Taxonomically Significant Colour Changes in *Brevibacterium linens* Probably Associated with a Carotenoid-like Pigment

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SUMMARY

Ninety-three coryneform bacteria were tested with various acids and bases and on the basis of these tests, five of the strains were identified as *Brevibacterium linens*. This identification correlated with that made by other methods, thus confirming the suggestion of Grecz & Dack (1961) that *B. linens* could be identified by certain colour reactions. Further studies indicated that these reactions are probably associated with the presence of a specific carotenoid-like substance located in the bacterial membrane.

INTRODUCTION

Grecz & Dack (1961) reported that *Brevibacterium linens* (78 strains) gave characteristic colour reactions when the yellow-orange surface growth was treated with various acids and bases. A characteristic red colour was produced with strong bases and various shades of green and blue with strong acids. No colour changes were produced with weak bases nor with weak acids, such as lactic or formic. Under certain conditions specific colour reactions were produced with acetic acid and aniline. Yellow pigmented strains of *Micrococcus*, *Mycobacterium*, *Sarcina* and *Staphylococcus* did not give the same reactions. Grecz & Dack suggested that the colour reactions produced with strong bases and acetic acid could be used for the rapid presumptive identification of *Brevibacterium linens*. However, they did not include in their survey any yellow coryneform bacteria which are frequently confused with *B. linens*, nor did they attempt to define the chemical basis of the reaction.

In view of the potential value of these tests for the identification of *Brevibacterium linens*, the reactions of a number of yellow, orange, pink and non-pigmented coryneform and related bacteria were investigated using the various acids and bases. An attempt was made also to characterize the pigment constituent responsible for the colour changes.

METHODS

Strains

The bacteria used were:* *Brevibacterium linens* ATCC9174; NCIB8546; *B. stationis* ATCC 14403; *B. leucinophagum* ATCC13809; *B. imperiale* ATCC8365; *B. acetylicum* ATCC953.

Arthrobacter pascens NCIB8910; *A. oxydans* NCIB9333; *A. aurescens* NCIB8912; *A. urea-fasciens* NCIB7811, Morris 8916 (from Professor J. G. Morris); *A. citreus* NCIB8915, Morris

* ATCC, American Type Culture Collection; NCIB, National Collection of Industrial Bacteria; NCTC, National Collection of Type Cultures; NCPPB, National Collection of Plant Pathogenic Bacteria.

8908 (from Professor J. G. Morris); *A. terregens* NCIB8909; *A. flavescens* NCIB9221; *A. nicotianae* NCIB9458; *A. simplex* Morris 8913 (from Professor J. G. Morris); *Arthrobacter* spp. G93, G109, G154, G162, J39, J52 (from Dr R. M. Keddie), Morris TG1 (from Professor J. G. Morris), 251, 365, 367, 368 (from Dr E. G. Mulder).

Cellulomonas uda ATCC491; *C. cellasea* NCIB8078; *C. fimi* NCIB8980; *C. gelida* NCIB8076; *C. flavigena* NCIB8073; *C. bibula* NCIB8142; *C. subalbus* NCIB8075.

Corynebacterium vesiculare ATCC11426; *C. fascians* ATCC12974, ATCC12975, NCPPB1488; *C. ilicis* ATCC14264; *C. michiganense* NCPPB1468; *C. betae* NCPPB363; *C. flaccumfaciens* NCPPB559; *C. rathayi* NCPPB797; *C. poinsettiae* NCPPB844; *C. renale* NCTC7448; *C. equi* NCTC1621; *C. aquaticum* NCIB9460; *C. manihot* NCIB9097; *C. mediolanum* NCIB7206; *C. rubrum* NCIB9433; *C. diphtheriae* NCTC3989; *Corynebacterium* spp. NCTC7510, EB/F64/100 (from Dr E. M. Barnes), 6-10, 7-11, 8-7, 11-10 from Dr H. E. Schefflerle), 3A-4302, 3A-4794 (from Miss C. Shaw), C206 (M.R.C. Collection, Leicester); 'Coryneform' NCIB8181, C311 (M.R.C. Collection, Leicester).

Kurthia zopfii NCIB8603, K2, S8, S9 (from Dr R. M. Keddie), Gardner 8 (from Dr G. A. Gardner).

Microbacterium flavum NCIB8707; *M. lacticum* NCIB8540, 8541; *M. thermosphactum* ATCC11509.

Mycobacterium phlei NCTC8151; *M. smegmatis* NCTC8159; *M. fortuitum* ATCC6841; *M. rhodocrous* NCTC8139, NCTC10210, 21, 417, 1240, 3408, 7698 (from Dr R. E. Gordon).

Nocardia vaccinii ATCC11092; *N. cellulans* NCIB8868; *N. hydrocarboxydans* NCIB9436; *N. petroleophila* NCIB9438; *N. saturnea* NCIB9437; *N. brasiliensis* 744 (from Dr R. E. Gordon); *Nocardia* sp. 245 (from Dr M. Goodfellow); *N. turbata* C192, C193, C194 (M.R.C. Collection, Leicester).

Propionibacterium shermanii NCIB8099.

Media

The bacteria were maintained on Nutrient Agar (Oxoid), and subcultured at 7 to 14 day intervals. The same medium was used for the culture of the bacteria to be tested except for *Arthrobacter terregens* which was grown on a soil extract medium.

Reagents

Aniline, acetic acid, potassium hydroxide, syrupy phosphoric acid (Analar grade, British Drug Houses Ltd, London); sodium hydroxide, sulphuric acid (Analar grade, Fisons Ltd, Loughborough, Leicestershire); lithium hydroxide (Laboratory Reagent grade, Fisons Ltd).

Colour reactions

A small sample of bacterial growth from the surface of an agar plate (incubated at 30 °C for periods up to 21 days) was transferred with a wire loop to a white tile to test for colour changes with sodium hydroxide (5 N), potassium hydroxide (5 N), lithium hydroxide (saturated solution), syrupy phosphoric acid and concentrated sulphuric acid. One or two drops of reagent were placed directly on the growth sample and colour changes noted at intervals up to 30 min; any reaction was usually detectable in less than 5 min. In testing with glacial acetic acid and aniline the bacterial growth was rubbed with a glass rod on to a piece of filter paper flooded with the reagent. Firm, smooth filter paper, e.g. Whatman no. 1, which does not tear easily during rubbing, was used.

Extraction of pigment

The following bacteria were used: *Brevibacterium linens* NCIB8546, ATCC9174; *Arthrobacter citreus* NCIB8915; *Arthrobacter* sp. 251; *Corynebacterium fascians* ATCC12974, ATCC12975, NCPPB1488; *Corynebacterium* spp. NCTC7510, NCIB8181, 8-7, 11-10, 3A-4302; *Mycobacterium phlei* NCTC8151; *M. smegmatis* NCTC8159; *Nocardia hydrocarboxydans* NCIB9436; *N. petroleophila* NCIB9438; *N. turbata* C192.

The bacteria were grown in 500 ml Nutrient Broth no. 1 (Oxoid) in 2 l conical flasks in an incubator shaker at 30 °C for 48 h (*Nocardia hydrocarboxydans* and *N. petroleophila* for 7 days). Bacteria were harvested by centrifuging at 6000 g for 20 min. The pellet was re-suspended in 10 ml of boiling methanol (65 °C), agitated by shaking and allowed to stand for 30 min. The debris was removed by centrifugation at 2000 g for 10 min. The supernatant was examined spectrophotometrically and by thin-layer chromatography.

Thin-layer chromatography

Hot methanol extracts (10 ml) prepared as described above were evaporated to 1 ml by heating in a dish over boiling water, and then spotted on Kieselgel G nach Stahl (E. Merck A.G. Darmstadt, Germany) plates which had been activated for 1 h at 80 °C. Plates were developed in one of three solvent systems: 30 % (v/v) ethanol/petroleum ether; 50 % (v/v) ethanol/petroleum ether; 80 % (v/v) ethanol/petroleum ether. R_F values were determined visually.

Spectral analysis

Methanol extracts prepared as described were examined without further treatment in a Shimadzu 'MPS-50L' spectrophotometer.

Distribution of pigment

Brevibacterium linens NCIB8546 was grown in Nutrient Broth no. 1 (Oxoid). Approximately 6 g wet wt bacteria were suspended in 10 ml 0.005 M-Na K phosphate buffer, pH 7.2, and sonicated for 5 min in 1 min periods with intervals for cooling. The sonicated material was centrifuged at 26000 g for 15 min to obtain a bacterial extract. The supernatant from the first centrifugation was recentrifuged at 157000 g for 1 h to obtain a membrane fraction. Absorption spectra were obtained for the pellet and supernatant from the second centrifugation in a Shimadzu 'MPS-50L' spectrophotometer. Relative concentrations of pigment in each fraction were determined as the $\Delta E_{450\text{nm}}$ /mg protein and $\Delta E_{480\text{nm}}$ /mg protein. Protein was determined by a modification of the biuret method (Gornall, Bardawill & David, 1949).

RESULTS

Colour reactions

Sodium hydroxide, potassium hydroxide, lithium hydroxide. Only 16 bacteria out of the 93 tested gave any colour change with NaOH, KOH, LiOH. All 16 gave a red colour but the pink-red colour produced by the two strains of *Brevibacterium linens* and three other cultures received as *Arthrobacter* sp. (1) and *Corynebacterium* spp. (2) was easily distinguished from the orange-red colour produced by the 11 strains received as *Corynebacterium fascians* (3), *Corynebacterium* spp. (3), *Mycobacterium phlei* (1), *M. smegmatis* (1), *Nocardia hydrocarboxydans* (1), *N. saturnea* (1), *N. petroleophila* (1). The orange-red colour did not appear to be specific for any particular taxonomic grouping. All the 16 bacteria which gave a red

Table 1. *Colour reactions and spectrophotometric analyses of some yellow pigmented and related bacteria*

Organisms	Colour reactions		$\lambda_{\max}$ (± 5 nm) in methanol							
	CH ₃ COOH	NaOH, KOH, LiOH								
<i>Brevibacterium linens</i>										
ATCC9174	SP	RP	—	400	—	—	450	475	—	—
ATCC8546	SP	RP	—	400	—	—	450	475	—	—
<i>Arthrobacter</i> sp. 251	SP	RP	—	400	—	—	450	475	—	—
<i>Corynebacterium</i> sp.										
8-7	SP	RP	—	400	—	—	450	475	—	—
11-10	SP	RP	—	400	—	—	450	475	—	—
C206	—	O	.	.	.	.	.	.	.	.
3A-4302	—	O	—	400	—	—	455	480	—	—
<i>Corynebacterium fascians</i>										
ATCC 12974	—	O	—	400	—	—	460	480	—	—
ATCC 12975	—	O	—	400	—	—	455	480	—	—
NCPPB 1488	—	O	—	400	—	—	460	480	—	—
<i>Mycobacterium phlei</i> NCTC8151	—	O	—	400	—	—	450	475	—	—
<i>M. smegmatis</i> NCTC8159	—	O	—	400	—	—	460	480	—	—
<i>Corynebacterium</i> sp. NCTC7510	—	O	—	400	—	—	—	—	—	560
<i>Nocardia hydrocarboxydans</i>										
NCIB9436	—	O	—	400	—	—	450	475	—	—
<i>N. saturnea</i> NCIB9437	—	O	.	.	.	.	.	.	.	.
<i>N. petroleophila</i> NCIB9438	—	O	—	400	—	—	450	475	—	—
<i>N. turbata</i> C192	—	—	—	400	420	440	—	470	—	—
<i>Arthrobacter citreus</i>										
NCIB8915	—	—	—	400	—	435	—	470	—	—
'Coryneform' NCIB8181	P	—	360	—	425	440	—	470	500	—

P, purple; SP, salmon pink; RP, red pink; O, orange; —, no change; ., not done.

colour produced orange or yellow pigment but in some cases the pigment was not fully apparent until the cultures were 14 days old. However, in every case, the colour was produced by 2- to 5-day cultures. The results are given in Table 1.

Sulphuric acid. The 93 bacteria tested gave a series of blue, green and brown colour reactions with sulphuric acid. The colours produced were not characteristic of any genus or species of bacteria, neither were they associated with the production of a pigment of any particular colour nor with the age of the culture.

Syrupy phosphoric acid. The results of testing with this reagent were not easy to interpret. Many of the 93 bacteria tested gave green reactions with syrupy phosphoric acid but in most cases the green colour became brown after 20 to 30 min. No blue colour such as that reported by Grecz & Dack (1961) was noted with any cultures even though the tests were allowed to stand overnight.

Acetic acid. Only five of the strains tested gave a positive salmon pink reaction when growth was rubbed onto filter paper flooded with glacial acetic acid. These were the same bacteria which gave the characteristic pink-red colour with strong bases. No significant colour change was noted with any of the other bacteria with the exception of the coryneform organism NCIB8181 which produced a purple colour (see Table 1).

Aniline. In our hands the reaction of the bacterial growth to aniline was not as clear-cut as that recorded by Grecz & Dack (1961). The brick-red colour noted by them was never seen. A very faint pink colour was shown by three strains only, these were *Brevibacterium linens* NCIB8546, *Arthrobacter* sp. 251 and *Corynebacterium* sp. 11-10.

Table 2. A comparison of thin-layer chromatography of methanol extracts of *Brevibacterium linens* and other bacteria

Bacteria	R_F values*				
<i>Brevibacterium linens</i> ATCC9174	0.91		0.81		
<i>B. linens</i> NCIB8546	0.91		0.80		
<i>Corynebacterium fascians</i> ATCC 12974		0.85		0.06	0.02
<i>Arthrobacter citreus</i> ATCC8915	Trailing yellow line from base				

* The developing system was 80 % (v/v) ethanol/petroleum ether.

Pigment studies

Spectrophotometric analyses. Spectrophotometric analyses of methanol extracts of 14 of the 16 strains which gave pink-red or orange-red reactions with strong bases are listed in Table 1. In addition the coryneform culture NCIB8181 and two organisms (*Arthrobacter citreus* NCIB8915 and *Nocardia turbata* C192) which gave no significant colour changes with any of the reagents were included for comparison. Lowering the pH of the methanol extracts to approx. 4 had no effect on the $\lambda_{\max}$. Raising the pH to approx. 9 resulted in no shifting of $\lambda_{\max}$ but did result in an intensification of the absorption in the 500 to 550 nm region which correlates with the red colour produced with strong bases.

Thin-layer chromatography. Four strains were examined: two gave the typical pink-red colour reactions with strong bases and the salmon pink colour with acetic acid (*Brevibacterium linens* ATCC9174 and *B. linens* NCIB8546), one gave the orange-red colour reaction (*Corynebacterium fascians* ATCC 12974) and one gave no colour reaction (*Arthrobacter citreus* NCIB8915). Although the relative positions of the spots varied with the developing system used (see Methods) the position of the two *B. linens* spots was always quite characteristic. Typical R_F values are given in Table 2. All the spots, with the exception of two of the spots given by *C. fascians* (ATCC 12974) were yellow. *Corynebacterium fascians* spot approx. R_F value 0.06 was blue and spot approx. R_F value 0.02 was red. These two spots were very near the base line but could be measured because of their characteristic colours. When all the spots on the plates were tested with KOH and NaOH only one spot given by each of the two strains of *B. linens* (R_F values 0.81 and 0.80) gave the characteristic pink colour.

Spectral investigation of the material eluted from these spots gave one peak at a $\lambda_{\max}$ value of 455 nm with a slight shoulder at 475 nm.

Distribution of pigment. Investigation of the distribution of the carotenoid pigment of *Brevibacterium linens* NCIB8546 as described under Methods indicated that the carotenoid was in the membrane fraction. ($\Delta E_{450\text{nm}}$ /mg protein was 0.075 for membrane fraction and 0.026 for the supernatant fraction; $\Delta E_{480\text{nm}}$ /mg protein was 0.062 for the membrane fraction and 0.019 for the supernatant fraction.) The wall fraction was unpigmented. These results agree with previous investigations of carotenoid location in bacteria (see for example, Mathews & Sistrom, 1959).

DISCUSSION

Of 93 coryneform strains tested only five gave the characteristic colour reactions reported by Grecz & Dack (1961) to be typical of *Brevibacterium linens*. Two were culture collection strains of *B. linens* (ATCC9174 and NCIB8546), but the other three were received as *Corynebacterium* spp. (8-7 and 8-11) and *Arthrobacter* sp. 251. Morphologically the three latter strains resembled other bacteria (especially *Corynebacterium* and *Arthrobacter* species)

amongst the 93 bacteria tested. Concurrently a numerical taxonomic survey was being conducted on 233 coryneform and related bacteria including the 93 strains used here (D. Jones and J. Watkins, unpublished). The results of the taxonomic work showed a close relationship (85 % similarity) between the five bacteria which gave the characteristic colour reactions. This close correlation between the colour reaction of the strains and the numerical taxonomic grouping reinforces the suggestion made by Grecz & Dack (1961) that the colour tests devised by them provide a simple and quick method for the identification of *B. linens*.

Spectroscopy (u.v. and visible) of hot methanol extracts indicated the presence of carotenoids in all bacteria tested (Table 1). Only those bacteria which gave a colour reaction with strong bases showed $\lambda_{\max}$ at about 450 nm and there were no obvious differences between the spectra of the bacteria (*Brevibacterium linens* group) giving a pink-red colour reaction and those giving an orange-red reaction, with the exception of NCTC 7510. However, thin-layer chromatographs of methanol extracts from representative bacteria (Table 2) showed a marked difference in relative R_F values between the two *B. linens* cultures (pink-red with strong bases) and *Corynebacterium fascians* (orange-red with strong bases). The carotenoids of *C. fascians* have been studied in detail (Prebble, 1968) but not the carotenoids of *B. linens*. We suggest that strains of *B. linens* contain a characteristic carotenoid in the cell membrane which after determination of its structure could be useful in the chemotaxonomic classification of this economically important group. However, until its chemical structure has been elucidated, the characteristic colour changes apparently due to this carotenoid found by treating intact bacteria with strong bases (KOH, NaOH, LiOH) and acetic acid provide a reliable and fast identifying test for members of the *B. linens* group even when colonies have not fully developed the orange-yellow pigment. This sort of test is especially valuable in detecting coryneforms which include so many morphologically similar yellow pigmented bacteria whose status can only be determined after making many tedious tests.

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