Botany. — *On Bacteria of Salted Fish.* By HELENA F. M. PETTER. (Communicated by Prof. F. A. F. C. WENT.)

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The following bacteria were isolated

1. From salted herring:
   a. a red rod
   b. a red sarcina and
   c. a colourless sarcina.
2. From dried cod-fish:
   a red rod.
3. From "Trapani" salt from a cannery at Bergen (Norway):
   an orange rod.

The isolation succeeded on pepton agar with 30 % NaCl, pure cultures were obtained by smears.

I. *Description of the Bacteria.*

a. The red rod isolated from salted herring and codfish.

The seven strains isolated from these materials had the following properties:

Non-sporulating rods: 2—11 $\mu \times$ 0.6—0.9 μ, motile, facultative aerobe. Gram-negative, catalase-positive. Does not grow on ordinary media.

Colonies on agar (15—30 % NaCl): round. In broth (15—30 % NaCl): turbid with pellicle. Forms no indol; optimum temperature at 37° C. Does not ferment (in 30 % NaCl), either saccharose, maltose or glucose. Nitrates reduced to nitrites.

The above characteristics and the properties of the pigment (see below) correspond to those of *Bacillus halobius ruber Klebahn* (KLEBAHN (6)). Because the organism is no spore-former I propose to change this name to *Bacterium halobium*.

Three strains formed transparent colonies on the agar, while the remaining four strains formed opaque colonies. In both transparent and opaque strains the colour of the strains varies from orange-red to purple.

The opaque colonies are originally hyaline, the opacity is due to the development of one or more bodies within the bacterial cell. (see figures I, II & III). One of the strains is almost pure white; in this strain the bodies fill the cell to such an extent that only a small amount of peripheral protoplasm remains. Usually the properties of the strains prove to be constant on transfer. In an opaque colony I once observed a transparent

area with a straight boundary. Transfers showed that the bacteria within this area had lost the power to form the typical intracellular bodies.

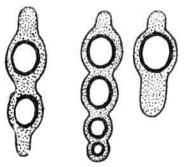

Fig. I
Bacteria with small "gas-vacuoles" in the cell

Fig. II
A young bacterium of the strain in which the "gas-vacuoles" fill the larger part of the cell-lumen

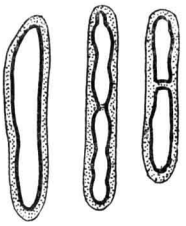

Fig. III
Older bacteria of the strain in which the "gas-vacuoles" fill the larger part of the cell-lumen

Similar sudden changes have been observed by various authors in *Bacillus prodigiosus Fl.*; *Bacillus violaceus Berg.* and others.

KLEBAHN has described, in the blue-green-algae, bodies with similar properties as formed in the Bacterium halobium (KLEBAHN (5) and (7)); the so-called gas-vacuoles. WILLE (14) and KOLKWITZ (8) observed similar structures in bacteria. The nature of these gas-vacuoles cannot be discussed here, let it suffice to point out that MOLISCH (11) doubts the gaseous nature of the contents of the vacuole and maintains that the "Schebekörperchen" contain either a viscous or a thin liquid. The following observations show the close resemblance of the bacterial structures with the "gas-vacuoles" or "Schebekörperchen".

1. The bodies disappear rapidly in 96 % ethylalcohol, in concentrated HCl and in ± 6 % phenol, before the bacterial body is destroyed.
2. The structures remain intact in water; they coalesce when the bacteria are destroyed.
3. They become very pronounced and distinct in olive-oil.
4. They appear red.
5. They disappear when the culture-fluid is subjected to a pressure of a few atmospheres.
6. They disappear when, while suspended in concentrated KOH, the coverslip is pressed closely to the slide.
7. They disappear after boiling a bacterial suspension for some time.
8. The "opaque" bacteria show remarkable power of flotation while the "transparent" bacteria may be divided throughout the liquid.
9. The bodies do not disappear in vacuo (one week in a vacuum-desiccator).

The fact mentioned under 9 and already observed by KLEBAHN in Bluegreen-algae is MOLISCH' chief argument against the gaseous nature of the "Schebekörperchen". It may be, however, that the peripheral protoplasm is impermeable to gas (KLEBAHN (7)).

The points mentioned under 3 and 4 support KLEBAHN's hypothesis. The refractive index of the bodies is apparently lower than that of protoplasm and the only substances known to have a lower index than water are gases and vapours or at least bodies in which gases and vapours are occluded in large quantities.

Points 5 and 6 also support the idea that the vacuoles contain gas. If opaque bacteria were subjected to pressure, and then the pressure is suddenly released, gasbubbles are liberated from the bacterial mass. Transparent bacteria do not generate gas under similar conditions.

If opaque bacteria are subjected to pressure in concentrated KOH (6) the colony becomes quite transparent while at the same time a great number of gasbubbles appears on the slide. The opacity of the cells, their white colour, may be accounted for by the presence of intracellular gas.

Whatever the nature of these corpuscles, their presence in Bacteria gives added evidence of the close relationship of the *Schizomycetes* and *Schizophyceae*.

b. The red sarcina.

Spheres: 0.9—2.7 μ in diameter, occurring singly, in pairs or in packets. Aerobe. Gram negative, catalase positive. Forms no indol. Indifferent to diminution of NaCl-concentration. Gelatin stab (15 % NaCl): surface-growth and liquefaction. Agar-colonies (20 % NaCl): circular, opaque. Agar slant (20 % NaCl): opaque layer. Optimum temperature 37° C. No fermentation with sugars (20 % NaCl). Nitrates reduced to nitrites.

The properties of this organism correspond closely to those of *Sarcina morrhuae Klebahn* as well as to those of *Micrococcus (Diplococcus) morrhuae* of the same author (KLEBAHN (6)). The pigments cannot be extracted by the usual solvents, as also observed by KLEBAHN (see below).

The organism is exceptionally variable; in liquid media (slight growth on the bottom of the vessel) the Sarcina appears as spheres of 1.5—2.7 μ diameter, while on solid media the cells generally are much smaller (0.9—1.5 μ diam.). On the latter media the "packets" often are poorly developed. KLEBAHN mentions 1.5—2.7 μ as the dimension of the *Sarcina* and 0.9—1.5 μ for the *Micrococcus*. Therefore both forms are most probably identical and designated by me as *Sarcina morrhuae Klebahn*.

c. The orange rod of the "Trapani" salt.

Non-sporulating rods: 0.6 $\times$ 1.5—3.5 μ, facultative aerobe, gram negative, catalase positive; agar colonies (30 % NaCl): small, circular, transparent. Agar-slant (30 % NaCl): turbid. Forms no indol. Optimum temperature 37° C. Nitrates reduced to nitrites.

New species: *Bacterium trapanicum*.

d. The colourless Sarcina.

Spheres: 3—6 μ in diameter occurring singly, in pairs or in packets.

Protoplasm homogeneous; becomes granular in water. Aerobe. Gram positive, catalase negative. Agar colonies (35—3 % NaCl): circular. opaque. Agar slant (35—3 % NaCl): opaque layer. Optimum temperature 20° C. No fermentation in sugars (30 % NaCl) or in acidified malt-extract (30 % NaCl). Brownish-yellow on some media.

New species: *Sarcina gigantea.*

II. *The properties of the bacterial pigments.*

The pigment of the red and orange bacteria described above does not diffuse into the agar. The potash-method of MOLISCH (10) showed that both *Bacterium trapanicum* and *Sarcina morrhuae* contained one carotinoid (needle-shaped crystals) while *Bacterium halobium* apparently contained two carotinoids (both needle-shaped and star-shaped crystals). The crystals show the well-known blue colour with H_2SO_4. The pigment of *Bact. halobium* and *trapanicum* may be extracted with alcohol, while none of the ordinary solvents proved effective on the pigment of *Sarcina morrhuae.*

The pigment of *B. halobium* was studied more in detail. No bacterio-chlorin or bacterio-erythrin could be isolated, either by the methods of MOLISCH (12) or BUDER (4). The organism does not belong to the purple bacteria.

In order to obtain macroscopical quantities of the crystalline pigment a large amount of fresh bacteria of the same strain was extracted with acetone (according to WILLSTÄTTER and STOLL (15)) until the bacteria were quite decolourized. The pigment was then passed into an equal mixture (by volume) of ether and petroleum-ether. This solution was kept over a 30 % KOH solution in methyl alcohol in order to saponify fats and other esters. After one day the intensively red liquid was separated; washed with water and dried with dry Na_2SO_4. The solution was then evaporated and petroleum-ether added. The pigment is badly soluble in this substance. After one night's sojourn at —10° C. a crystalline precipitate appeared, which was finally purified by repeated precipitation from a CS_2 solution with petroleum-ether. The crystals were dried and kept under nitrogen in the dark; they are very dark red.

A few other precautions may be mentioned here: the solutions were kept under nitrogen in the dark, the temperature at the operations never exceeded 45° C. and only peroxyde-free ether was used.

The chromatographic method of TSWETT (13) showed that the crystalline mass consisted of two closely-related substances, both adsorbed by $CaCO_3$ from a CS_2 solution. As far as could be ascertained the properties of the isolated substances did not agree with any of the known carotinoids.

The pigment which is most actively adsorbed by $CaCO_3$ I will call *Bacterio-ruberin* α, the other pigment *Bacterio-ruberin* β. α occurs in larger quantities in the bacterial body than β.

The carotinoid-reactions of VAN WISSELINGH (16) are positive for both pigments. Their solubilities are almost similar, however, β is more yellowish than α. The best solvent is methylalcohol, followed by acetone, ether, CS_2, chloroform, toluene. They are fairly soluble in ethylalcohol, poorly soluble in 96 % ethylalcohol, and slightly soluble in olive-oil. Only traces dissolve in petroleum-ether and benzene. The pigments are insoluble in water and in dilute acid.

In relation to the system petroleum-ether — methylalcohol (80—92 %) the pigments behave like xanthophyll and are, therefore, hypophasic. The preference for alcohol is not as great as in fucoxanthin, which substance dissolves is the methylalcohol in a system consisting of 70 % methyl-alcohol-ether + petroleum-ether (1 : 1). *Bacterio-ruberin* α and β are divided over both phases; α, however, prefers the methylalcohol while β is chiefly present in the upper layer.

The absorption maxima in the spectrum of both substances was measured by means of a double monochromator and a vacuum-thermo-couple. This instrument was kindly given at my disposal by Prof. Dr. L. S. ORNSTEIN.

Bacterio-ruberin α in methylalcohol			Bacterio-ruberin β in methylalcohol		
Band	Range	Maximum	Band	Range	Maximum
I strong	527—518 m μ	522 m	I strong	508—499 m	504 m
II very strong	503—478 m μ	490 m	II very strong	487—475 m	482 m
III weak	469—464 m μ	467 m	III weak	—	453 m

Crude crystalline mixture ($\alpha + \beta$)				Ethylalcohol extract of *B. halobium* according to KLEBAHN (6)	
In CS_2		In CH_3OH		Band	Max.
Band	Max.	Band	Max.		
I strong	561 m μ	I strong	525 m μ	I strong	528 m μ
II very strong	528 m μ	II very strong	492 m μ	II very strong	493 m μ
III strong	497 m μ	III strong	462 m μ	III weak	462 m μ

The observations of KLEBAHN agree quite closely with measurements obtained from a mixture of both pigments.

Only one strain being hitherto investigated, the pigments from other

strains were studied by means of adsorption-analysis. They all gave the same picture. The difference in colour of the various strains cannot be due to a difference in acidity for the pigment is not markedly influenced by this factor. Changes of a pigment with acidity have been observed in *Bacillus prodigiosus* (KRAFT (9)) and this pigment shows, therefore, no relation to the pigments of the red brine bacterium. Neither is there a close relation between *Bacterium halobium* and the purple-bacteria.

III. *Culture experiments.*

Both *Sarcinae* and *Bacterium trapanicum* could be grown in a purely synthetic medium (with 1 % asparagin or 1 % glycocoll as a source of nitrogen). *Bacterium halobium,* however, only developed in peptone, yeast-extract or fish-bouillon. The inorganic medium of *Bacterium halobium* however, is more variable.

The control-medium consisted of:

NaCl 4.5 mol, $MgSO_4$ 0.02 %, K_2HPO_4 0.02 %, Peptone "Poulenc" 1 %. A good development took place at pH 5.6—8.0. In this mixture osmotic replacement of NaCl was made. In this way it could be shown that the organism was tolerant to KCl 3.1 mol (concentrated), $(NH_4)_2SO_4$ 0.5 mol, LiCl 0.5 mol, KBr 0.5 mol, $CaCl_2$ 0.1 mol, $CaCl_2$ 0.2 mol + $MgCl_2$ 0.1 mol, $BaCl_2$ 0.05 mol. No development took place in KNO_3 2.3 mol, KJ 0.5 mol or $Na_2B_4O_7$ 0.01 mol. Both kations and anions may be toxic; we do not find here the pronounced kation-toxicity which is present in another brine-organism: *Dunaliella viridis Teodoresco* (BAAS BECKING (1)). The protoplasmic colloids in *Bacterium halobium* may be, therefore, closer to their isoelectric point.

The Ca-Mg antagonism of the bacterium is also quite different from that of *Dunaliella*. While in the former 0.1 mol $MgCl_2$ may counteract the toxic effect of 0.2 mol $CaCl_2$ ($Mg : Ca = 1/2$) in *Dunaliella* this relation is, at 4 mol NaCl $Mg : Ca = 20$ (BAAS BECKING (2)). The toxicity of Lithium for *Bacterium halobium* may be compared with the toxicity of Li and K for the nauplii of the brine-shrimp: *Artemia salina* (BOONE and BAAS BECKING (3)); while neither Na, K or Li are toxic to *Dunaliella* (BAAS BECKING (1)).

I want to express here my best thanks to Prof. Dr. A. J. KLUYVER and Prof. Dr. L. G. M. BAAS BECKING for their helpfull advise.

This is a preliminary communication. An extensive publication will follow later.

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