MICROCOCCUS NIGER, A NEW PIGMENT-FORMING ANAEROBIC COCCUS RECOVERED FROM URINE IN A CASE OF GENERAL ARTERIOSCLEROSIS

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The obligately anaerobic cocci are so difficult to isolate in pure culture and to maintain after isolation that they still remain one of the least studied groups of all bacteria. There is no question, however, that they are quite common in nature and it is only the inadequacy of our technic and our general lack of knowledge of them that causes us frequently to overlook them in mixed cultures, where, if anaerobic bacilli are present, the anaerobic cocci are almost certain to be lost in the process of isolation by heating and other selective methods particularly designed for sporulating bacteria.

In some cases, however, where the anaerobic bacilli are absent and none of the aerobic organisms recovered produces gas as the original mixed culture does, one may suspect the presence of non-sporulating anaerobes, and frequently, although of course by no means always, these prove to be anaerobic cocci, for many of the anaerobic cocci, unlike the aerobic cocci, produce gas in suitable media. *M. gazogenes* (Lewkowicz) is one of the commonest, particularly in the mouth (Hall and Howitt, 1925) and it frequently occurs in the naso-pharynx (Branham, S. E., 1927, 1928; Noble and Brainard, 1928) and in the genito-urinary system (Schmid and Kamniker, 1926).

In the present instance, a specimen of catheterized urine was examined from an elderly psychotic woman suffering from general arteriosclerosis and ergot poisoning, in whom gaseous gangrene caused by *Bacillus Welchii*, *Micrococcus aureus* and *Streptococcus*...
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Ignavus infection of one hand following an amputation required by endarteritis obliterans had been averted by the timely use of antigangrene serum and thorough drainage. The patient died a few weeks later from general sepsis but no autopsy could be secured.

The urine was slightly turbid. Upon centrifugation no pus cells were seen but there were numerous Gram positive diplococci, streptococci, and a few long Gram negative filaments.

Primary cultures were made upon lactose-eosin-methylene blue plates, blood agar plates, and in milk (constricted tubes) and deep brain medium. No coliform colonies appeared upon any of the plates, and B. coli, which is so common in urinary infections, was not isolated. Streptococcus fecalis and Micrococcus albus were isolated from the blood agar plate. The milk culture was coagulated after twenty-four hours and showed gas production at forty-eight hours but no stormy fermentation such as might suggest the possible presence of B. Welchii. The deep brain culture also produced gas but the microscopic examination showed only Gram-positive diplococci, streptococci and Gram-negative rods. Being unable to isolate any Gram-negative rod by aerobic means, I assumed that an anaerobic organism of this type might be responsible for the gas production in the primary aero-anaerobic cultures, and the usual deep agar dilution cultures in Burri tubes were prepared for its isolation (Hall, 1929). Numerous well separated colonies were fished from these and several of them showed gas production in brain medium, but, upon checking, all were found to consist of the aerobic cocci above mentioned mixed with some unknown anaerobic gas former. The Gram negative filaments and rods never appeared, however, in any of the isolation cultures and were no doubt lost.

The daily manipulations designed to isolate the gas former lasted more than six weeks and this fact is mentioned only to indicate the very great difficulty of isolation and to emphasize the point that if some other gas former had been isolated the present organism would perhaps have been entirely overlooked. Early in this period my attention was drawn to a few small densely black colonies appearing in certain deep agar cultures that had been
incubated for about five days, which is longer than we usually incubate them before picking colonies. These suggested the possible presence of the *Bacterium melaninogenicum* of Oliver and Wherry (1921) and of Burdon (1928) although no reference to gas production is made by these authors. Finally, after many failures and by repeated picking of black colonies from deep agar to brain medium, checking those showing gas for aerobic contamination (always *M. albus* or *Strep. fecalis*) and again making deep agar dilution cultures, three colonies were picked which gave gas in brain medium and no aerobic growth on plain agar slants. All of them proved to be pure cultures of the organism here designated "*Micrococcus niger,*" species novum.

**MORPHOLOGY AND TINCTORIAL REACTIONS**

*M. niger* is a small Gram-positive coccus measuring about 0.6μ in diameter and forming irregular masses resembling staphylococci. Diplococci occurred occasionally but no true chains were formed.

**CULTURAL PROPERTIES**

*M. niger* is obligately anaerobic. Numerous check tests upon plain and blood agar slants failed to show any aerobic growth. The usual methods of securing anaerobiosis are quite adequate, however, for its culture. Growth was slow upon all media at 37°C, requiring at least two to four days before becoming visible. At room temperature (about 25° to 30°C.) in controlled experiments with deep agar about seven days were required.

*Deep 1 per cent meat infusion agar with 2 per cent peptone, pH 7.0.* Colonies appeared first at 37°C after about two days incubation. If thickly seeded, small bubbles of gas sometimes appeared in the agar. The colonies were at first colorless, but about the fourth day began to show a brownish color, turning to dull black about the fifth or sixth day. The pigment was strictly localized in the colonies; there was never any discoloration of the medium suggesting diffusion or solubility. Even though well isolated the colonies rarely exceeded 0.5 mm. in diameter and were irregularly globular, smooth and dense. Owing to their strictly anaerobic re-
quirements and slow growth, no colonies were usually found nearer than 3 cm. to the surface. Curiously, as the medium dried out after a few weeks, admitting air alongside the agar plug, the colonies so exposed faded to a dull gray.

The addition of several cubic centimeters of ascitic fluid to each tube of deep agar apparently made no difference in the rapidity or character of growth.

Brain medium. Single colonies picked pure from some deep agar into brain medium showed turbidity first only after four or five days incubation at 37°C., and gas production uniformly after the sixth day. Heavily seeded cultures sometimes showed gas after one day's incubation. I tried but was unable to prove acceleration of gas production in brain medium due to synergy or symbiosis with *Streptococcus fecalis*. Pure cultures showed only a slight blackening of deep brain containing iron wire after prolonged incubation, due presumably to the production of sulfuretted hydrogen. The fact that the discoloration in this medium is not marked suggests that the pigment formed in agar is probably not directly connected with the production of sulfuretted hydrogen and the formation of iron sulfide as in the case of the putrefactive anaerobic bacilli.

Blood agar slants under alkaline pyrogallol. No growth visible to the naked eye appeared until the fourth or fifth day. Then minute black colonies arose like tiny black pearls, round, smooth and glistening, reaching next day their maximal size of 0.5 mm. They were non-hemolytic even after two weeks.

*Loeffler's coagulated blood serum under alkaline pyrogallol.* I was unable to note any growth until the eighth day when minute brown colonies appeared discretely over the white surface of the serum. The largest of these later reached a maximal size of 0.5 mm. but did not become as black as in agar. There was no liquefaction of the serum in five weeks.

*Gelatin.* In the constricted tube the gelatin remained clear throughout the entire period of observation, but a dark sediment became noticeable on the fifth day, gradually becoming more and more intensely black. There was no liquefaction after twelve days incubation at 37°C. as shown by the hardening of the gelatin in the ice box. Identical results were obtained under vaspar seal.
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Milk. There was no evidence of any growth or change on ten days incubation at 37°C., either in the constricted tube or under vaspar seal.

Fermentation tests. Glucose, levulose, galactose, maltose, glycerol and xylose, tested in constricted tubes with marble seals showed turbidity first on the second day, and a trace of gas on the third day. A black sediment collected in the bottom of the tubes. There was no production of acid; the cultures uniformly became more alkaline. It seems probable that no carbohydrates are fermented.

NON-PATHOGENICITY FOR ANIMALS

*M. niger* is non-pathogenic for guinea pigs inoculated subcutaneously and for rabbits inoculated intravenously. I tried 2 cc. of a four-day-old glucose broth culture in a 200-gram guinea pig without any result. I also inoculated a 2500-gram rabbit intravenously with 5 cc. of a five-day glucose broth culture without effect.

DISCUSSION

There are only a few references in the literature to brown or black pigment forming cocci. One of the earliest was that of Sternberg (1900) who described an obligately anaerobic long chained streptococcus from sputum in a case of human pulmonary actinomycosis, which was pathogenic for rabbits, and formed dark brown or black colonies upon sugar agar plates.

In 1903, Löw having observed a dark colored microbial growth upon the surface of a solution of chinchic acid neutralized with chalk, Emmerling and Abderhalden (1903) repeated the conditions and isolated a coccus, which they called "*Micrococcus chinicus*" and which reproduced the pigment upon chinchic acid neutralized with chalk by the oxidation of calcium chinate to protocatechuic acid; under anaerobic conditions naturally no oxidation occurs and the colonies were colorless. This phenomenon was subsequently studied by Beijerinck (1911) who showed that various bacteria, notably *Bacillus prodigiosus*, *Aerobacter aerogenes*, *Pseudomonas aromatic*ica, *P. fluorescens* non-liquefaciens, *P.*
fluorescens liquefaciens, P. pyocyaneus, and a coccus, closely related to M. chinicus, but according to Beijerinck distinct, and named by him "M. calco-aceticus," all oxidize chinic to protocatechetic acid, as it is now called. Beijerinck further showed that brown or black colored compounds may be formed by other aerobic bacteria by other oxidations, for example, quercite to pyrogallic acid and its colored compounds in the presence of alkali and oxygen by Pseudomonas aromatica, tyrosine to homogenetic acid and melanin by Microspira tyrosinatica, and possibly gelatin or peptone to chinon by Acetobacter melanogenum. It seems obvious, however, that none of these oxidations can account for the production of pigment by an obligately anaerobic organism such as M. niger.

In 1907, Gräf and Wittenben isolated two species of anaerobic streptococcus forming brown or black colonies in deep agar. Their Streptococcus "Sch" (Schwartzenbek), from an actinomyecoid swelling in the neck of a peasant, formed long chains of 30 to 50 elements and liquified gelatin; their Streptococcus "K" (Kiel) from a brain abscess, coagulated and peptonized milk, so that M. niger is easily distinguished from these.

In 1910, Schottmüller recovered a long chained obligately anaerobic streptococcus from puerperal infections, which he named S. putridus. The same germ was again recovered by Marwedel and Wehrsig (1915) from war wounds. According to Weinberg and Seguin (1917) it turns blood broth black and produces a putrid odor. Schwartz and Dieckmann (1926) (1927) suggested that the blackening of blood cultures by S. putridus might be due to melanin, but I believe there is no proof for this. Numerous studies (Abderhalden, 1909; Kastle, 1909; Gortner, 1911) have shown that melanin is formed by the oxidation of tyrosin to homogenetic acid by tyrosinase, and while some aerobic bacteria apparently produce melanin in this manner, according to Gessard (1898, 1901), Lehman (1902), Lehman and Sano (1908), and Beijerinck (1911), under aerobic conditions, no pigment is formed by any of these organisms when they are grown anaerobically in the presence of tyrosine, and it is impossible to explain the pigment production of an obligate anaerobe in this manner.
There is no question of the separate identity of *S. putridus* and *M. niger*; the long chains and putrid odor of the former clearly distinguish it.

On three different occasions, Gilmer and Moody (1914) observed black pigment forming organisms in old anaerobic cultures from alveolar abscesses but did not succeed in isolating them. I have had similar experiences in dealing with cultures from the mouth; we do not know whether these are cocci or not.

Castellani and Chalmers (1919) placed the black pigment forming cocci in a separate genus, "Nigrococcus," with *Nigrococcus nigrescens* (Castellani 1911) as a type. Castellani (1926) has since described *Micrococcus (Nigrococcus) nigrescens* in detail. The relation of this organism to air was not definitely stated but the impression was left that it is aerobic. Castellani regards it as an important symbiont (or better synergist?) with *Nocardia tenuis*, in the etiology of Trichomycosis nigra.

The possession of so striking a character as the production of black pigment makes it unnecessary here to discuss the other anaerobic cocci that do not have it. For more general reviews of these organisms the interested reader may refer to the works of Jungano and Distaso (1910), Prevot (1925), Ford (1927), Weinberg and Ginsbourg (1927), Thomson and Thomson (1927), and Taylor (1929).

As to the nature of the pigment in *M. niger*, I have been unable as yet to cultivate the organism in sufficient quantity to make any determination of its character, but it is permissible, *a priori*, from its strict localization within the colonies, to suggest that it will be found to be insoluble in aqueous solutions, and from the fact of its production under anaerobic conditions that the mechanism of its elaboration differs strikingly from those chromogenic processes for the production of black pigments by aerobic bacteria, where oxidation plays so important a rôle. Furthermore the fading of the anaerobic colonies on exposure to the air suggests that studies of this pigment will be complicated by the necessity of keeping it protected from the atmosphere.
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