THE OCCURRENCE AND CHARACTERISTICS OF CHITINOCLASTIC BACTERIA IN THE SEA

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Chitin is a tough leathery insoluble substance of indeterminate chemical structure somewhat resembling cellulose. It is generally believed to be a polymer of glucosamine in which each amino group is acetylated. Chitin is the chief constituent of the exoskeleton of Arthropods and it occurs in some Mollusks, Coelenterates and Protozoa as well as in certain fungi. There may be more than one kind of chitin but the observations of Diehl (1936), van Iterson *et al.* (1936) and others indicate that animal and fungoid chitins are identical.

Large quantities of chitin are produced in the oceans of the world each year. From data given by Johnstone (1908) on the abundance of Copepods and their chitin content it is estimated that just this one sub-class of planktonic crustacea, some of which form 10 to 12 chitinous casts in their development stages, produces several billion tons of chitin annually. Most of this is probably utilized by biological agents because little accumulates in marine sediments and moreover, if it were not decomposed, it would soon become a serious drain upon carbon and nitrogen in the cycle of these elements.

The fragmentary and contradictory literature on the subject fails to indicate to what extent chitin may be utilized as a source of food by animals which may ingest it. Some workers claim to have detected chitinase in the alimentary tract of certain animals while other investigators find no evidence that animals can digest chitin. However, the possibility of symbiotic bacteria aiding animals in the digestion of chitin should not be overlooked.

Bacteria are probably responsible for the disintegration of much chitin in the sea. Bertel (1935), Bucherer (1935), Waksman *et al.* (1933), ZoBell and Anderson (1936) and others have reported chitin digestion by mixed cultures from marine sources but have not described the bacteria involved. These observations, together with those of Stuart (1936) on the occurrence of halophilic chitinovorous bacteria in marine salt from Africa, S. America, Spain, California and the West Indies, indicate a worldwide distribution of chitin-digesting bacteria in the sea. It is doubtful if *Bacillus chitinovorus* which Benecke (1905) isolated from Kiel harbor, where there is much terrigenous contamination and dilution of the harbor water, is a true marine species, particularly in view of the fact that Rammelberg (1931) isolated an identical organism from soil. Chitinovors from terrestrial sources have been reported by Rammelberg (1931), Folpmers (1921), Jensen (1932), Johnson (1932) and others.

This paper is concerned primarily with the demonstration of chitinoclastic bacteria in the sea and a study of their physiological characteristics. The term *chitinoclastic* is applied to bacteria which in any way attack the chitin molecule. It includes the so-called *chitinovors* besides others revealed by these studies which split certain radicals or side-chains from the chitin molecule although they do not *devour* chitin, either in the sense of deriving nutrients from it or causing its dissolution.

EXPERIMENTAL METHODS

Chitinoclastic bacteria were detected by inoculating chitin medium with samples of raw sea water, bottom sediments and other marine materials. The chitin medium was prepared by partly covering 1 x 5 cm. strips of purified chitin in test tubes with sea water and sterilizing at 124°C. for 20 minutes. The chitin strips were prepared from lobster shells by methods similar to those used by Benton (1935). After successive prolonged treatments with 1 per cent hydrochloric acid, 2 per cent potassium hydroxide, several changes of boiling alcohol and finally water, the chitin was colorless and reacted positively to the tests for chitin listed by Buchanan and Fulmer (1928). Simple

nitrogen and carbon compounds were added to some of the media as sources of readily available nutrients. All media were prepared with sea water.

The inoculated media were incubated at 21°C. and examined periodically for evidence of chitinoclastic activity. The visible dissolution of chitin, the liberation of ammonia, acids or reducing sugars from chitin, or the growth of bacteria in the medium consisting of only chitin and sea water were used as criteria of chitinoclastic activity. In some of the inoculated media such activity became manifest within two or three days but, in others, not until the cultures had been incubated five or six months. In the preliminary work all cultures were held at least six months before being discarded as negative. It was found that two months' incubation was adequate to detect most chitinoclastic species.

Pure cultures of chitinoclastic bacteria were obtained by inoculating a second tube of chitin medium with a loopful of material from the positive primary raw cultures. Growth from the enriched cultures was then streaked on nutrient agar plates. The different types of colonies which developed were isolated, retested for their ability to digest chitin and checked for purity by examining stained smears and colony characteristics. About one-fourth of the pure cultures thus isolated proved to be chitinoclastic.

OCCURRENCE IN THE SEA

In preliminary surveys, chitinoclastic bacteria were demonstrated in nearly all 5- to 6-gram portions of bottom sediments which were inoculated into chitin media. Included in the survey were several sediment samples collected from the beach or shallow water along the coast of southern California. Many others were collected at stations occupied by the boat SCRIPPS, some of which were nearly 200 miles from the mainland and from water depths as great as 2000 meters.

Estimating their relative abundance by the minimum dilution method, chitinoclastic bacteria were found to be very unevenly distributed in bottom sediments. For example, a given sample may contain several hundred chitinoclasts per gram while in a similar sample immediately adjacent there may be less than

one per gram. This lack of uniformity in the distribution of bacteria within a limited area has been discussed by ZoBell and Anderson (1936) who attribute it to the unevenness in the distribution of nutrient particles and to the tendency of bacteria to colonize.

Chitinoclasts are most numerous in the topmost layers of mud, where as many as a thousand per gram have been found. The number decreases sharply with core depth although some have been recovered from the bottom of mud cores exceeding 60 cm. in length. No relationships were noted between the abundance of chitinoclastic bacteria in sediments and the depth of the overlying water or the distance from the mainland. The largest chitinoclastic populations were usually associated with coarser sedimentary materials like sand, with which the chitinous particles are concentrated by the assorting action of the forces of sedimentation.

Between 0.1 and 1.0 per cent of the bacteria found in sea water are chitinoclastic to some degree, the total bacterial population of the topmost 25 to 50 meters of water being from a few to a few thousand per cubic centimeter. Below this depth the number of bacteria in the water drops off sharply to 200 meters, below which, only occasionally, can bacteria be demonstrated at all except in the immediate proximity of the bottom. Following initial isolation most of the bacteria will grow in sea water media but not in corresponding fresh-water media which, together with the fact that they have been isolated at places remote from possibilities of terrestrial contamination, is regarded as evidence that they are species which are indigenous to a marine environment.

The richest source of chitinoclastic bacteria is the remains of decomposing crabs, lobsters or other crustacea found on the bottom or along the sea shore. Thirteen different kinds of chitinoclastic bacteria have been isolated from this source. Johnson (1932) isolated several kinds of chitin-destroying bacteria from the shells of fresh-water crabs undergoing decomposition, and recently Hess (1937) recovered several strains of chitin digesters from living lobsters having a shell disease.

From a hundred to more than a thousand chitinoclastic bacteria per cubic centimeter were found in the stomach contents of squid and other cephalopods which ingest chitinous food. This suggests the possibility that such bacteria may play an important rôle as symbionts which aid animals in the digestion of chitin. Benton (1935) recovered chitinoclastic bacteria from the intestines of fishes, frogs and bats.

Chitinoclastic activity was exhibited by 12 of the 85 pure cultures which constitute the Scripps Institution stock collection and which are believed to be representative of the aerobic heterotrophs inhabiting the sea. The cultures have been isolated at random during a period of several years from various marine materials without deference to any special physiological function. This observation gives further evidence of the widespread distribution of chitinoclastic bacteria in the sea, and since the cultures have been cultivated for several years in the absence of chitin, it shows that chitinoclastic ability is not limited to bacteria which have been cultivated in the presence of chitin.

CHARACTERISTICS OF MARINE CHITINOCLASTS

To date, 31 different pure cultures of chitinoclastic bacteria have been isolated from various marine materials. Several were isolated from two or more sources thereby indicating a widespread distribution of these species. While all of them are believed to be new and undescribed species, they have not been studied completely enough to warrant classifying them as such, and even if all of the important characteristics had been determined, the authors would hesitate to assign generic names to the organisms in the face of the present chaotic condition of systematic bacteriology.

The problem is further complicated by the fact that nearly all of these bacteria grow either preferentially or exclusively in sea water media. Therefore in characterizing the bacteria it is usually necessary to deviate from Standard Method procedures to the extent of substituting sea water for fresh water in the differential media. Few of the marine species show any growth on the conventional potato and milk media, which are still widely

used to characterize bacteria in spite of their highly variable composition. Following prolonged laboratory cultivation or acclimatization procedures in which the salt content of the media is gradually reduced with each successive transfer, most of the cultures slowly develop the ability to grow in fresh-water media but it is not known how many other characteristics are changed during the adaptation process. It has been noted that the tendency to produce pigment is lessened, the temperature range of growth is widened and the ability to liquefy agar is lost during such acclimatization.

Table 1 gives the morphological and physiological characteristics of 14 representative chitinoclastic bacteria most of which have been recovered two or more times from various marine materials. Gram-negative, slender to ovoid rods varying only slightly in size and shape predominate. Only one chitinoclastic coccus and two species of vibrio have been found to date. Neither staining procedures nor thermotolerance tests indicated endospore formation in any of the cultures. Flagellation was not determined, but all except four of the 31 cultures were found to be actively motile. Encapsulation is a common property of the chitinoclasts but one which is highly variable under different conditions of cultivation. Involutionary forms appeared in most of the cultures, their abundance increasing with the age of the cultures. Chains of four or more cells were noted in none of the cultures and pairs occurred only infrequently.

In view of the structural similarity of chitin and cellulose it is noteworthy that none of the chitinoclastic bacteria digest cellulose. With few exceptions they are only feebly saccharolytic, nearly half of them failing to ferment any of the simple sugars and none forming gas from carbohydrates. Although chitin is probably a glucosamine, many of the chitinoclasts are unable to utilize glucose. Interestingly enough, some of them which attack neither glucose, maltose, sucrose nor lactose hydrolyze starch with acid production. Most of the pure cultures are actively proteolytic as indicated by their ability to liquefy gelatin and to produce ammonia from various proteinaceous substrates.

The chitinoclasts differ greatly in their nitrogen and carbon or

TABLE 1

Morphological and physiological characteristics of sixteen representative marine chitinoclastic bacteria as determined in sea water media

CULTURE NUMBER	AVERAGE SIZE	GRAM STAIN	MOTILITY	PIGMENTATION ON CHITIN MEDIUM	GLUCOSE	LACTOSE	SUCROSE	GLYCEROL	MANNITOL	STARCH HYDROLYSIS	CELLULOSE DIGESTION	GELATIN LIQUEFACTION	H_2S PRODUCTION	AMMONIA UTILIZATION	ACETATE UTILIZATION	NITRATE REDUCTION	MATERIAL FROM WHICH ISOLATED*
	microns																
2	2.1 x 0.6	−	−	Yellow	A	−	−	−	−	+	−	+	+	+	+	−	3, 5
3	3.2 x 1.3	−	−	None	−	−	A	−	−	+	−	+	−	+	+	−	2, 3
6	2.4 x 1.0	−	−	None	A	−	A	A	A	+	−	+	+	+	+	+	1, 2, 3, 4, 5
18	1.5 x 0.4	−	−	Yellow	−	−	−	−	−	+	−	+	+	+	+	−	2, 3, 4, 5
24	1.2 x 0.4	−	−	None	−	−	−	A	−	+	−	−	−	−	−	+	2, 3
29	3.0 x 0.6	−	−	Orange	−	−	A	−	−	−	−	+	+	+	+	−	1, 2
32	3.2 x 1.4	−	−	None	A	−	A	−	A	+	−	+	−	+	+	−	1, 3, 5
34	1.7 x 0.9	−	−	Yellow	−	A	−	−	−	+	−	+	−	+	+	−	1, 4
37	2.0 x 0.5	−	−	Pink	−	−	A	−	−	−	−	+	−	+	+	+	4, 5
47	0.8 x 0.6	−	−	Brown	A	−	−	A	A	−	−	+	−	+	+	+	3, 4, 5
50	1.4 x 1.1	−	−	Yellow	−	−	−	−	−	−	−	−	+	+	+	−	2, 3, 4
71	2.5 x 0.5	−	−	None	A	−	−	−	−	+	−	−	+	−	−	−	1, 2, 3, 4, 5
75	3.9 x 0.4	−	−	Brown	−	−	−	−	−	+	−	+	−	+	+	−	3
77	2.9 x 0.6	−	−	Brown	−	−	A	−	−	−	−	+	−	+	+	+	4, 5

* 1 = Bottom sediment; 2 = Crustacea; 3 = Squid stomach; 4 = Sea water; 5 = Tide pools.

A = Acid production.

energy requirements as well as in their action on chitin. Some multiply freely in a medium consisting of only pure chitin in sea water. They may not otherwise detectably effect the chitin, they may dissolve it, or their growth may be accompanied by the liberation of ammonia (table 2) or other decomposition products. Another group of chitinoclasts can derive their nitrogen from chitin when an available carbon or energy source such as the salt of an organic acid or a simple carbohydrate is added, after which some of them may merely multiply without otherwise detectably effecting the chitin while others cause its dissolution. A third

TABLE 2

Liberation of ammonia from chitin medium by representative chitinoclastic bacteria after varying periods of incubation at 21°C. and the elapsed time before there was any visible disintegration of the chitin

CULTURE NUMBER	AMMONIA REACTION AFTER				CHITIN DISINTEGRATION VISIBLE AFTER
	2 days	4 days	9 days	16 days	
5	−	−	++	+++	None after 5 months
6	−	−	+	++	28 days
8	−	+	+	+++	9 days
9	−	−	+	++	None after 5 months
10	−	+	++	+++	18 days
18	−	+	++	+++	19 days
28	−	−	+	+++	13 days
37	−	+	++	+++	34 days
63	+	++	+++	++++	16 days
Control	−	−	−	−	None after 5 months

type has been isolated from the sea which attacks chitin only in the presence of both carbon and nitrogen nutrients such as peptone. Further studies must determine whether the soluble decomposition products are inadequate for the carbon and nitrogen nutrition of such cultures or if they produce a chitinase only after multiplying for some time in the presence of chitin. More credence is placed in the former explanation in view of the fact that some of the stock cultures referred to above which have been cultivated on nutrient agar for several years started to attack the chitin within two or three days after being transferred to a chitin medium.

Observations on certain mixed cultures indicate that there are also symbiotic relationships in which two or more bacteria together can attack chitin although neither alone is endowed with this property. The investigation has revealed no obligate chitinovors, or bacteria which require chitin. The presence of peptone and other simple nutrients in chitin medium accelerates the chitinoclastic activity of most bacteria probably due to the growth-promoting properties of the peptone.

No pure cultures have been observed which can obtain their energy and carbon but not their nitrogen requirements from

TABLE 3

pH of sea water-chitin medium inoculated with chitinoclastic bacteria after different periods of incubation at 21°C. as measured with a glass electrode and Beckman pH meter

CULTURE NUMBER	INITIAL pH	pH OF MEDIUM AFTER				CHITIN DISINTEGRATION VISIBLE AFTER
		4 days	8 days	16 days	33 days	
8	7.5	7.3	6.9	6.7*	5.5	9 days
14	7.6	7.6	7.2	6.7	7.0	None after 5 months
17	7.6	7.3	7.0	6.4*	5.3	11 days
18	7.5	7.5	7.4	7.6	7.8	19 days
28	7.5	7.3	6.9	5.9*	5.0	13 days
37	7.5	7.3	7.0	6.6	6.0*	28 days
56	7.5	7.7	7.5	7.6	7.7	33 days
63	7.5	7.5	7.4	7.6	7.9	16 days
Control	7.6	7.6	7.7	7.7	7.6	None after 5 months

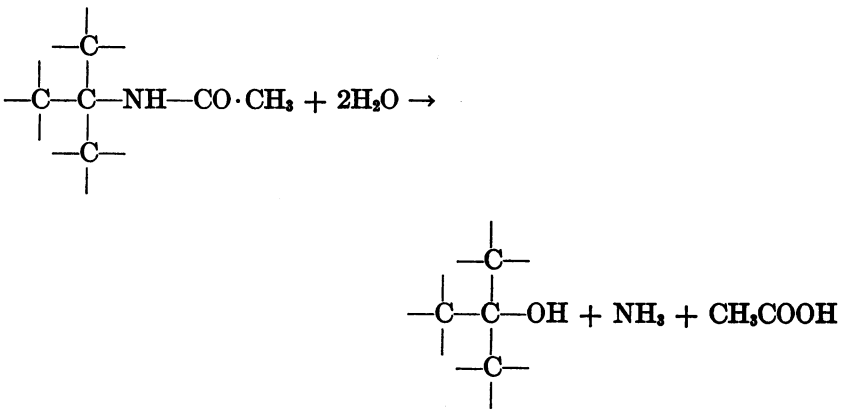
* Tests indicate the presence of acetic acid.

chitin. This is attributed to the vulnerability of the amino group to hydrolysis in the chitin molecule and to the fact that most of the chitinoclasts utilize ammonia nitrogen (table 1). Nearly all of the bacteria which attack chitin liberate an excess of ammonia therefrom. The data in table 2 show that the ammonia reaction becomes positive before there is any physical evidence that the chitin is being attacked by such bacteria; and some cultures never dissolve the chitin strips although ammonia is liberated in abundance.

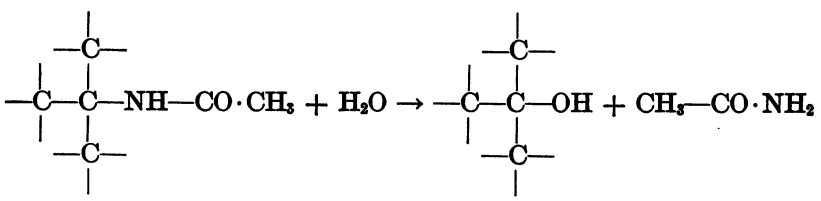
Some chitinoclasts produce enough acid from chitin to change

the reaction of the sea-water menstruum from an initial pH 7.6 to pH 5.0 as illustrated by the data in table 3. Also it will be noted that some cultures form acid before the chitin undergoes visible dissolution. All of the cultures tested which produced acid likewise liberated ammonia from chitin but there were many cultures which liberated ammonia without changing the pH of the chitin medium. Acetic acid has been identified as one of the products of the bacterial decomposition of chitin and the odor of some cultures resembles butyric acid. Folpmers (1921) has reported butyric acid formation by mixed cultures of chitinovors.

The production of acetic acid and ammonia suggests that the chitinoclasts attack the chitin molecule by hydrolyzing the acetylated amino groups:



The reaction probably takes place in two steps, with acetic acid first being liberated after which deaminization occurs. Or, it is possible that the carbon-nitrogen linkage is hydrolyzed first, thereby liberating acetamide:



Hydrolysis of the acetamide would give rise to acetic acid and ammonia. Some chitinoclasts may merely attack the acetylated amino side-chains leaving the essential carbon and oxygen linkages in the chitin molecule intact, which would account for the fact that some grow freely in chitin medium and even liberate acetic acid and ammonia in excess without dissolving the chitin. Incidentally, most of the marine chitinoclasts are able to derive their nitrogen and carbon or energy requirements from ammonia and acetic acid respectively (table 1). Of course, many of the chitinoclasts attack the essential carbon and oxygen linkages of the chitin molecule as manifested by the complete disintegration of the chitin strips and the liberation of reducing sugars. Reducing sugars were detected in four of the ten different cultures grown in chitin medium when tested with Benedict's reagent.

Several cases of obligate periphytism (ZoBell, 1937) have been observed among the chitinoclasts. For example, one culture covered the chitin strip with a heavy orange growth while the surrounding menstruum remained quite colorless. When a loopful of the liquid was transferred to another tube of chitin medium no growth developed. However, when a bit of the orange growth scraped from the original chitin strip was used as the inoculum, bacteria began to develop at once on the new chitin strip. This indicates that most of the bacteria were growing attached to the chitin, with few or none in the menstruum.

About one-fourth of the initial raw cultures were definitely pigmented and more produced various tinctorial changes, including fluorescence on the chitin strips. Yellow, orange, green fluorescence and light brown predominated in the order given. Some of the cultures produced an abundance of yellow or orange water-soluble pigment which diffused throughout the medium. Pigmentation has proved to be very ephemeral; so, until more is learned concerning the factors which influence pigment production by marine bacteria, this characteristic must be used with extreme caution for differentiating species.

Nearly all of the organisms included in this study are strict aerobes although a few of them are facultative anaerobes. This does not mean that most marine chitinoclasts are aerobic because

the isolation procedures were designed to obtain only aerobes. Marine anaerobes which digest chitin have been demonstrated in oval tube deeps (Rittenberg, *et al.*, 1937) containing a chitinous medium from which oxygen was excluded by a leuco-methylene blue agar seal. Indications are that there are many anaerobic chitinoclastic bacteria in the highly reduced bottom deposits of the sea.

Since over 80 per cent of the ocean floor is perpetually colder than 4°C., it is significant that chitin digestion by marine bacteria has been demonstrated at refrigeration temperatures (0 to 4°C.). ZoBell (1934) has shown that while few, if any, of the bacteria isolated from the sea are obligate psychrophiles, most of them are slowly, yet definitely, biochemically active at 0 to −2°C. and perhaps even lower temperatures. Johnson (1932) isolated chitinovors growing on crabs packed in ice.

SUMMARY

Chitinoclastic bacteria have been found to be quite widely distributed in marine sediments, animals and sea water off the coast of southern California.

Thirty-one different pure cultures isolated from marine materials have been studied.

Marine chitinoclasts are described which can derive their complete carbon or energy and nitrogen requirements from chitin. Others require supplementary carbon compounds but can utilize nitrogen from chitin. Still others attack chitin only in the presence of simple carbon and nitrogen sources.

Many chitinoclastic bacteria liberate ammonia or acid from chitin; processes which may or may not be accompanied by its dissolution. Reducing sugars have been detected in some cultures as a decomposition product.

REFERENCES

BENECKE, W. 1905 Über *Bacillus chitinovorous*, einen Chitin-zersetzenden Spaltpilz. Bot. Ztg., Abt. 1, **63**, 227–242.

BENTON, ANNE G. 1935 Chitinovorous bacteria. A preliminary survey. Jour. Bact., **29**, 449–463.

BERTEL, R. 1935 Les bactéries marine et leur influence sur la circulation de la matière dans la mer. Bull. Inst. Oceanograph., **672**, 1–12.

BUCHANAN, R. E., AND FULMER, E. I. 1930 Physiology and Biochemistry of Bacteria. The Williams & Wilkins Company, **1**, 98.

BUCHERER, H. 1935 Über den mikrobiellen Chitinabban. Zentralbl. f. Bakt., Abt. II, **93**, 12–24.

DIEHL, J. M. 1936 Pflanzlichen Chitin. Chem. Weekblad., **33**, 36–38.

FOLPMERS, T. 1921 Der Zersetzung des Chitins und des Spaltungsproduktes desselben, des Glucosamins, durch Bakterien. Chem. Weekbl., **18**, 249–258.

HESS, E. 1937 Chitinovorous bacteria on live lobsters. Progress Reports, Biol. Board of Canada, No. **19**, 10–11.

ITERSON, G. VAN, MEYER, K. H., AND LOTMAR, W. 1936 Über den Feinbau des pflanzlichen Chitins. Rec. trav. chim., **55**, 61–63.

JENSEN, H. L. 1932 The microbiology of farmyard manure decomposition in soil. Jour. Agric. Sci., **22**, 1–25.

JOHNSON, DELIA E. 1932 Some observations on chitin-destroying bacteria. Jour. Bact., **24**, 335–340.

JOHNSTONE, J. 1908 Conditions of Life in the Sea. Cambridge Univ. Press, pp. 332.

RAMMELBERG, G. 1931 Beitrag zur Kenntnis des Chitins der Pilze und Krabben. Bot. Arch., **32**, 1–37.

RITTENBERG, S. C., ANDERSON, D. Q., AND ZOBELL, C. E. 1937 Studies on the enumeration of marine anaerobic bacteria. Proc. Soc. Exper. Biol. and Med., **35**, 652–653.

STUART, L. S. 1936 A note on halophilic chitinovorous bacteria. Jour. Amer. Leather Chem. Assoc., **31**, 119–120.

WAKSMAN, S. A., ET AL. 1933 Studies on the biology and chemistry of the Gulf of Maine. III. Bacteriologic investigations of the sea water and marine bottoms. Biol. Bull., **64**, 183–205.

ZOBELL, C. E. 1934 Microbiological activities at low temperatures with particular reference to marine bacteria. Quart. Rev. Biol., **9**, 460–466.

ZOBELL, C. E., AND ANDERSON, D. Q. 1936 Vertical distribution of bacteria in marine sediments. Bull. Amer. Assoc. Petrol. Geol., **20**, 258–269.