CLXXXIX. BACTERIOLOGICAL AND BIO-CHEMICAL RELATIONSHIPS IN THE PYOCYANEUS-FLUORESCENS GROUP.

I. THE CHROMOGENIC FUNCTION IN RELATION TO CLASSIFICATION.

By GEORGE EDGAR TURFITT.

From the Department of Biochemistry, University College, Nottingham.

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IN the system of bacterial classification proposed by a committee of the Society of American Bacteriologists [Bergey, 1934], the very closely related green fluorescent bacteria occurring chiefly in soil and water are grouped together as the genus *Pseudomonas*. In common with most other bacterial characteristics pigmentation has, from time to time, been considered a variable cultural factor. According to Gessard [1890], a given strain of *B. pyocyaneus* may, by suitable cultural methods, be induced to form the fluorescent pigment alone, pyocyanine alone or the two pigments simultaneously. Charrin & Phisalix [1892] record the persistent abolition of chromogenic function with *B. pyocyaneus*. More recently, Baerthlein [1918] has obtained, by selection of colonies, six variants from a single strain of *B. pyocyaneus*. His original strain gave two types of colony, (1) dry, opaque colonies, containing short, stumpy cells, (2) moist, translucent colonies of long slender bacteria. Each of these gave three further variants (a) in colourless colonies, (b) in pale green colonies, producing the green fluorescent pigment but not the blue pyocyanine, and (c) in deeper green colonies, producing both fluorescent pigment and pyocyanine. From this it would seem a matter of extreme difficulty, if not actually impossible, to decide definitely whether a given bacillus is one of the fluorescent type or merely a strain of *B. pyocyaneus* which has lost its typical property of producing the blue pyocyanine, or whether it belongs to the group at all.¹ Previous observations (unpublished) in connexion with another problem have led the author to believe that pigment production in this group might probably be much more constant than has hitherto been supposed. The investigations described in this paper were undertaken in an endeavour to ascertain to what extent the chromogenic function can be regarded as invariable and of sufficient fundamental importance to act as the basis for the classification of these bacteria.

EXPERIMENTAL.

(1) *Bacteriological examination of fluorescent bacteria.*

The bacteria used (100 strains) were isolated as follows: 25 strains from water (River Trent, canal water, reservoirs, etc.) 25 from fresh faeces (man, animal,

¹ A comprehensive survey of the literature is included in a thesis, "The Metabolic Pigments of the *Pyocyaneus-Fluorescens* Group," to be presented by the author for the degree of Ph.D. (Lond.), 1936.

fowl), 25 from soil and the remainder from various widely differing sources; in every case growth on agar slants was accompanied by diffusion of a greenish fluorescence through the medium, this being the criterion for inclusion amongst the stock strains. In addition, pure cultures of *B. pyocyaneus*, *B. fluorescens liquefaciens* and *B. fluorescens non-liquefaciens* were obtained from the National Collection of Type Cultures, Lister Institute, London (Catalogue Nos. 1999, 964, 3247 respectively). The standard bacteriological examinations were conducted in each case from 24-hour cultures on nutrient agar slants, and are briefly summarized:

The cells, in every case, were small rods $0.5 \times 2.0 \mu$; the variation in size was remarkably small, and rods longer than 2.0μ were found in only 7 strains.

All were actively motile, but in several cases (chiefly from acid clay soil) the staining of flagella was unsuccessful. In the other strains, staining by the method of Gray [1926] showed 1-3 polar flagella.

Endospores and capsules were never encountered.

The strains were invariably Gram-negative.

Both liquefaction of gelatin and formation of indole occurred in about 70% of the strains. This figure is considerably higher than that of Tanner [1918] who, however, worked with fluorescent bacteria solely from water.

Approximately the same percentage of strains was found to be able to grow under anaerobic conditions in presence of nitrate or nitrite. This facultative anaerobic growth has been described very thoroughly in the case of *B. pyocyaneus* by Quastel *et al.* [1925].

No gas was obtained from carbohydrates, but in many cases a small amount of acid was produced from glucose.

Investigations on the pathogenicity of the various strains were not attempted.

From a purely bacteriological standpoint the above examination shows that these strains form a homogeneous group of very closely related individuals, and on these characteristics alone little more can be said concerning their classification.

(2) *Cultural requirements for pigment production.*

Investigations on this much-debated subject [Gessard, 1890; 1892; Noesske, 1897; Kuester, 1899; Jordan, 1899, 1, 2; Sullivan, 1905; *et al.*] seemed to have culminated with the painstaking researches of Benecke [1907], who showed that the essentials for production of fluorescent pigment were Mg, phosphate, sulphate, and a very small amount of K^+ ions, together with suitable sources of C and N. The subject was again reopened by Georgia & Poe [1931; 1932] and by Robinson [1932]; these workers agree that in synthetic media requirements for production of fluorescence are available C and N, with Mg and phosphate. For the present work this conclusion has been accepted, and attention has been directed towards the discovery of media by means of which the stock strain of typical *B. pyocyaneus* might be induced to form (a) both pyocyanine and fluorescent pigment, (b) pyocyanine alone, (c) fluorescent pigment alone, (d) neither pigment. The media used by Gessard [1890; 1891] were tried, but with the exception of the glycerol-peptone-agar described below they did not give altogether consistent results. It was thought better to use, wherever possible, media of definite chemical composition in order that the exact conditions in any particular case might be repeated. Experiments conducted on many different media, involving numerous permutations, gave the following as optimum conditions.

(a) The presence of peptone and of glycerol seemed to favour production of pyocyanine, whilst gelatin caused considerable formation of fluorescent pigment.

Ordinary double-strength broth, with the addition of 1 % glycerol, gave abundant production of both pigments. (No attempt was made to find a definite chemical medium here, since production of both pigments simultaneously was not required in connexion with the classification.)

(b) On the following glycerol-peptone-agar medium [Gessard, 1891]:

Medium A.	Glycerol	...	...	5 %
	Peptone	...	...	2 %
	Agar	...	...	3 %

which should be neutral to phenolphthalein and sterilized by autoclaving for 15 min. at 10 lb. pressure, there was profuse formation of pyocyanine but little fluorescent pigment. On slightly increasing the peptone constituent (2.5 %), fluorescence was undetectable. No chemical medium tried gave pyocyanine at all comparable in quantity with that obtained on this medium. Various makes of peptone and agar were used, the best results being obtained from "Difco" Brand Bacto-Peptone and B.D.H. powdered agar; these products were used exclusively in the later work.

(c) The medium recommended by Georgia and Poe [1931]:

Asparagine	...	...	0.3 %
K ₂ HPO ₄	...	...	0.05 %
MgSO ₄ , 7H ₂ O	...	...	0.05 %
Distilled water			

proved to be satisfactory in inhibiting pyocyanine production, whilst at the same time giving good yields of fluorescent pigment. In view of proposed attempts on the isolation of the green fluorescent pigment, and the enormous quantities of culture medium required, it was decided to find if possible a less expensive substitute for asparagine. After the trial of many C and N sources, the following liquid medium was evolved which gave very intense green pigment production and no pyocyanine detectable by extraction with chloroform:

Medium B:	NH ₄ NO ₃	...	...	0.1 %
	K ₂ HPO ₄	...	...	0.025 %
	MgSO ₄ , 7H ₂ O	...	...	0.025 %
	Distilled water			

This medium was sterilized by steaming in a Koch sterilizer at 100° during 1 hour on each of three successive days, after which 0.1 % freshly-boiled alcohol was added by means of a sterile pipette. Better results were obtained by the use of larger quantities than the usual culture tubes. Two-litre quantities of the medium, contained in plugged 2500 ml. flat-bottomed flasks, were inoculated from 24-hour agar slant cultures of *B. pyocyaneus* and incubated at 25°. After 12 days the medium showed a green fluorescence and a cloudiness due to bacterial growth. On transferring the contents of one of the flasks to a 2000 ml. beaker, adding 5 ml. of a concentrated NaOH solution and warming to about 85°, a thick white coagulum of protein formed on the surface of the liquid, which now was transparent and showed a bright green fluorescence.

(d) If, from medium B, Mg or phosphate is omitted, growth still occurs and a cloudiness develops throughout the medium, but no colour is obtained. Similarly, if a trace of some heavy metal salt be added there is complete inhibition of all pigment formation, although growth proceeds. The minute amounts of salts present in tap-water will completely prevent the development of any colour if such water is used instead of distilled water in preparing the culture medium.

Medium C: NH_4NO_3	...	...	0.1 %
K_2HPO_4	...	...	0.025 %
$\text{MgSO}_4, 7\text{H}_2\text{O}$	...	...	0.025 %
$\text{FeSO}_4, 7\text{H}_2\text{O}$	...	...	0.0001 %
EtOH	...	...	0.1 %.

B. fluorescens liquefaciens and *B. fluorescens non-liquefaciens* were both cultured on the above media A, B, C, but the results obtained were widely different from those described for *B. pyocyaneus*. In no case was growth accompanied by any pigmentation, although on broth and in the asparagine medium a greenish fluorescence developed.

Each of the strains isolated from natural sources was subcultured three times at intervals of 7 days on each of the three media A, B, C, and incubated at 25°. They were found to fall, with respect to their chromogenic functions, into two sharply defined groups corresponding to (a) *B. pyocyaneus*, (b) *B. fluorescens*.

(3) *Constancy of the chromogenic function in B. pyocyaneus and B. fluorescens.*

According to Sullivan [1905] varieties of *B. pyocyaneus* producing pyocyanine alone and fluorescent pigment alone cannot be made to take up the production of the other pigment, although a variety producing both pigments may be made to exhibit either or both. This view is somewhat at variance with Gessard's [1890] results already quoted, and with the report of Meader *et al.* [1925] that all bacteria isolated by them from natural sources gave definite production of pyocyanine after subculture on Gessard's glycerol-peptone-agar.

During the past 18 months, intensive selective cultivation in this group has been carried out by the author. The stock strain of *B. pyocyaneus* has been subcultured repeatedly at intervals of 7 days upon each of the media A, B and C and incubated at 25°. It was thought reasonable to assume that if pigment production were a readily variable feature such treatment would modify to some extent this characteristic of the organism. At the end of the time, however, there appeared to have been no change in this respect, the other chromogenic features being readily recovered by subculture on the appropriate medium, thus substantiating the results obtained by Gessard [1890]. Spontaneous degeneration with loss of chromogenic power [see Jordan, 1899, 1, 2; *et al.*] has not been encountered in this investigation.

These experiments were carried out in addition with *B. fluorescens* and also with certain selected natural strains (No. 3 from canal water; No. 37 from human faeces; No. 69 from uncultivated soil; No. 81 from snow), but no permanent change in the colour-producing properties was ever observed. *B. fluorescens* and Strains 69, 81 could not at any time be induced to form pyocyanine, whereas Strains 3, 37 behaved exactly as the stock strain of *B. pyocyaneus*. In view of these results, the observation suggests itself that the bacteria used by Meader *et al.* [1925] were, without exception, strains of *B. pyocyaneus*, especially as they report that their organisms were isolated from environment in close proximity to man.

DISCUSSION.

The very unsatisfactory present state of bacterial classification may be accounted for almost entirely by the ever increasing records of variation. Biological characters have, in various instances, been supplemented for purposes of classification by consideration of purely chemical features. The genus *Lacto-*

bacillus, for example, is composed of organisms producing a common metabolic product, lactic acid, on carbohydrate media. The formation of a metabolic product, whether it ultimately serves some useful purpose or whether it is merely a waste product, is a relatively constant property and as such is more especially suited to act as the basis for the classification of the organism producing it. So distinctive a property as the production of a diffusible, green fluorescent pigment in the culture medium, would seem a very convenient basis for classification of the responsible organism, subject to the provisions that, firstly, the green pigment is of the same chemical composition in each case; secondly, the organisms are not widely differentiated by their morphological and cultural characteristics; and thirdly, the chromogenic function is sufficiently invariable. Emphasis is placed on the second point, since certain fluorescent organisms of the genus *Phytomonas* are almost identical morphologically and culturally with those of the genus *Pseudomonas*. Even should subsequent research indicate the identity of the green pigment from both groups, the differentiation of the two genera would perhaps still be satisfactorily accomplished on the pathogenicity for plants displayed by the former organisms. There still remains the possibility that the *Phytomonas* and *Pseudomonas* organisms would be better considered as distinct species of a genus whose generic characteristic is the production of a diffusible, green fluorescent pigment. In the present paper, the work has been concerned with the second and third of the points outlined. An investigation on the isolation of the green fluorescent pigment, and the comparison of its composition in a number of selected bacteria is nearing completion. The results, which show the pigment to have an identical composition in the cases examined, will be communicated as a second part to this paper.

The allocation, then, of the green fluorescent bacteria in a single genus (*Pseudomonas*) is strongly supported by definite biochemical evidence. Whilst it is not suggested that the chromogenic function shall serve as more than the generic character, there remains the evidence that within the group the media quoted appear to differentiate between two constant forms:

- (1) *B. pyocyaneus* (*Pseudomonas aeruginosa* (Schröter) Migula).
- (2) *B. fluorescens* (*Pseudomonas fluorescens* (Flügge) Migula).

The conclusion that the fluorescent group of bacteria contained these two constant forms had been reached on different evidence by various workers [Ruzicka, 1899; Niederkorn, 1900], many years before the introduction of the genus *Pseudomonas*.

Whether the chromogenic function really provides a basis for the further differentiation into species is a problem which must, for the present, remain undecided. The possibility of the discovery of other media giving a finer separation of species within the genus cannot be overlooked.

SUMMARY.

1. Evidence has been obtained for the classification of the pyocyaneus-fluorescens group of bacteria into a single genus on the basis of their chromogenic function.

2. Media have been elaborated upon which a very marked constancy in pigment production is exhibited by these bacteria.

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