mals, aquatic animals, and exotic animal species, as well as strains grown in chemically defined media, diploid strains from a variety of species, and strains with special chromosomal configurations, biochemical markers, and virus susceptibilities, will be placed in the repository within the next year. Investigators who wish to submit or suggest cell strains to the collection in these or other categories are requested to write to the chairman of the committee.


### References and Notes

1. The committee was called together by the late J. T. Syverton under grant auspices and by authorization of the former Viruses and Cancer Panel, National Advisory Council, National Cancer Institute, after conferences held at the National Cancer Institute, 15 May 1959, and the Rockefeller Institute, 3 June 1959.
2. Laboratories currently participating in the program are: Child Research Center of Michigan, Detroit (C. S. Stulberg); South Jersey Medical Research Foundation, Camden (L. L. Coriell); Naval Biological Laboratory, University of California, Berkeley (S. H. Madin); and Cell Repository, American Type Culture Collection, Rockville, Md. (J. E. Shannon).
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27 May 1964


## A System for Studying Microbial Morphogenesis: Rapid Formation of Microcysts in Myxococcus xanthus


Abstract. *A method has been found for inducing the rapid, quantitative, and relatively synchronous conversion of vegetative rods of a fruiting myxobacterium to microcysts. The conversion is induced by the addition of 0.5*M *glycerol to a dispersed, growing, liquid culture of* Myxococcus xanthus. *The vegetative rods are converted to microcysts in about 120 minutes.*


Part of the life cycle of the fruiting myxobacterium, *Myxococcus xanthus*, involves the conversion of vegetative rods to round refractile cells called microcysts (*1*). Adye and Powelson (*2*) were the first workers to describe a method for inducing this conversion under relatively defined conditions in liquid medium. Under these conditions, however, the conversion is highly asynchronous and requires about 3 days.

As part of our interest in describing this conversion in biochemical terms, we have sought a method for carrying out the conversion in liquid medium rapidly, quantitatively, and synchronously. We need not elaborate on the value of such a system for examining microbial morphogenesis. This report describes such a technique.

*Myxococcus xanthus*, strain FB, was grown at 30°C with shaking in liquid CT medium (Bacto-Casitone, 2 percent; $MgSO_4$, 0.008*M*; $K_2HPO_4$–$KH_2PO_4$, 0.01*M*, *p*H 7.6). Cells from the exponential phase of growth were centrifuged at 3°C; washed once with cold 1 percent Casitone containing 0.008*M* $MgSO_4$; inoculated at a cell density of about $3 \times 10^8$ cells per milliliter (200 Klett units with a No. 54 filter) into a liquid medium containing 1 percent Casitone, 0.008*M* $MgSO_4$, and 0.5*M* glycerol; incubated at 30°C. The presence of glycerol caused the conversion of the vegetative rods to microcysts.

Figure 1 illustrates the sequence of events during a typical experiment. There is invariably a sharp increase in turbidity immediately after addition of glycerol. Within 1 minute the turbidity returns to the original value or goes below it. [Mager *et al.* (*3*) have described similar changes in optical density resulting from the addition of solutes to cultures of a variety of gram-negative bacteria.] For the first 35 minutes there is no visible morphological change. Then, as indicated in Fig. 1, the cells convert to short rods, ovoids, nonrefractile spheres, and refractile spheres in sequence. The entire conversion requires about 120 minutes and is relatively synchronous. Total cell counts during this period indicate no decrease in cell numbers.

Factors which affect the conversion are as follows: (i) Aeration is required (20 ml of medium in a 250-ml erlenmeyer flask subjected to about 250 strokes per minute on a shaker is sufficient). (ii) $MgSO_4$ is required, while 0.01*M* potassium partially inhibits the conversion. (iii) The conversion will take place when Casitone is replaced by the amino acid mixture which supports growth of *M. xanthus* (*4*). Under these conditions, however, the process requires about 5 hours and is highly asynchronous. (iv) Exponentially growing cells convert much more satisfactorily than lag or stationary phase cells.

Microcyst formation may also be induced by the addition of 0.5*M* glycerol directly to the growth medium during

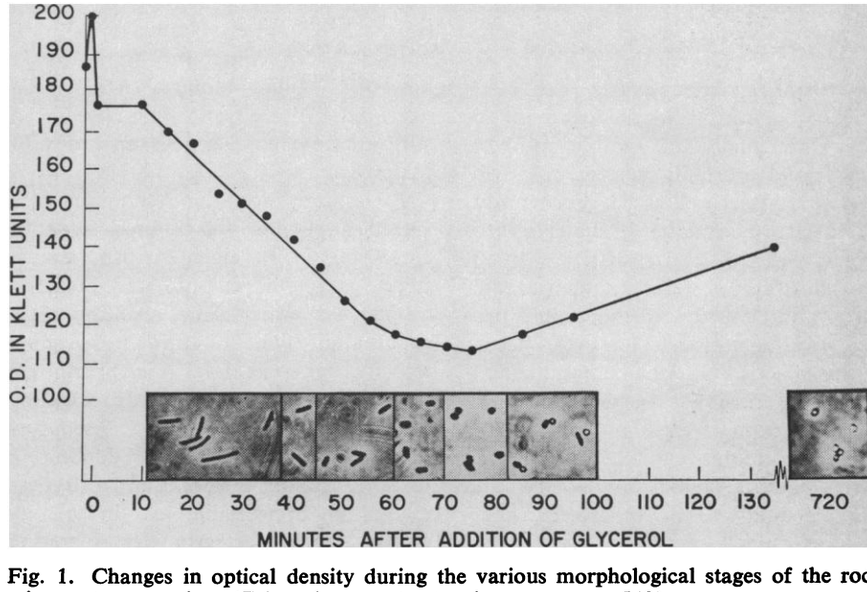


Fig. 1. Changes in optical density during the various morphological stages of the rod-microcyst conversion (Zeiss phase-contrast microscope, × 540).

exponential growth. However, the conversion under these conditions is neither as rapid nor as synchronous as it is under the conditions already described. Furthermore, although glycerol gives optimum results, 0.012*M* phenethyl alcohol, under the same conditions, also induces formation of microcysts.

While the refractile spheres produced by the glycerol technique are microcysts from a morphological point of view and are capable of almost 100 percent germination efficiency, other parameters indicate that, physiologically, they are not mature microcysts for at least an additional 12 hours; for example, the ability to respire on Casitone does not disappear until about 12 hours have passed.

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5. Supported by NSF grant GB-9 of the Developmental Biology Program and a PHS career development award (I-K3-GM-5869-10) to M.D.

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## Ostracitoxin: An Ichthyotoxic Stress Secretion of the Boxfish, Ostracion lentiginosus

Abstract. *Boxfish under stress produce an ichthyotoxic, hemolytic, nonprotein poison in the mucous secretions of their skin. This heat-stable, nondialyzable "ostracitoxin" foams profusely in aqueous solutions and is toxic to various biological systems. It is apparently unique among known fish poisons; it is toxic to boxfish and resembles red tide and sea cucumber toxins in general properties.*

The boxfish, *Ostracion lentiginosus*, is a member of the trunkfish family (Ostraciontidae), a group of tropical marine teleosts characterized by a rigid dermal carapace encasing the body. Trunkfishes are often confused with their close relatives, the puffer fishes (*1*), but, whereas the poisonous nature of puffers is well known (*2*), information on the toxic nature of trunkfishes is sketchy.

It has been suspected that trunkfishes under stress exude a substance poisonous to other fishes (*3, 4*). Introduction of newly captured, highly excited trunkfish into aquaria with other fishes often results in the death of all other fish inhabitants within minutes. Such rapid mortality cannot be attributed to anoxia, and it has been postulated that a poisonous substance is produced by distressed trunkfish.

Boxfish out of water secreted a copious, watery mucus which foamed profusely on agitation. The toxic nature of this mucus was convincingly demonstrated by rinsing skin secretions into the aquarium water of other reef fishes. Symptoms exhibited by fish so exposed were initial irritability and gasping, followed by quiescence characterized by decrease in the rate of opercular movements; loss of equilibrium and locomotion and sporadic convulsions and death. No recovery occurred after the initial symptoms appeared.

Mucous secretions of the skin of distressed boxfish were collected by placing newly captured, living boxfish into small containers, adding 10 to 50 ml of a distilled water rinse, and swirling the fish around for five minutes. Such stress caused the boxfish to secrete a foamy mucus which was collected in the rinse water. A standard bioassay was established by adding a portion of the total rinse volume to 100 ml of sea water containing four to six newborn sailfin mollies, *Mollienesia latipinna*, 10 to 12 mm long. The mean survival time was used as an index of toxicity.

Preliminary isolation techniques (*5*) included pooling the filtered aqueous rinses of six to eight boxfish, centrifuging at 16,000 rev/min, and dialyzing against tap water. The resulting clear supernatant fluid retained its original toxicity, whereas both the precipitate and dialyzate were nontoxic. Heating the toxic supernatant fluid caused further precipitation but no reduction in toxicity. Boiling the solution to dryness caused no appreciable loss in toxicity of the residue, clearly demonstrating the nonprotein nature of the poison.

The crude toxic residue was soluble in water, methanol, ethanol, acetone, and chloroform, but insoluble in diethyl ether and benzene. It was stable in acid (*p*H 2.0) and mildly basic (*p*H 11.0) solutions, but was rapidly detoxified upon excess addition of a strong base (KOH). Before heating, the toxicity of fresh secretions gradually decreased at refrigeration temperatures, while boiled solutions could be maintained for months at these temperatures with little loss in toxicity, indicating that bacterial detoxification was occurring.

Repeated extraction of the dried residue with acetone or chloroform and diethyl ether resulted in a particulate substance which formed stable foams in aqueous solutions and was toxic to the assay fish at concentrations of 1 : 1,000,000. Approximately 50 to 100 mg of this crude toxin could be obtained at one time from the skin secretions of a single adult boxfish (*6*). The toxic principle of these secretions will be referred to as "ostracitoxin," using the generic (or familial) name of the boxfish in accordance with the naming of other toxins such as tetrodotoxin-tarichatoxin, bufotoxin, and holothurin.

Aqueous and ethanolic extracts of the skin, viscera, and muscles of freshly killed boxfish were, surprisingly, nontoxic. Ostracitoxin was detected only in the epidermal mucous secretions of living, distressed boxfish. This is in sharp contrast with tetrodotoxin, which is found in the skin and viscera of many species of puffer fish and was only recently found in large amounts in the skin secretions of a few puffers (*7, 8*). The boxfish paradox suggests that ostracitoxin is "activated" during secretion. The susceptibility of the boxfish to its own toxin also supports this hypothesis: intramuscular injections of fresh mucous secretions caused almost immediate loss of balance, and death occurred within a few minutes. And although boxfish are much more resistant to ostracitoxin in solution than are other fishes, they become moribund when left in sea water with a high concentration of ostracitoxin (*9*). Puffer fishes, on the contrary, are immune to tetrodotoxin.

A survey of the effects of ostracitoxin solutions on various biological systems indicated that ostracitoxin has high biologic activity. For example, ostracitoxin caused desensitization of sea anenome and hydroid tentacles, inhibit-