CULTURAL REQUIREMENTS FOR THE PRODUCTION OF BLACK PIGMENTS BY BACILLI

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Spore-bearing aerobic bacteria[1] capable of producing striking black pigments have been known since the early reports of Gorini (1896) on "*Bacillus lactis-niger*" and of Biel (1896) and Lunt (1896) on "*Bacillus mesentericus-niger.*" Although these types have become established in the bacteriological literature under the respective binomial designations of *Bacillus niger* (Migula, 1900) and *Bacillus aterrimus* (Lehmann and Neumann, 1896), there has been little agreement upon their characterization or upon the cultural conditions necessary for their pigmentation.

Ford (1927) noted that *B. aterrimus* blackened potato very strikingly, in contrast to the brown color produced by *B. niger*. On the other hand, the differential key in Bergey's Manual (1934) employed the blackening of potato in the reverse order for separating the two species—*B. aterrimus*, white to pink; *B. niger*, black. Levine and Soppeland (1926) found both species capable of blackening potato, but only *B. niger* capable of fermenting lactose. This character was therefore used to separate the two species. Lehmann, Neumann and Breed (1931) considered the pigment-producing abilities of these organisms to be variable or inconstant, and grouped them together with forms closely related to *Bacillus vulgatus*.

[1] The authors wish to express their appreciation to all those who so kindly furnished the cultures which made this study possible and to Charles Thom, principal mycologist in charge, for his interest and coöperation during the course of this work.

Other species capable of producing a black coloration have been described. Carbone and associates (1921) refer to *Bacillus tyrosinogenes* of Rusconi; Fabian and Nienhuis (1934) found *Bacillus nigrificans* in spoiled pickles; and Cameron, Esty and Williams (1936) described *Bacillus betanigrificans* capable of blackening beets and culture media in the presence of iron. The relationships of these proposed species to *B. niger* and *B. aterrimus* have not hitherto been defined.

CULTURES STUDIED

Observations reported in this paper were made upon a collection of 40 cultures, 12 of which were received as named species, 7 were received unnamed but known to produce pigment, and 21 were isolated by the authors. These cultures are listed below under the three species into which they naturally fall because of their physiological characteristics. The name under which the culture was received and the history as far as known, is given opposite the number of the culture as it exists in our collection.

I. *Bacillus niger*

220 *B. mesentericus*, var. *niger*, from AMNH (733) in 1923; Kral.
229 Black bacillus, Smith, isolated in 1912.
254 *B. lactis-niger*, from Gorini (2) in 1936.
264 *B. niger*, from Hall (799) in 1936; Ford.
265 Black bacillus, from Hall (1509) in 1936.
650 *B. niger*, from Cameron in 1937; Breed; NCTC 2736; Gorini.
651 *B. niger*, from Cameron in 1937; Breed; NCTC 2592; Ford (6).
655 *B. lactis-niger*, from Cameron in 1937; Breed; Gorini (2).
18 isolates from soil and air, 1936.

II. *Bacillus aterrimus*

230 Black bacillus, from Thom in 1936 (isolated in 1912).
259 Black bacillus, from Hall (581) in 1936.
260 Black bacillus, from Hall (620) in 1936.
261 Black bacillus, from Hall (621A) in 1936.
262 Black bacillus, from Hall (622) in 1936.
353 *B. tyrosinogenes* (Rusconi), from Hall in 1937; Istituto Sieroter., Milan.
624 *B. nigrificans*, from Porter in 1937; Fabian.

653 *B. aterrimus*, from Cameron in 1937; Breed; NCTC 2591; Ford (5B)
659 *B. nigrificans*, from Cameron in 1937; Fabian.
3 isolates from soil, 1936.

III. *Bacillus betanigrificans*

648 *B. betanigrificans*, from Porter in 1937.
649 *B. betanigrificans*, from Cameron in 1937.

In addition to these cultures, 6 cultures were received as *B. aterrimus* and 6 as *B. niger* (or *B. lactis-niger*), which could not be identified as belonging to either of these species.

INFLUENCE OF THE MEDIUM ON PIGMENTATION BY B. NIGER

The importance of the substrate in the development of pigment by *B. niger* was evident when cultures, previously coal-black, failed to produce pigment when transferred to another lot of nutrient agar. These cultures, however, readily produced pigment when returned to the original medium. To overcome the supposed deficiency in the one medium, various modifications, such as the addition of carbohydrates or mineral salts, singly and in combination, or the changing of the pH value, were made without success. Finally, all brands of commercial peptones available in this laboratory were used in agar. On 5 peptone agars, *B. niger* produced blackening (see table 1) but failed to do so on the remaining 4; *B. aterrimus* failed on all. The addition of beef-extract paste to 2 of the 4 peptone agars not blackened by *B. niger* resulted in blackening; the growth of *B. niger* upon beef-extract agar alone, however, was colorless.

The addition of glucose or maltose inhibited the production of pigment on those peptone agars which were normally blackened by *B. niger*. This suggested that certain break-down products of protein were necessary for the blackening and that fermentable carbohydrates spared the protein from decomposition. Since the various commercial peptones are known to vary in their amino-acid content as well as in other respects, correction of those peptones unsuitable for pigmentation was attempted. Glycine, alanine, aspartic acid, leucine and tyrosine were added singly to

nutrient agar prepared from peptone "A"[2] *B. niger* produced a black pigmentation only on the nutrient agar to which tyrosine had been added. The addition of glucose or maltose to this medium did not interfere with the development of the pigmentation as it did in the cases mentioned above.

In addition to those media already mentioned, milk agar (equal volumes of skim milk and 4 per cent agar-agar) is suitable for *B. niger*, the black pigment usually appearing in a few days.

TABLE 1

Pigmentation responses of B. niger and B. aterrimus on various peptone agars

PEPTONE	1.0% PEPTONE		0.5% PEPTONE 0.3% BEEF EXTRACT		0.5% PEPTONE 0.3% BEEF EXTRACT 1.0% GLUCOSE	
	B. niger	*B. aterrimus*	*B. niger*	*B. aterrimus*	*B. niger*	*B. aterrimus*
A	−*†	−‡	−†	−‡	−†	+
B	+	−	+	−	−	+
C	+	−	+	−	−	+
D	+	−	+	−	−	+
E	+	−	+	−	−	+
F	−	−	+	−	−	+
G	−	−	−	−	−	+
H	+	−	+	−	−	+
I	−	−	+	−	−	+

* + black pigment present; − black pigment absent.
† Blackening with free tyrosine added.
‡ No blackening with free tyrosine added.

RÔLE OF FERMENTABLE CARBOHYDRATES IN THE PIGMENTATION OF B. ATERRIMUS

The reports of many previous workers that carbohydrate compounds are especially desirable for the black pigmentation of *B. aterrimus* have been confirmed. *B. aterrimus* cultures failed to show blackening on any of the peptone, peptone-beef, or tyrosine-peptone-beef agars blackened by *B. niger*. However, the addi-

[2] None of our cultures of *B. niger* blackened nutrient agar containing this peptone (Difco's Bacto-Peptone). Of the 9 peptones studied, nutrient agars prepared with Difco Bacto-Tryptone or Bacto-Proteose Peptone were the most readily blackened by *B. niger*.

tion of a fermentable carbohydrate to any type of peptone agar that would support at least a moderate growth of *B. aterrimus* rendered that medium favorable for pigmentation by this species, regardless of whether or not the peptone was a type blackened by *B. niger*. The differences in the types of media which permit pigmentation of *B. aterrimus* and *B. niger* are shown in table 1.

Broquin-Lacombe (1913) recognized that *B. aterrimus* had the ability to blacken an inorganic nitrogen medium containing a fermentable sugar, whereas *B. niger* lacks this ability. We have noted blue or black pigments in *B. aterrimus* cultures upon monobasic ammonium phosphate agar with such carbon sources as xylose, arabinose, glucose, levulose, maltose, sucrose, starch, dextrin, salicin, or mannitol.

All cultures of *B. aterrimus* and the majority of cultures of *B. niger* blackened sterilized potato. In view of the differences obtained on other media, it is probable that *B. aterrimus* produces pigment on potato by means of a different enzyme system than does *B. niger*. Muschel (1922) expressed the opinion that *B. aterrimus* produces a polyphenyloxydase blackening of carbohydrate condensation products. It is probable that *B. niger* produces a melanin-type pigment by means of a tyrosinase system. For either system, potato, or peptone agar containing both fermentable sugar and free tyrosine, serve as satisfactory substrata for pigment production.

Although we are in agreement with Levine and Soppeland (1926) that either species may produce blackening on potato, we do not agree that lactose fermentation by *B. niger* suffices to separate it from *B. aterrimus*. For this determination the synthetic carbohydrate agars with brom-cresol-purple indicator were used as recommended in the Manual of Methods for Pure Culture Study, edited by the Committee on Bacteriological Technique of the Society of American Bacteriologists. Growth and more or less acid formation within 2 weeks indicated a positive reaction, whereas no growth or a very scant growth and no acid indicated a negative reaction. All of our isolations of *B. niger* are lactose negative, as was the *B. niger* (culture 254) received directly from Gorini in 1936. On the other hand, cultures of *B. niger* from the

American Type Culture Collection (culture 220) and from Hall (culture 264) were lactose positive. All cultures of *B. aterrimus* were lactose negative. Therefore, lactose fermentation does not appear an adequate differential criterion. It has been previously reported (Clark and Smith, 1938) that several species of aerobic spore-formers regularly produce slow or mutant fermentations on certain sugars. For instance, *Bacillus megatherium* was noted to vary on mannose, *Bacillus cereus* and *Bacillus mycoides*, on sucrose, and *Bacillus vulgatus*, on lactose.

The cultures of *B. nigrificans* and of *B. tyrosinogenes* (Rusconi) were identical in every respect to type cultures of *B. aterrimus*; they produce similar blackening on sugar media, and are considered to be synonymous.

CULTURES DISTINCT FROM B. NIGER AND B. ATERRIMUS

B. betanigrificans exhibited morphologic and cultural differences, especially the production of gas on carbohydrate media which readily separates it both from *B. niger* and *B. aterrimus*. It was not found to produce a black pigment on potato, milk agar, or ordinary peptone or sugar agars, but it does produce a brownish or black pigment in the presence of metallic iron in various media, as originally reported by Cameron, Esty and Williams (1936).

As stated in the forepart of this paper, a total of 12 cultures received as *B. aterrimus* or *B. niger* were identified as species other than either of these. The donor of certain cultures stated that he had never seen any pigmentation with those particular cultures, but that they undoubtedly did produce color when they were isolated. This apparent variability in pigment production has led some bacteriologists[3] to believe that this character is transient, whereas others believe it a stable character, although at the moment lacking proof for their opinion except as they have observed their own cultures. Of these 12 mislabelled cultures, only 3 were identified as *Bacillus vulgatus*, a closely allied species, whereas 9 were distinctly different in their reactions.

Cultural similarities between *B. vulgatus*, *B. niger* and *B.*

[3] Personal communications.

aterrimus made it desirable to determine whether pigment production was a stable character and whether variants might be developed. Consequently, cultures of *B. vulgatus* were subcultured repeatedly in enrichment broths without any pigmented strains being obtained. Attempts were also made to produce non-pigmented strains from cultures of *B. niger* and *B. aterrimus*. Cultures were aged in acid, neutral or alkaline broths (pH 5.5, 7.0, 8.0) and plated out at varying intervals. Smooth and rough colony forms were picked from these platings for further aging and plating. In no case was it found possible to obtain a non-pigmented daughter strain from an originally pigmented parent culture. Finally, it may be stated that *B. niger* (number 229) and *B. aterrimus* (number 230) have been maintained in this laboratory for 26 years without any special care and without loss of their characteristic type of pigmentation.

SUMMARY

Bacillus niger produces a black pigment upon protein media which contain free or metabolically available tyrosine. Some commercial peptones containing no readily available tyrosine are not blackened unless free tyrosine is added. The addition of a fermentable sugar to many protein media normally blackened by *Bacillus niger* inhibits pigmentation unless free tyrosine is added.

Bacillus aterrimus blackens media containing fermentable carbohydrates, either in the presence or absence of tyrosine, but does not blacken sugar-free peptone media which are readily blackened by *Bacillus niger*. Carbohydrate media containing mineral nitrogen are also blackened by *Bacillus aterrimus* but not by *Bacillus niger*.

Cultural requirements for the production of pigment by *Bacillus aterrimus* and *Bacillus niger* are believed sufficient to warrant their recognition as separate species. Since non-pigmented variants could not be developed and since a culture of each has been maintained for 26 years without loss of pigmentation, it seems that this is a stable character.

Bacillus nigrificans (Fabian) and *Bacillus tyrosinogenes* (Rus-

coni) were found similar in all respects to *Bacillus aterrimus*, and are believed to be synonymous with that species.

Bacillus betanigrificans produces black coloration in the presence of metallic iron, but not upon the media readily blackened by the species considered above. It possesses morphological and cultural properties that easily separate it from *Bacillus niger* and *Bacillus aterrimus*.

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