

PRELIMINARY STUDIES OF THE PIGMENTS OF
FLAVOBACTERIUM BREVE NCTC 11099 AND FLAVOBACTERIUM ODORATUM NCTC 11036

O.B. Weeks

Arts and Sciences Research Center
New Mexico State University
Las Cruces, New Mexico 88003, USA

Summary

The pigments of Flavobacterium breve NCTC 11099 and Flavobacterium odoratum NCTC 11036 are extractable with acetone and each consists of a major constituent and three or more minor pigments. The electronic absorption spectrum of the major pigment of each species shows a single maximum absorption and lack of fine structure. The principal pigment of F. breve shows maximum absorption at 435 nm in diethyl ether or 442 nm in ethanol. That of F. odoratum occurs at 437 nm in diethyl ether or 450 nm in ethanol. Each pigment demonstrates an alkaline-catalyzed bathochromic spectral shift which is completely reversed by acid. F. breve shows a bathochromic shift of 36 nm and F. odoratum, 28 nm. The pigments are not carotenoid in nature.

Introduction

Flavobacterium breve and F. odoratum recently have been subjected to extensive studies which resulted in revised descriptions for the species and the selection of neotype strains [4,5]. F. breve has also been proposed as the type species of the genus to replace F. aquatile [6]. Chemical studies of the pigments of the two species have been commenced using the neotype designates F. breve NCTC 11099 and F. odoratum NCTC 11036.

Results

Preliminary work to select a growth medium for cultivation of F. breve and F. odoratum showed that a medium containing proteose peptone (0.5%), beef extract (0.2%), yeast extract (0.1%), all Difco products, dissolved in 0.01M phosphate buffer, pH 7.0, was suitable and superior to media containing glucose. The culturing system was 1ℓ medium contained in a 2ℓ flask, and incubated at 30°C, 48 hr., with continuous shaking, 250 rpm. The total culture volume used was 8ℓ. Each culture flask was inoculated with 100 ml of a 24 hr.-old primary culture grown in the same medium.

The F. breve culture grew readily, growth after 48 hr. being equivalent to 1.2 OD units at 580 nm (Spectronic 20, Bausch and Lomb). F. odoratum was more difficult to culture seldom producing growth equivalent to 1.0 OD unit. On one occasion the culture lysed after 30 hr. The two bacterial strains have quite different morphologies when grown as in this study (Figs. 1,2).

The bacterial cells were easily removed from the cultures by centrifugation (Sharples Super Centrifuge, Pennsalt Chemicals Co.). The cells were placed in acetone and then stirred for 1 hr., room temperature. The pigment apparently was completely removed by this treatment. The amount of pigment was estimated spectrally using an $E_{cm}^{1\%}$ value of 2000. On this basis F. breve yielded 0.9 mg total pigment from the 8ℓ and this is equivalent to 0.09 mg/OD unit·ℓ. F. odoratum yielded 3.75 mg from the 8ℓ which was equivalent to 0.47 mg/OD unit·ℓ. F. odoratum produced almost 6 times more pigment than F. breve.

The pigment extracts were subjected to phase separation and successive column chromatographic treatments to separate and partially purify the constituents. The acetone was removed (Büchi Rotovapor) and the residue dissolved in chloroform. This solvent was filtered through activated alumina (neutral alumina AG-7, Bio-rad Laboratories) before use to remove the stabilizing ethanol. The chloroform solutions were converted into Bligh and Dyer systems by the addition of methanol and water [3]. After shaking each system was allowed to stand overnight for complete separation of the two phases. The pigment partitioned into the chloroform phase.

The chloroform solutions were partially purified using a silicic acid system [7]. In each instance the entire pigment fraction was eluted with chloroform. The second column system was alumina activity III developed with hexane-acetone mixtures. The chromatographic behavior of the pigments from the two bacterial species was nearly identical. In both instances five colored fractions separated and were eluted from the column. The principal fraction for each species, comprising about 90% of the total pigment separated and eluted with 12% acetone in hexane. Two minor fractions eluted prior to this and another, dark pink, with 15% acetone. Each column chromatographic fraction was chromatographed on Silica Gel G thin-layer plates, 0.25 mm thickness developed with either chloroform:methanol (9:1) or benzene-ethyl ace-

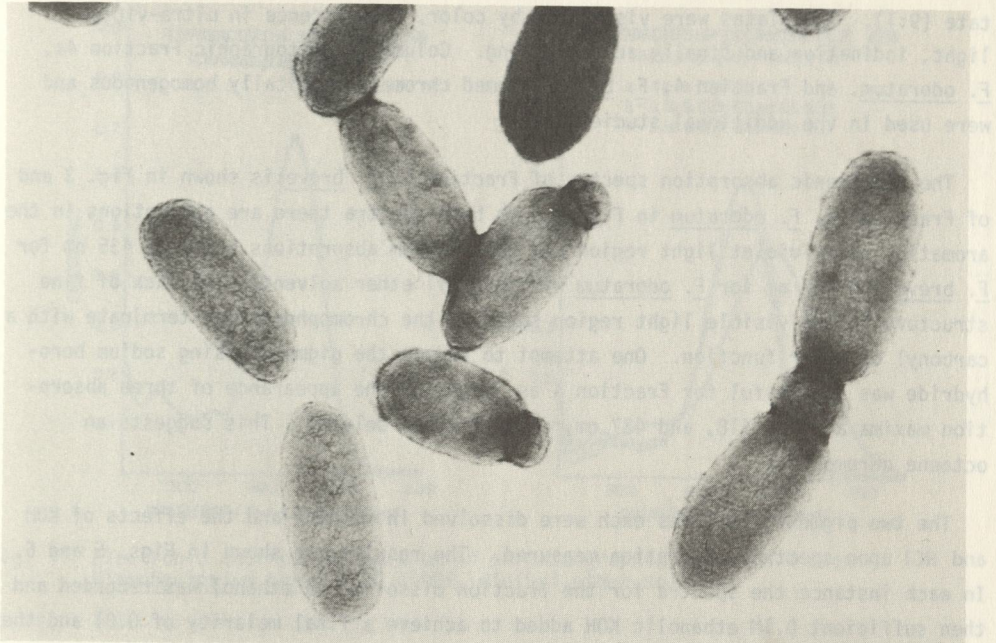


Fig. 1. Electron micrograph of *Flavobacterium breve* NCTC 11099 stained with phosphotungstic acid, 20,000X magnification.

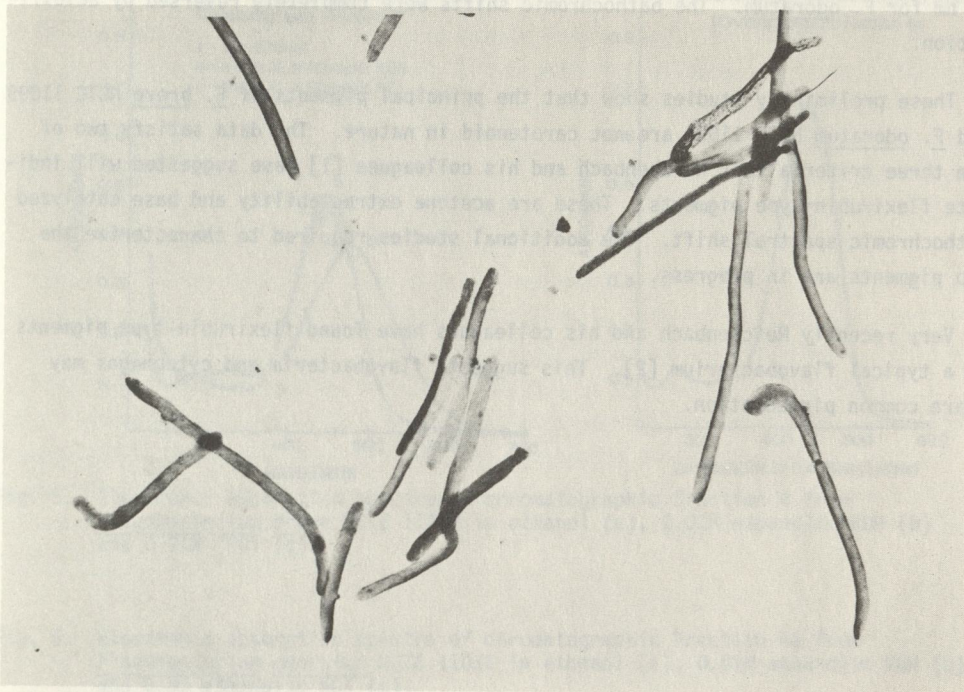


Fig. 2. Electron micrograph of *Flavobacterium odoratum* NCTC 11036, 42,000 X magnification

tate (9:1). The plates were visualized by color, fluorescence in ultra-violet light, iodination and finally acid charring. Column chromatographic Fraction 4a, F. odoratum, and Fraction 4, F. breve, seemed chromatographically homogeneous and were used in the additional studies.

The electronic absorption spectra of Fraction 4, F. breve is shown in Fig. 3 and of Fraction 4a, F. odoratum in Fig. 4. In both spectra there are absorptions in the aromatic, ultra-violet light region and the maximum absorptions occur at 435 nm for F. breve and 437 nm for F. odoratum with diethyl ether solvent. The lack of fine structure in the visible light region suggests the chromophores may terminate with a carbonyl or ester function. One attempt to reduce the pigments using sodium borohydride was successful for Fraction 4 as judged by the appearance of three absorption maxima at 395, 418, and 437 nm, diethyl ether solvent. This suggests an octaene chromophore.

The two pigment fractions each were dissolved in ethanol and the effects of KOH and HCl upon spectral absorption measured. The results are shown in Figs. 5 and 6. In each instance the spectra for the fraction dissolved in ethanol was recorded and then sufficient 0.1M ethanolic KOH added to achieve a final molarity of 0.01 and the spectra again recorded. Finally the alkaline solutions were acidified using 0.5M ethanolic HCl and the spectra measured. The results show that the absorption maximum for each pigment fraction was bathochromically displaced, 36 nm for F. breve and 28 nm for F. odoratum. The bathochromic shifts were completely reversed by acidification.

These preliminary studies show that the principal pigments of F. breve NCTC 11099 and F. odoratum NCTC 11036 are not carotenoid in nature. The data satisfy two of the three criteria that Reichenbach and his colleagues [1] have suggested will indicate flexirubin-type pigments. These are acetone extractability and base catalyzed bathochromic spectral shift. The additional studies required to characterize the two pigments are in progress.

Very recently Reichenbach and his colleagues have found flexirubin-type pigments in a typical flavobacterium [2]. This suggests flavobacteria and cytophagas may share common pigmentation.

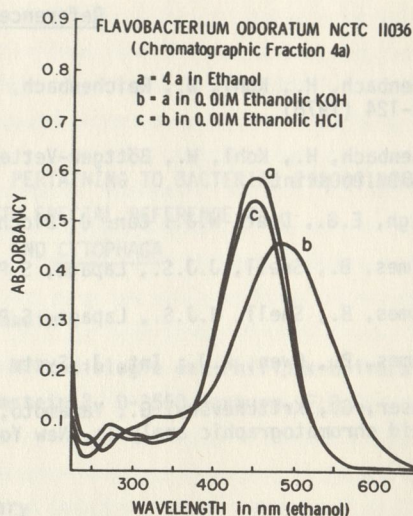
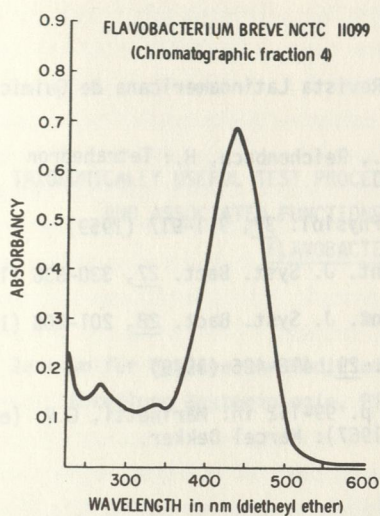


Fig. 3. Electronic absorption spectrum of chromatographic Fraction 4 from *Flavobacterium breve* NCTC 11099, diethyl ether solvent.

Fig. 4. Electronic absorption spectrum of chromatographic Fraction 4a from *Flavobacterium odoratum* NCTC 11036, diethyl ether solvent.

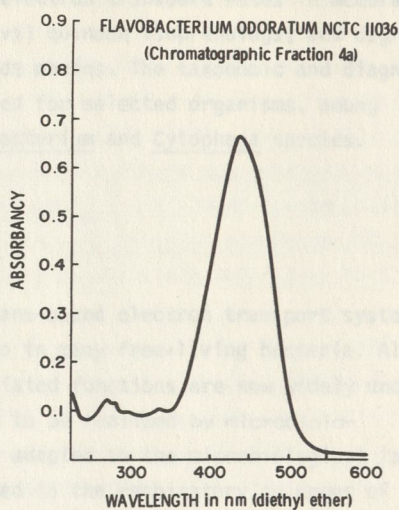
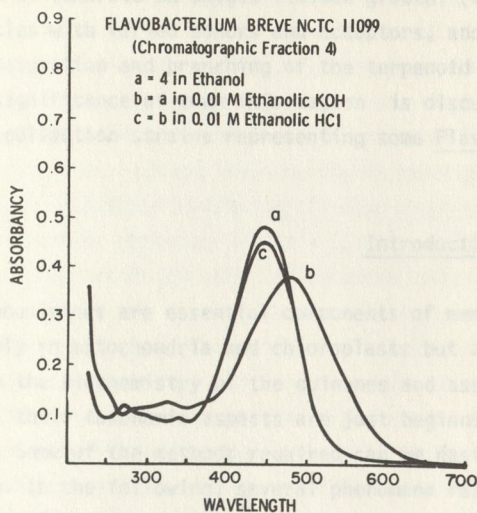


Fig. 5. Electronic absorption spectra of chromatographic Fraction 4 from *Flavobacterium breve* NCTC 11099 in ethanol (a), 0.01M ethanolic KOH (b) and 0.01M HCl (c).

Fig. 6. Electronic absorption spectra of chromatographic Fraction 4a from *Flavobacterium odoratum* NCTC 11036 in ethanol (a), 0.01M ethanolic KOH (b) and 0.01M ethanolic HCl (c).

References

- [1] Achenbach, H., Kohl, W., Reichenbach, H.: *Revista Latinoamericana de Quimica* 9: 111-124 (1978)
- [2] Achenbach, H., Kohl, W., Böttger-Vetter, A., Reichenbach, H.: *Tetrahedron* 1981, in print
- [3] Bligh, E.G., Dyer, W.J.: *Can. J. Biochem. Physiol.* 37, 911-917 (1959)
- [4] Holmes, B., Snell, J.J.S., Lapage, S.P.: *Int. J. Syst. Bact.* 27, 330-336 (1977)
- [5] Holmes, B., Snell, J.J.S., Lapage, S.P.: *Int. J. Syst. Bact.* 28, 201-208 (1978)
- [6] Holmes, B., Owen, R.J.: *Int. J. Syst. Bact.* 29, 416-426 (1979)
- [7] Rouser, G., Kritchevsky, G., Yamamoto, A.: p. 99-162 in: Marinetti, G.V. (ed.), *Lipid chromatographic analysis*. New York (1967): Marcel Dekker.