COLOURED *BACILLUS* SPECIES RESEMBLING *B. FIRMUS*

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Bacillus species resembling *B. firmus*, which produce carotenoid pigments, were isolated from the surface of freshwater fish and from a sample of activated sludge. The absorption spectra of the pigment components of four strains were similar and were influenced by the age of the culture. The pigment(s) of the fifth strain was different in its spectral behaviour in that it did not vary with the age of the culture; it resembled the pigment from young cultures of the four strains. Diphenylamine inhibited pigmentation at concentrations that did not affect growth. Changes in the temperature of incubation and the degree of aeration did not affect the degree of pigmentation of the cells.

Introduction

While investigating the bacterial flora of fish from polluted waters we isolated spore-forming bacilli that were markedly chromogenic. A survey of the literature showed that with the exception of the pulcherrimin producing species (Canale-Parola 1963) not much is known about other pigmented *Bacillus* species. We, therefore, undertook a study of the coloured strains isolated by us and the results are given in this paper.

Materials and Methods

Organisms.—Strains 4, 8 and 11 were isolated from nutrient agar plates on which suspensions of scales of *Puntius* species had been streaked (Dias *et al.* 1965). Strain 4, when plated on nutrient agar 12 months after isolation, showed two colony types, the dominant form resembling the parent in colour, and the other, which comprised less than one per cent of the colonies, had a different hue; the latter strain is referred to as 4*a*. It has not been possible to isolate the 4*a* type again from the parent strain or to obtain the latter from the variant type 4*a*. The strain referred to as ACSL was isolated from a nutrient agar plate on which activated sludge, which had been heated in a boiling water bath for 10 min., had been plated. Attempts to obtain more strains of the chromogenic *Bacillus* species from activated sludge, sewage and soil have not been successful.

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Media.—The media and methods used in the characterization of the isolates were those of Smith, Gordon and Clark (1952). Oxoid peptone was used in all experiments unless stated otherwise.

Pigment extraction and fractionation.—The cells were harvested by centrifugation from nutrient broth cultures (500 ml broth in 1L Erlenmeyer flasks, incubated on a rotary shaker—250 rev./min., 2·5 cm eccentric throw) at the requisite growth phase. The pigment was extracted with methanol as described by Starr and Stephens (1964). The cell mass after extraction was white. The methanol extract was then partitioned between solvents to separate the pigments (Starr and Stephens 1964). The spectra of the extracted pigments were measured with a Beckman spectrophotometer (model DU).

Estimation of growth and pigmentation.—Cells from a known volume of medium were harvested by centrifugation and washed once with distilled water. To estimate growth the cells were resuspended in a known volume of water and the optical density at 600 mμ was measured on a Bausch and Lomb Spectronic 20 colorimeter. The optical density was converted to dry weight from a previously prepared standard curve. To estimate pigmentation the centrifuged cells were extracted with boiling methanol till all the pigment was extracted from the cells. The debris was centrifuged out and the methanol extract made up to 5 ml. The optical density of the clear methanol extract was measured at 465 mμ on a Spectronic 20 colorimeter. The amount of pigment produced is expressed as the optical density (465) of the methanol extract of 10 mg cells (dry weight).

Results

Characterization of the isolates.—The morphological attributes of the isolates were extremely variable and were markedly affected by the growth medium. All strains were gram positive rods (varying in length from 1·7 to 6 μ and about 0·5 μ in breadth). When grown on nutrient agar for 24 hours, strains 4 and 4*a* occurred as long chains while the cells of the other strains occurred singly or in pairs. Strains 4 and 4*a* occurred as filamentous cells in older cultures. On glucose agar the cells were extremely pleomorphic, especially strain 8 which showed a large number of spiral cells. The cells contained granules of fat and volutin. All the strains produced oval spores which were located centrally or subterminally. The spores of strains 8 and 11 and particularly those of strain ACSL imparted a slight bulge to the sporangium giving the cells a spindle shape. On continued cultivation for about six months strains 8 and 11 became asporogenous and all attempts to induce sporulation by changing the conditions of growth were unsuccessful. However, after about 16 months a nutrient agar stock of strain 8 was found to have regained the ability to sporulate. The sporogenous character of all

isolates was established both by staining the spores with malachite green (Society of American Bacteriologists 1957) and by testing their ability to withstand boiling for 10 minutes.

With the exception of strain 8, the isolates produced colonies which were flat and dry and had irregularly lobed margins and dry wrinkled surfaces. Strain 8, on the other hand, produced smooth umbonate colonies. The colonies of strains 4, 8, 11 ACSL were orange in colour; those of strain 4*a* were lemon yellow. Growth in nutrient broth of strains 8 and 11 resulted in a uniform turbidity; the other strains grew as pellicles. With the exception of strain 11, the isolates grew better on nutrient agar than on glucose agar; strain 11 grew equally well on both media.

The extent of growth of the isolates in nutrient broth was influenced greatly by the quality of the peptone employed (Table I). With the exception of strain 11, the coloured bacilli showed a marked preference for Oxoid bacteriological peptone followed by Difco Bacto peptone; Difco Proteose peptone, Difco tryptone and Oxoid tryptone allowed only limited growth, if any. Strain 11 showed no marked preference, growing well on all peptones. The nutritional significance of this observation has not been elucidated.

TABLE I

Growth of the coloured Bacillus *species in nutrient broth made up with different peptone brands*

Bacillus strain	Growth (mg/ml)				
	A*	B*	C*	D*	E*
4	4·4	0	1·6	0	4·9
4*a*	4·8	1·5	0	0	5·5
8	3·8	0·7	0	0	4·8
11	5·1	5·6	5·6	4·9	5·2
ACSL	4·1	0·6	0·9	0	6·3

* A: Difco Bacto peptone
B: Difco Proteose peptone
C: Difco tryptone
D: Oxoid tryptone
E: Oxoid Bacteriological peptone.

All the strains produced acid but no gas from glucose, galactose, fructose, maltose, sucrose, raffinose, dextrin, starch and glycerol; no strain produced acid from xylose, arabinose, lactose or inositol; only strains 8 and 11 utilized mannitol. No growth occurred in glucose broth or glucose nitrate broth under anaerobic conditions. All strains hydrolysed starch, gelatin and casein.

Only strain 11 grew on potato plugs. None of the strains produced indole, acetyl methyl carbinol, urease or utilized citrate. All strains grew in 5 per cent NaCl broth; strains 8, 11 and ACSL grew in 7 per cent NaCl broth; only strains 8 and 11 tolerated 10 per cent NaCl. All strains produced a wide zone of hydrolysis on milk agar. The coloured bacilli did not grow in a medium containing ammonium salts as the sole nitrogen source.

Pigmentation.—The pigments of all strains were hypophasic and behaved as 'alcohols' in the fractionation procedure of Starr and Stephens (1964). None of the other fractions contained any pigment. The pigments produced by strains 4, 8, 11 and ACSL were similar and differed from that of 4*a*. The spectrophotometric behaviour of the pigments of the former strains changed with the age of the culture. Figure 1 shows the absorption spectrum obtained

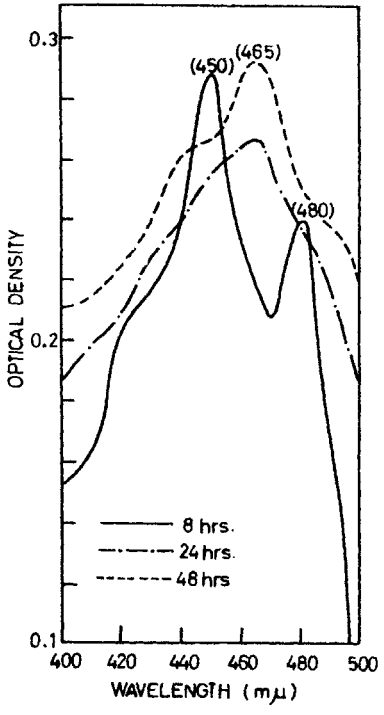

FIG. 1. Absorption spectra of pigment of *Bacillus* strain ACSL at various stages of growth (in methanol).

with the pigment of strain ACSL at various stages of growth. The spectral absorption curve of the pigment of strain 4*a*, on the other hand, was the same irrespective of the age of the culture (Fig. 2). It resembled the spectrum of the pigment from young cultures of the other four strains (peaks in the region 450 and 480 mμ, in methanol). The absorption maxima of the pigments

in various solvents are given in Table II. All the pigments gave a blue colour with concentrated H_2SO_4 but not with HCl. It may be pointed out that the above data are based on the fractions obtained after portioning between solvents and not on purified pigments. It may be mentioned that, though

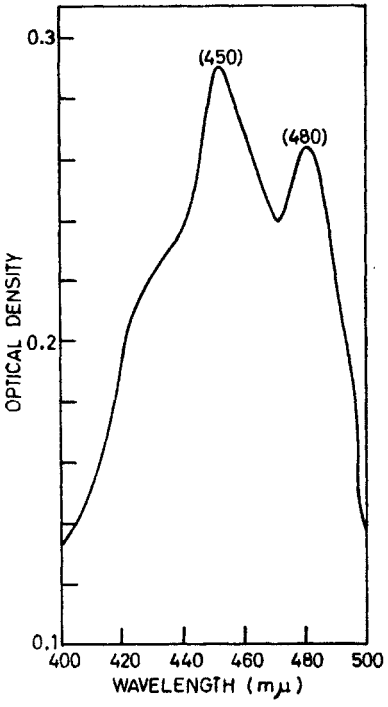

FIG. 2. Absorption spectrum of pigment of *Bacillus* Strain 4*a* (in methanol).

TABLE II

Absorption maxima of* Bacillus *pigments*

Solvent	Pigment from young cultures (mμ)	Pigment from old cultures (mμ)
Benzene	440, 462, 495	(455), 480 †
Carbon disulphide	(435), 455, 480	(474), 495, (525)
Ethyl alcohol	425, 452, 495	(445), 465, (485)
Hexane		(445), 465, (485)
Methanol	(435), 450, 480	(445), 465, (485)

* Parentheses indicate shoulder centred around that approximate wavelength.

† Not measured beyond 500 mμ.

no concerted effort was made, work of a preliminary nature, using chromatography on celite-magnesia columns, seemed to indicate that the carotenoid 'alcohol' from a 72-hour culture of strain ACSL contained a single pigment.

Cells harvested at various stages of growth contained essentially the same amount of pigment (Fig. 3) though, as has been mentioned, the spectral

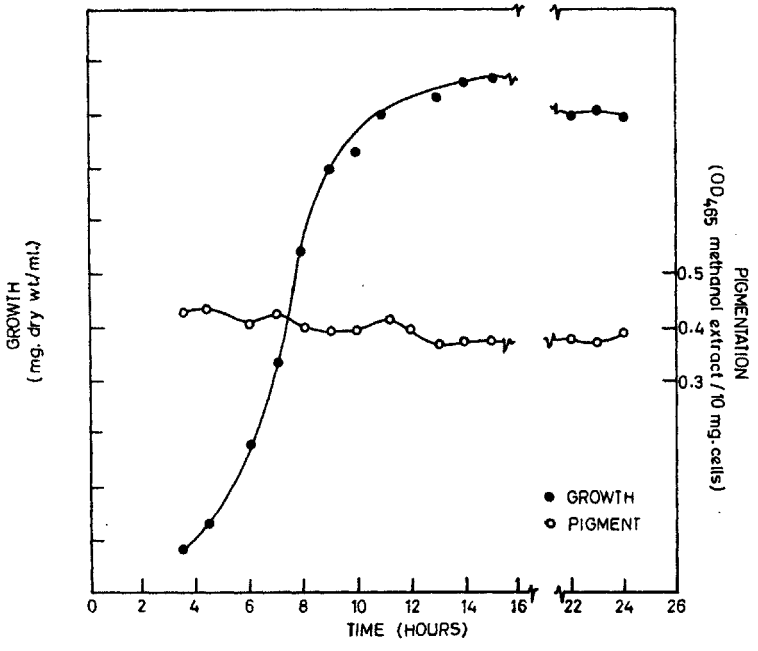

FIG. 3. Growth and pigmentation of *Bacillus* strain ACSL in nutrient broth (shake culture 250 rev./min. at 25° C).

behaviour of the pigment changed. Shaking (on a rotary shaker, 250 rev./min.) speeded up growth; the total cell yield, however, was the same in shake or static cultures. The extent of pigmentation remained constant (Table III). Growth and pigmentation were the same whether the cultures were incubated at 25° C or 37° C (Table III). Diphenylamine, in concentrations that did not affect growth, completely suppressed pigmentation (Table III) (Jensen 1965; Turian and Haxo 1952).

DISCUSSION

The ability of four of the coloured bacilli to grow better on nutrient agar than on glucose agar points to their belonging to the *firmus-lentus* group, one of the less studied groups of the genus *Bacillus*. Their inability to hydrolyse urea and their ability to hydrolyse casein point to *B. firmus*. However, differences are evident: the ability of some of our isolates to grow in 10 per cent NaCl broth, for example. It is of interest to note that Bergey's Manual

(Breed *et al.* 1957) describes the growth of *B. firmus* on agar slants as 'rarely pink' and that Smith, Gordon and Clark (1952) report that 3 out of their 19 strains were pink. The results presented in this paper point to the occurrence of carotenoid pigments in *Bacilli* resembling *B. firmus*, but whether it is the same as the pink pigment remains to be proved. None of our isolates can be frankly described as pink. The identity of strain 11 is somewhat uncertain. Its ability to grow equally well on nutrient agar and glucose agar does not permit its being included in the *firmus-lentus* group. On the other hand, its similarity to the other four strains makes us loath to consider

TABLE III

Effect of temperature, shaking and diphenylamine on growth and pigmentation of Bacillus *strain 11*

Variable*	Maximum Growth (mg dry wt/ml)	Pigmentation (O.D. 465 methanol extract/10 mg cells)
A: *Temperature of incubation* †		
25° C	5·3	0·47
37° C	5·5	0·5
B: *Shaking* ‡		
Shake culture	5·6	0·49
Static culture	5·3	0·47
C: *Diphenylamine*		
0·01 mg/ml	5·7	0
0·02 mg/ml	5·5	0
0·04 mg/ml	5·7	0
None	5·9	0·51

* In all cases nutrient broth was the medium.
† Static culture.
‡ Temperature of incubation: 25° C.

it a different species though we do not consider it impossible that more than one species of *Bacillus* contains the same pigment. At the same time, it is possible that variants of *B. firmus* able to grow at low *p*H might exist.

The present study does not permit an identification of the *Bacillus* carotenoids. However, we hope that this report will serve as an incentive to a more detailed study of the carotenoids and other pigments of the genus *Bacillus*. Though several of the *Bacillus* species are reported to produce coloured variants (Breed *et al.* 1957) nothing is known about these pigments or the factors that favour their production. Pulcherrimin, as was mentioned, is a notable exception.

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REFERENCES

Breed, R. S., Murray, E. G. D., and Smith, N. R. (1957). Bergey's Manual of Determinative Bacteriology, 7th Ed. The Williams and Wilkins Co., Baltimore.

Canale-Parola, E. (1963). A red pigment produced by aerobic spore-forming bacteria. *Arch. Mikrobiol.*, **46**, 414–427.

Dias, F. F., Agate, A. D., Bhat, J. V., Pillai, S. C., and Aswathanarayana, N. V. (1965). *Aeromonas punctata* in *Puntius* species. *Curr. Sci.*, **34**, 81-82.

Jensen, S. L. (1965). Biosynthesis and function of carotenoid pigments in micro-organisms. *A. Rev. Microbiol.*, **19**, 163–182.

Smith, N. R., Gordon, R. E., and Clark, F. E. (1952). Aerobic spore-forming bacteria. U.S. Dept. of Agriculture, Agriculture monograph No. 16.

Society of American Bacteriologists. (1957). Manual of microbiological methods. McGraw-Hill Book Co., Inc., N.Y.

Starr, M. P., and Stephens, W. L. (1964). Pigmentation and taxonomy of the genus *Xanthomonas*. *J. Bact.*, **87**, 292–302.

Turian, G., and Haxo, F. (1952). Further use of diphenylamine for the study of carotenoid biosynthesis in *Mycobacterium phlei*. *J. Bact.*, **63**, 690-691.