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MARINE CHITIN-DECOMPOSING BACTERIA*

By

CHARLES W. HOCK

Woods Hole Oceanographic Institution,
Woods Hole, Mass.

Chitin is found in various members of the plant and animal kingdoms. It forms the main skeletal material of the Insecta and Crustacea in the animal kingdom, and is present also in tissues of fungi. Chitin has an elementary composition of $C_{32}H_{54}O_{21}N_6$ (*6*), On hydrolysis with concentrated acid it yields glucosamine and acetic acid in proportions which indicate that there is an acetyl group for each glucosamine residue. It is a tough leathery substance, insoluble in water, in concentrated alkalis, and in the common organic solvents. No essential differences have been found in the chitin from animal and vegetable sources (*4*, *8*).

In the course of a study of the decomposition of chitinous material in natural marine media, a number of bacteria capable of decomposing chitin were isolated from marine sand, mud, and water (*7*). Chitin-decomposing bacteria were isolated also from the intestinal contents of several common marine animals. The following methods were used in the isolation of the bacteria from their respective habitats.

Purified chitin was prepared from horseshoe crab shells according to the procedure of Benton (*2*), and ZoBell and Rittenberg (*11*). The shells were treated successively for prolonged periods with 1 percent hydrochloric acid, 2 percent potassium hydroxide, and 95 percent

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ethyl alcohol. The snowy white material which remained reacted positively to simple qualitative tests for chitin (*3*).

In preparing liquid culture media, 1 by 5 cm. strips of the purified chitin were placed in test tubes, and partly covered with sea water, or with sea water containing additional nutrients (Fig. 12). The medium best suited for the detection of chitin-decomposing bacteria

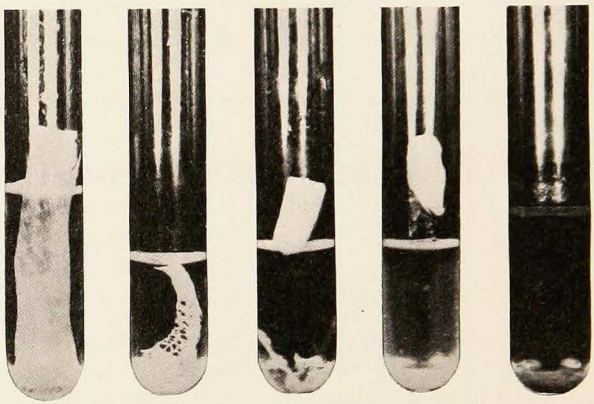

Figure 12. Decomposition of purified chitin in liquid culture medium. The tube on the left is the control; the other tubes show the chitin in various stages of breakdown.

consisted of a strip of chitin in a solution used by Waksman (*10*). This basic medium had the following composition:

glucose	1 gram
peptone	1 gram
K_2HPO_4	0.05 gram
Sea water (filtered)	1000 ml

By elimination and by substitution in this basic medium, the metabolic requirements of the bacteria were studied.

Solid media were prepared from the liquid culture media either by the addition of 15 grams of agar to each liter of solution or by diffusion of the nutrient solutions through silica gels. Where solid media were employed strips of sterile purified chitin were placed on the surface of the hardened gel (Fig. 13). The silica gel plates were prepared under as nearly aseptic conditions as possible; all other media were sterilized by autoclaving for 20 minutes at 120° C.

One additional solid medium was found useful for the rapid detection of chitin-decomposing bacteria. It was prepared by uniformly

dispersing chitin in agar according to the following procedure. Fifteen grams of purified chitin were added to 150 ml. of 1 : 1 sulfuric acid and kept overnight in the refrigerator. The chitin was dissolved by this treatment but analyses showed that it was not hydrolyzed. The following day the chitin was precipitated by the addition of approximately 140 ml. of 20 percent potassium hydroxide. The precipitate was placed on a Büchner funnel and washed repeatedly with distilled water until the filtrate was neutral to litmus. Thirty-five grams of the chitin, as removed from the filter, were then added to the basic liquid

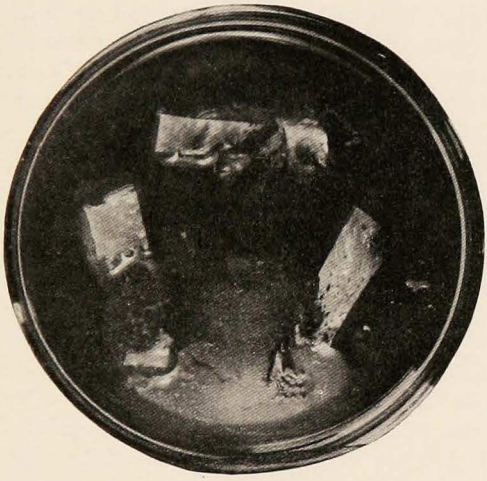

Figure 13. Breakdown of strips of purified chitin on the surface of a silica gel plate inoculated with a sample of marine mud.

medium. After autoclaving, the agar was thoroughly shaken to redistribute the chitin, and Petri plates were poured. In this condition the medium was of a uniform milky-white opacity. When chitin-decomposing bacteria grew on the surface of this agar they dissolved the chitin, thereby producing a clear halo around each colony (Fig. 14). By noting the length of time necessary for the appearance of halos the rates of bacterial activity could be estimated. Bacteria which decomposed chitin rapidly often produced a halo after two days' growth whereas others required eight to ten days for a positive test. Although bacteria which were unable to decompose chitin often grew well on the medium, they did not alter it in appearance (Fig. 14).

The media were inoculated with sea water, marine sand or mud, a piece of decomposing horseshoe crab shell, or with a loopful of ma-

terial from the intestinal tract of a fish or other marine animal. In order to test a given sample for chitin-decomposing bacteria, several different media were inoculated. These were incubated at room temperature and examined periodically for evidence of chitin breakdown. Growth was slow in the medium consisting of only chitin in sea water, whereas the same bacteria decomposed the chitin more rapidly when additional nutrients were present.

In preliminary work cultures of bacteria which showed no evidence of chitin-decomposition were kept one year before being discarded as

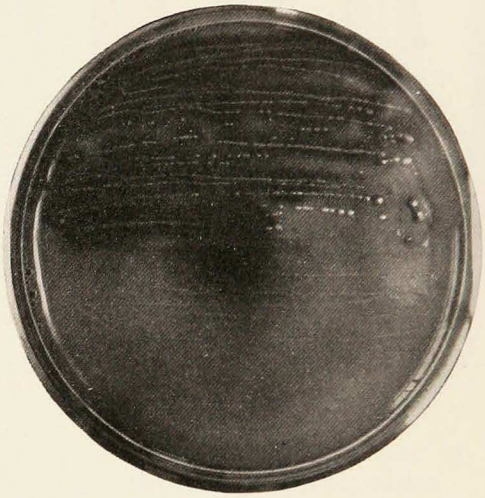

Figure 14. Growth of marine bacteria on agar containing precipitated chitin. Upper half inoculated with a pure culture of chitin decomposing bacteria: lower half inoculated with a culture incapable of attacking chitin.

negative. It became evident, however, that several weeks were adequate for detecting the chitin-decomposing capacity of most species. After bacterial development took place a second tube of enriched medium was inoculated with a loopful of material from an original positive culture. Growth from the second tube was then streaked either on nutrient agar plates or on agar medium containing precipitated chitin. In either case the colonies which developed were again retested for their ability to attack chitin. In this way pure cultures of the bacteria were obtained.

Visible dissolution of the chitin, either in liquid medium or on a solid agar surface was indisputable evidence that the chitin was being attacked. To detect incipient decomposition a hand lens was helpful.

During advanced stages of decomposition the shell was frequently observed to be "eaten" away in spots. The liberation of ammonia from media lacking peptone or nitrate, reducing substances from media lacking glucose, or a pronounced change in acidity, were further reliable evidence of chitin-decomposition.

The chitin-decomposing bacteria isolated from the sea were found to be Gram negative, asporogenous, motile rods. Among the readily demonstrable products resulting from the decomposition of purified chitin by pure cultures of the bacteria were ammonia, reducing substances, and organic acids. All of the bacteria liquefied gelatin rapidly, but failed to digest cellulose even after incubation for many weeks. Most of the bacteria were unable to hydrolyze starch: about half of them were able to reduce nitrates, but none were able to produce hydrogen sulfide. Likewise, the various cultures of bacteria differed in their ability to ferment simple sugars and alcohols. Some of the bacteria produced pigments while others did not. They also varied in the rapidity of decomposition of chitin. None of the cultures was able to grow in fresh water media. No attempt was made, however, to acclimate the bacteria by reducing the salt content of the media gradually with each successive transfer.

A description of two types of bacteria, selected as representing the marine chitin-decomposing bacteria isolated in the present investigation, is reported here. Five strains of the first type, *Bacterium chitinophilum* are described. These strains vary in their ability of reducing nitrate and of utilizing several sugars and other carbohydrates, but appear to be sufficiently similar to warrant placing them together as one type. From the description given by Benecke (*1*), his *Bacillus chitinovorous* might be identical with either Strain I, II, or III. Rammelberg (*9*) and Folpmers (*5*) each isolated a bacterium, the former from harbor water, the latter from the soil, which apparently correspond to the one described by Benecke. Benton (*2*) described 17 types of chitin-decomposing bacteria, but was unable to identify any of these with *B. chitinovorous*. Two of her groups, according to the description given, could be identical with Strain I or Strain IV of *B. chitinophilum*. Except for color, Strain IV is identical with one of 14 types described by ZoBell (*11*). *Bacterium chitinochroma*, the second type isolated in this study, is represented by a single strain.

Bacterium chitinophilum, n. sp.

Short rods, .35 to .65 by .95 to 1.5 microns in size. Gram negative. Motile.

Basic agar plate: colonies circular, smooth, entire, raised, white.

TABLE I

Name	*Average Size (microns)*	*Color on Chitin Medium*	*Gram Stain*	*Motility*	*Nitrate Reduction*	*Starch Hydrolysis*	*Gelatin Liquefaction*	*H_2S Production*	*Cellulose Digestion*	*Dextrose*	*Sucrose*	*Lactose*	*Glycerol*	*Mannitol*	*Sources*
Bacterium chitinophilum *Strain I*	0.5 × 1.2	White	−	+	+	−	+	−	−	+	+	−	+	+	Mud, intestines of horseshoe crab and dogfish.
Strain II	0.5 × 1.4	White	−	+	+	−	+	−	−	+	+	+	+	+	Water, mud, intestines of puffer.
Strain III	0.5 × 1.2	White	−	+	+	−	+	−	−	+	+	−	−	−	Water, sand, mud.
Strain IV	0.5 × 1.2	White	−	+	+	−	+	−	−	+	−	−	+	+	Intestines of quahaug and sand crab
Strain V	0.5 × 1.0	White	−	+	−	−	+	−	−	+	+	−	−	+	Mud, water.
Bacterium chitinochroma	0.5 × 1.2	Yellow	−	+	−	+	+	−	−	+	+	−	−	−	Sand, water, intestines of squid.

Basic liquid medium: moderate growth, sometimes with formation of ring or pellicle. Scant granular sediment.

Sea water gelatin: liquefaction, growth absent in stab but abundant in liquefied zone.

Does not hydrolyze starch nor produce hydrogen sulfide. Four of five strains reduce nitrates. The strains vary in their ability to ferment sugars. All are unable to digest cellulose.

Aerobic.

Optimum temperature 20° C.

Habitat: Common in marine sand, mud and water. Also isolated from the shell of a decomposing horseshoe crab, *Limulus polyphemus*, and from the intestinal tract of *Venus mercenaria mercenaria*, *Ovalipes ocellatus ocellatus*, *Mustelus mustelus* and *Spheroides maculatus*.

Bacterium chitinochroma, n. sp.

Short rods, .45 to .75 by .90 to 1.4 microns in size. Gram negative. Motile.

Basic agar plate: colonies circular, smooth, entire, raised, varying in color from lemon to deep orange.

Basic liquid medium: abundant growth with production of pellicle. Scant granular sediment increasing with age of culture.

Sea water gelatin: active liquefaction, no growth in stab, but thick bright yellow growth throughout the liquefied zone.

Does not reduce nitrates: hydrolyzes starch: does not produce hydrogen sulfide, ferments dextrose and sucrose, but not lactose, glycerol and mannitol. Does not digest cellulose.

Aerobic.

Optimum temperature 20° C.

Habitat: marine sand, mud, and water. Also isolated from the intestinal tract of the squid, *Loligo pealeii pealeii*. Common occurrence.

The characteristics of the bacteria are summarized in Table I.

SUMMARY

Bacteria capable of decomposing chitin were isolated from sea water, marine sand and mud, and the intestinal contents of several marine animals.

Two species of marine chitin-decomposing bacteria are described. All of the bacteria are Gram negative, asporogenous, motile, short rods. Ammonia, reducing substances, and organic acids are readily demonstrable products resulting from the decomposition of purified chitin by pure cultures of the bacteria.

These bacteria decompose chitin not only in purified preparations but also in natural chitinous material such as horseshoe crab shell.

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