for these strains for white mice (2).

Liquid medium used for algal growth was the synthetic medium of Gaffron (N.R.C. Medium No. 11). The bacterial contaminants were grown in nutrient broth or in pepticase corn steep medium. The latter bacteriological media were added to Gaffron medium in supplements of one per cent when necessary to favour the growth of bacteria.

Solid media for both algae and bacteria were prepared by adding two per cent agar to their respective liquid media. In preparing Gaffron solid medium it was necessary to add the chelating agent *after* autoclaving to prevent darkening of the medium.

All liquid cultures were grown on rotary shakers under fluorescent illumination of adjustable intensity. Growth on solid medium was in moist jars under similar illumination.

Nutrient broth and pepticase corn steep plates streaked with cultures of each of the strains of algae were found to be contaminated with bacteria. Incubation for two days was carried out aerobically and anaerobically, at room temperature and at 37° C.

Fifteen pure cultures of bacteria were isolated from the four algal strains on the basis of differences in colony morphology, cell morphology, sugar reactions and other biochemical tests. Nutrient broth cultures of these bacteria, incubated on a rotary shaker, were injected into mice. Each mouse (25gm.) received 1 ml. of a three-day culture intraperitoneally.

RESULTS

Eight of the 15 cultures were found to be toxic when injected into mice. The similarities among these eight toxic contaminants were more striking than the differences.

The colonies of most of them were 1 mm., bluish, translucent, with entire edge. The cells were Gram negative, motile rods, 0.5μ by 2-3μ, usually in a matrix of slime. Few sugars, usually none, were fermented, no indole or H_2S was formed, and gelatin was not liquified.

The contaminants differed in several respects. Some cultures (Table I) produced mutants, which were more opaque, slightly rough, and non-toxic for mice. Contaminants 1a and 6b failed to reduce nitrate to nitrite. Three contaminants, 1a, 6a and 6b, with more mucoid colonies, became more toxic for mice after anaerobic incubation.

Except for the three bacterial strains mentioned, toxicity was not increased by anaerobic incubation, growth in pepticase corn steep medium, or incubation at 37°C. It was observed, however, that after several subcultures were grown in broth medium, the toxicity of three contaminants decreased due to the increase in numbers of non-toxic mutants in the cultures. Mutant colonies were distinguishable on plates as they were more opaque and less smooth. In Table II the designation "O" refers to the non-toxic mutant in contrast to "T", the toxic form.

* Collected from natural waters and identified by Dr. D.A. McLarty, Dept. of Botany, University of Western Ontario.

Mice died in not less than 3 hr. and not more than 48 hr. Symptoms in nearly all cases were: partial paralysis of hind legs, difficulty in breathing, and death with convulsive twitching of legs.

TABLE I
Summary of Characteristics of Eight Toxic Bacterial Strains

Designation of Bacteria	Algae Source	Mouse Mortality	Description (Summary of Similarities and Differences)
1a	*Nostoc sp.*	0/5	**Similarities** 1. Gram neg rods, motile, 0.5 μx 2-3 μ in matrix of slime 2. Colonies bluish, translucent, entire 3. Biochemical tests (a) few sugars fermented (b) no indole, H_2S (c) gelatin not liquified **Differences** 1. Cultures 11b, 11b anaerobe 16c produced non-toxic mutants. 2. Cultures 1a, 6a and 6b increased toxicity after anaerobic incubation. 3. Cultures 1a and 6b failed to reduce nitrate to nitrite.
6a	*N. ellipsosporum*	0/5	
6b	*N. ellipsosporum*	1/5	
11b	*Anacystis montana*	5/5	
11b anaerobe	*Anacystis montana*	5/5	
16a	*M. aeruginosa* (Western)	5/5	
16c	*M. aeruginosa* (Western)	5/5	
20b	*M. aeruginosa* (Western)	5/5	

TABLE II
Mortality of White Mice Injected Intraperitoneally with Toxic and Non-toxic Mutants

	1a	6a	6b	11b O	11b T	11b anaer. O	11b anaer. T	16a	16c O	16c T	20b
Still culture	0/2	0/2	1/2	0/2	2/2	1/2	2/2	1/2	0/2	2/2	1/2
Shaken culture				0/2	2/2	0/2	2/2	2/2	2/2	2/2	2/2
Shaken culture (1st trans)				0/3	1/3	0/3	3/3	3/3	0/3	3/3	3/3
Shaken culture (2nd trans)					2/3		3/3	3/3		3/3	3/3
Anaerobe culture	3/3	1/3	2/3	0/3	2/3	0/3	3/3	3/3	1/3	3/3	3/3

O = opaque (non-toxic) T = translucent (toxic)

Isolation of algae (*M. aeruginosa* Western strain) uncontaminated with Bacteria

The four strains of blue-green algae, previously mentioned, were grown on solid Gaffron medium on stock slants as well as on streaked plates, from which isolated colonies could be selected. To prevent excessive drying during the relatively long growth period, incubation was carried out in moist jars.

Larger quantities of the four strains of algae were grown more conveniently in Erlenmeyer flasks of Gaffron medium on rotary shakers operating continuously. Medium, pH, incubation time, temperature, and

light intensity adjustments could be made as required. Although Gaffron algae medium was not a good medium for the growth of bacteria, all cultures and plates were found to be contaminated with bacteria.

To obtain at least one strain free from bacteria, *M. aeruginosa* (Western strain) was treated with ultra-violet irradiation, sonic vibration, heat, chemicals, and antibiotics.

The irradiation was carried out using a General Electric germicidal lamp with an emission peak at a wave-length of 2537° A. Agitated cultures in petri plates were treated for periods up to one hour.

Liquid cultures were subjected to sonic oscillations for periods up to 30 min. with a 10-kilocycle Raytheon oscillator (Model D.R. 101).

Supplements were added to Gaffron medium and omissions made of ingredients listed in the standard formula. Finally, mechanical micromanipulation was carried out to obtain an inoculum of purified algae.

The purified algal culture, when obtained, was centrifuged and freeze-dried and weighed doses were resuspended in distilled water. Some mouse doses were incubated at 37°C. and some were injected into mice without incubation.

Controls were freeze-dried cells of the same strain unpurified (1) grown in Gaffron medium (2) grown in Gaffron medium supplemented with one per cent pepticase corn steep. The supplement was added to favour greater multiplication of bacteria present in the algae.

RESULTS

Purification of the algal strain by such means as ultra-violet irradiation, ultrasonic vibration, heat, chemicals, and antibiotics resulted in the differential destruction of algal cells rather than an elimination of the bacteria. Besides a relatively high degree of natural resistance to these agents, some protection was afforded by sticky slime in the cultures. It has not been shown whether the bacteria constitute an actual infection of the algae. If the bacteria grow intracellularly they would be protected against the treatments outlined.

Micromanipulation of washed cells was a more successful procedure, but single-cell inocula were not enough to initiate growth. This may have been due, in part, to technical difficulties. Small inocula (10-20cells) of washed algae were transferred to each of many small Erlenmeyer flasks of fresh medium. When routine bacteriological sterility tests showed that a particular culture was sterile, or appeared to remain so for a few days, small inocula were transferred from it to fresh medium. This procedure was continued until loopfuls of the culture could be transferred to nutrient broth, incubated at room temperature and at 37°C. and show no growth of bacteria.

To obtain an algal culture which remained bacterially sterile, several hundred transfers were necessary. Phase microscopy was a convenient additional test of sterility in the later stages of purification.

To provide enough material for mouse injections, the algae were grown in one-litre Erlenmeyer flasks under the conditions noted in Table III.

The contaminated algae grown in Gaffron medium produced no killing. The same contaminated algae grown in supplemented medium, more suitable for bacterial growth, were toxic for mice, but the bacterial count was much greater.

TABLE III

Toxicity of M. *aeruginosa* (Western strain) Grown Under Illumination

(50 Foot Candles, on Rotary Shaker at pH 9.8, for 15 days. Dose 480 mg./kgm.)

Growth Medium	Freeze-dried cells	Incubation after resuspending	Bacterial counts	Mortality of mice
Gaffron	purified	none	none	0/10
Gaffron	purified	18 hrs. at 37°C.	-	0/10
Gaffron	contaminated	none	7.2×10^6	0/10
Gaffron	contaminated	18 hrs. at 37°C.	3.4×10^7	0/10
Gaffron + 1% pepticase cornsteep	grossly contaminated	none	2.4×10^8	7/10
Gaffron + 1% pepticase cornsteep	grossly contaminated	18 hrs. at 37°C.	5.0×10^9	10/10

Results similar to those reported in Table III were obtained when algae was grown under adjusted conditions. Purified algae was not toxic when grown under illumination of 50, 100, and 200 foot candles. Mice were inoculated with purified cells grown for 15 days and for 30 days, at pH 9.8 (adjusted daily) and at pH 7.0.

None of the purified algae grown under any of these conditions was toxic for mice in doses as high as 480 mg./kgm.

DISCUSSION

In the preliminary investigation of four strains of algae, bacteria toxic to mice were isolated from each strain. The toxic bacteria exhibited similarities which would indicate that they belonged to the same taxonomic group. All produced a sticky slime which interfered with the purification of the algae. The toxic factor, present in the bacterial cultures, was a "slow-acting" neurotoxin which produced neurological signs and death in not less than three hours. No evidence of a "fast-acting" toxin was found.

It has been demonstrated that *Microcystis aeruginosa* (Western strain), when freed completely from bacteria, is non-toxic for mice in doses as high as 480 mg./kgm. In order to eliminate the possibility that the purified algae would be toxic if grown under other conditions, many variations were tried but no toxicity was produced.

It is quite possible that other strains of blue-green algae are toxic in the purified state but rigid bacteriological tests should be carried out on the material injected. Before laboriously attempting to obtain complete purification in other algal strains, this investigation has shown that reduction of the numbers of bacteria to an ineffective level may be used as a preliminary screening procedure. Algae that appear toxic *per se*, eventually could be purified.

Even complete purification does not eliminate the possibility that the algal cells play a role in modifying a "slow-acting" bacterial toxin to a "fast-acting" toxin. Isolated reports of toxin capable of causing death of mice in one-half hour have appeared in the literature (1,2).

The following theories of "association" of algae and bacteria that might give both these components roles in growth and toxin production are advanced.

Contamination Theory of Toxin Production (Theory A)

Bacteria contaminate the algal cultures, remain embedded in a polysaccharide, and are therefore resistant to many agents used in attempted purification. The bacteria also have a natural resistance to these agents, greater than the resistance of the algal cells. Washing and transfer of small inocula leads to the removal of much of the polysaccharide and eventual purification of the algae.

Purified algal cells are non-toxic for mice. All of the toxicity is due to a neurotoxin produced by the bacteria. The enzymatic action of the algal cells may result in the modification of the bacterial toxin and result in the modification of the toxin and result in a faster-acting toxin, which has been described.

However, the role of the algae as producer of a "toxin-altering" enzyme has not been demonstrated experimentally. The toxicity of the bacteria has not been increased significantly by mixing bacteria with algal extract or algal cells, nor has it been increased by mixed growth of bacteria and algae.

If the algae have no such role in toxin production, they appear to play an indirect role in toxin production by supplying some metabolites which increase the population of toxin-producing bacteria. This role in promoting bacterial growth has been demonstrated experimentally by growth in Gaffron medium, which, admittedly, is not a good medium for bacterial growth.

The role of the algae *after* freeze-drying may be quite a different matter. Freeze-dried material was resuspended in water - not a growth medium. The toxicity of the purified algae did not increase when incubated. The mixed algae and bacteria produced much greater toxicity after even 18 hr. incubation. During incubation the algal cells do not multiply but the bacterial population increases. The algae or algal products constitute the only nutritive source so that it must have a role in bacterial growth. Initial population studies show that the increased bacterial population may account for the increased toxicity.

Infection Theory of Toxin Production (Theory B)

The algal cells are "infected" with bacteria in contrast to being contaminated. Polysaccharide may interfere with purification but the more important factor in purification is the *selection* of non-infected algal cells. Cultures of these non-infected cells are not toxic for mice. In the infected toxic cells the bacteria grow intracellularly but on the death of the algal cell, or during cell division free bacteria are released and are present in the culture.

This "infection" theory is supported by some of the observations made in the process of purification. Transfers of small inocula of algae to fresh flasks of medium often resulted in cultures which were negative to routine sterility tests initially but became positive after two or three weeks (by release of free bacteria). Only after many transfers was an algal culture obtained which remained bacteria-free.

Besides the difficulty in purifying algae, two observations suggest a close association. The bacteria were capable of both aerobic and anaerobic growth, but several bacterial strains were more toxic when grown anaerobically. Associated with the algae the environment could have been more anaerobic, so that the bacteria were maintained in a more toxic form.

Pathogenic bacteria, in general, are more virulent when freshly isolated from the natural host. During laboratory transfer in other media they tend to become less virulent. Five bacterial strains isolated from Anacystis and Microcystis lost their toxicity by mutation to a non-toxic form when they were cultivated *in vitro*. Growth in association with the host algae appeared to be necessary to maintain their toxigenicity.

Whether we consider the algae and bacteria in close physical contact (Theory A) or associated by way of infection (Theory B) the bacteria appear to be necessary for toxin production in *Microcystis aeruginosa* (Western strain).

Without a careful study of other strains of blue-green algae, we would be presumptuous to assume that bacteria are involved in toxin production, even though they are present in algal samples that are taken from their natural environment.

Experience with the purification of one strain of algae has shown the need of reducing the bacterial counts to such a level that they could not be involved in toxin production. If any toxicity is demonstrated under these conditions, complete purification is justified.

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