Cultural and Biochemical Characteristics of the Genus *Chromobacterium*

By P. H. A. SNEATH

*Microbiological Research Department, Ministry of Supply, Porton, Wiltshire,
and now National Institute for Medical Research, Mill Hill, London, N.W. 7*

SUMMARY: The cultural and biochemical characteristics of 38 strains of *Chromobacterium* are described. They are non-sporing aerobic Gram-negative rods which produce a violet pigment and possess both polar monotrichous and peritrichous flagella. They produce little acidity from carbohydrates and utilize citrate.

Two main groups of strains were distinguishable, a mesophilic and a psychrophilic group. The former usually ferment glucose anaerobically, are markedly proteolytic, facultatively anaerobic, and give a positive egg-yolk reaction, but do not hydrolyse aesculin. The psychrophilic organisms produce acid from glucose but only aerobically, are poorly proteolytic, strictly aerobic, and hydrolyse aesculin but do not produce an egg-yolk reaction.

Chromobacterium viscosum Grimes, *C. iodinum* Davis and *C. ianthinum* Gilman have been re-examined; they should be excluded from the genus. The nomenclature of the genus is confused. Proposals have therefore been made to the International Committee on Bacteriological Nomenclature that the mesophilic species should be called *Chromobacterium violaceum* and the psychrophilic species *C. lividum*, and neotype strains have been suggested.

The genus *Chromobacterium* comprises a group of bacilli producing on ordinary media a characteristic violet pigment which is soluble in ethanol but not in water or chloroform. The genus was defined in these terms by Buchanan (1918): this is a narrow definition of the genus, excluding the *Bacillus prodigiosus* group (*Serratia*) which is not considered here. These organisms are generally known as *Chromobacterium violaceum* (in the older literature *Bacillus violaceus* or *Bacterium violaceum*). They are a poorly known group, and the classification and taxonomy are in confusion. About 70 blue or violet chromogens are described in the literature, but authentic strains of the great majority of them are not available. The work reported here is an examination of available strains which can reasonably be placed in this genus, or which have been described under this generic name. The most extensive work on the genus has been that of Bampton (1918), Calderini (1925) and Cruess-Callaghan & Gorman (1933). Early in the present work it was noted that the difference in growth temperature, emphasized by the last-named authors, divided the strains into two groups, those which grow at 37° but not at 4° and those which grow at 4° but not at 37°. This division has proved to be fairly sharp, and is correlated with a number of other characteristics. For brevity therefore they are referred to as the mesophils and the psychrophils respectively.

METHODS

Variable results are obtained with different techniques and growth temperatures and times, and for this reason the techniques used here are described in considerable detail. All tests, except where otherwise stated, were carried out at 25°, at which temperature the growth rates of the mesophils and the psychrophils are about equal. The inability of single organisms to multiply on nutrient agar, the rapid dying-out of strains in culture and the inability of some strains to grow at high temperatures upon over-dried plates, necessitated setting up many controls, and preferably inoculation into condensation water on slopes of solid media. Many out-dated tests were performed as an aid to the revision of the nomenclature of the genus.

The source of the strains studied is shown in Table 1. Besides the 38 strains of *Chromobacterium*, three bacteria, which have in the past been placed in this genus, *Chromobacterium viscosum* Grimes 1927, *C. iodinum* Davis 1939, and *C. ianthinum* Gilman 1953, were studied since they are believed to be authentic strains. They are referred to here under the names *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI respectively (see Table 1) to avoid confusion with the true *Chromobacterium* strains, without implying that these are their correct names. Another organism which has been placed in this genus is *Chromobacterium prodigiosum* Topley & Wilson 1929 (*Serratia marcescens*), here referred to as 'Bacillus prodigiosus'; two strains (NCTC 1377 and NCTC 9493) were studied together with two strains of *Pseudomonas aeruginosa* (NCTC 2000 and NCTC 6749) for comparison. In my opinion neither organism should be classified in the same genus as *Chromobacterium*, and the results with them are only given where they are of interest. Another strain of *C. iodinum* (strain RE) which is the authentic culture of Davis, was obtained late in the investigation; it was not fully investigated, but is clearly the same organism as ATCC 9897. Strain SH is that of Black & Shahan (1938); strains SL and RV are those of Sippel, Medina & Atwood (1954); strains BH and BN are from cases 11 and 12, strains FH, MK and LG are the Malayan water strains and strain TV is the Trinidad strain of Sneath, Whelan, Singh & Edwards (1953); strain EC is strain 16 of Cruess-Callaghan & Gorman (1933); strain DK is that of Darrasse, Mazaud, Giudicelli & Camain (1955). Strains AM and TI are believed to be the strains called *C. amethystinum* and *C. ianthinum* by Gilman (1953).

Staining and morphology

Strains were grown upon nutrient agar at 25° and examined after 18 hr. and 4 days. For general morphology and measurements of size by an eye-piece micrometer, heat-fixed films were stained with Löffler's alkaline methylene blue. Heat-fixed films were stained by the methods of Gram, Ziehl-Neelsen, Albert and Hiss according to the details given in Mackie & McCartney (1948, pp. 82, 86, 88, 90, 94). For Gram staining, both the ethanol method of decolorizing followed by neutral red as counterstain, and the acetone method followed by safranin, were used. Fat staining was by the technique of Burdon (1946)

using Sudan Black B (G. T. Gurr Ltd., London, S.W. 6). Neisser staining was as follows: 0.1 g. methylene blue was dissolved in 2 ml. ethanol, and 95 ml. distilled water and 5 ml. glacial acetic acid were added. Heat-fixed films were stained in this solution for 2 min., rinsed with tap water and counterstained for 1 min. with 0.2% aqueous Bismarck brown. The flagella stain of Leifson (1951) was used.

Table 1. *Source of strains*

Strain letters	NCTC no.	From whom received and date	Name or no. as received	Details of isolation where known			
				Investigator	Source	Country	Date
FH	9373	Whelan, 1952	Frazer's Hill	Whelan	Water	Malaya	1952
MK	9757	Whelan, 1952	Mentekab	Whelan	Water	Malaya	1952
LG	9374	Whelan, 1952	Lake Gardens	Whelan	Water	Malaya	1952
BH	9696	—	—	Sneath	Human urine	Malaya	1951
BN	9694	—	—	Sneath	Human fatal case	Malaya	1952
AM	9370	BSC, 1952	<i>C. membranaceum amethystinum</i>	—	From NCTC in 1935	—	—
MW	9371	MWB, 1952	—	Prof. Thomas	Water	England	—
NT	7917	NCTC, 1952	NCTC 7917	Collins, FBA, strain 2	Lake water	England	1949
TV	—	HCC, 1952	<i>C. violaceum</i>	HCC	Forest soil	Trinidad	1950
LW	8683	ATCC, 1952	ATCC 7461	—	—	—	—
SH	8684	ATCC, 1952	Lewitus strain ATCC 6357	Shahan	Human infection	Florida, U.S.A.	1937
RT	8685	ATCC, 1952	Shahan strain ATCC 553	L. F. Rettger, Yale University	—	—	—
SL	9695	Sippel, 1954	Rettger 4/22 strain	Sippel	Swine infection	Georgia, U.S.A.	1953
RV	9372	Sippel, 1954	Sealy	Sippel	Bovine infection	Georgia, U.S.A.	1952
DK	9376	Darrasse, 1955	Reeves	Sippel	Human infection	Dakar, Africa	1954
MH	9377	U.M., 1952	135	Darrasse	—	—	—
EA	—	Eltinge, 1955	60	Eltinge	Soil	New Hampshire, U.S.A.	1953
EB	—	Eltinge, 1955	30	Eltinge	Dust	Massachusetts, U.S.A.	1952
EC	—	Eltinge, 1955	47 (ATCC 6915)	Prof. Kluyver, strain L91	—	—	—
RU	—	—	—	Sneath	Soil	England	1955
GA	—	—	—	Sneath	Soil	England	1955
DA	—	—	—	Sneath	Soil	England	1955
NC	—	—	—	Sneath	Soil	England	1955
TA	—	HCC, 1955	<i>C. violaceum</i>	Morris, HCC	Water	Trinidad	1955
TB	—	HCC, 1955	<i>C. ianthinum</i>	—	(From Institut Pasteur, Paris)	—	—
MA	—	MWB, 1955	Ch 25	MWB	Water (sand filter)	England	1955
MB	—	MWB, 1955	Ch 27	MWB	Water (sand filter)	England	1955
MC	—	MWB, 1955	Ch 28	MWB	Spring water	England	1955
PT	—	Sippel, 1955	Porter	Sippe	Swine infection	Florida, U.S.A.	1955
HA	—	Hans, 1955	H-4	Hans	Soil	Michigan, U.S.A.	1952
HB	9796	Hans, 1955	H-24	Hans	Soil	Michigan, U.S.A.	1952
HC	—	Hans, 1955	6-15	Hans	Soil	Michigan, U.S.A.	1952
HD	—	Hans, 1955	6-20	Hans	Soil	Michigan, U.S.A.	1952
HE	—	Hans, 1955	Traunstein	Hans	Soil	Germany	1955
HF	—	Hans, 1955	Ulm	Hans	Soil	Germany	1955
IN	—	Hans, 1955	Indiana X	Indiana University	Soil	—	—
CA	—	Hans, 1955	Corpe	Corpe	Soil	U.S.A.	—
PB	—	Hans, 1955	Institut Pasteur Paris, 52227	—	Water	—	1952
GR	2416	NCTC, 1955	NCTC 2416	Grimes	Butter	Ireland	1927
			<i>Corynebacterium viscosum</i>				
TE	9742	ATCC, 1955	ATCC 9897	Davis	Milk	England	1938
			<i>Pseudomonas todtii</i>				
RE	—	NIRD, 1955	<i>Chromobacterium indium</i> Davis	Davis	Milk	England	1938
TI	—	HCC, 1952	<i>C. ianthinum</i>	HCC	Forest soil	Trinidad	1950

Abbreviations. The addresses of workers and of the institutes (indicated above by abbreviations) that provided strains are given in the acknowledgements. Strains RU, GA, DA and NC were isolated by the method of Corpe (1951).

To exclude the formation of heat-resistant spores, 5 ml. nutrient broth cultures and nutrient agar slopes, incubated for 7 days at 25°, were heated at 56° for 30 min. and after adding 5 ml. of fresh broth and incubating at 25° for 4 days, were plated for sterility.

Cultural methods

The peptone used throughout was Bacteriological Peptone from Evans Medical Supplies Ltd., Liverpool. Nutrient broth was prepared according to the method of Hartley (1922): the nutrient agar was this broth solidified with 1.5% (w/v) Japanese agar. The potato slopes were plugs of potato resting on wet cotton wool, steamed on 3 successive days. Gelatin stabs and gelatin plates consisted of 15 g. Bacto-Gelatin (Difco) dissolved in 100 ml. nutrient broth at 80°, cleared by holding at 80° after adding 5 ml. white of egg and filtering. The pH value was adjusted to 7.2 and the medium sterilized by steaming on 3 successive days. The stabs were inoculated from broth cultures with a needle, and both pour plates and surface-streaked plates were made; they were incubated at 20° for 14 days.

Löffler's inspissated serum slopes were prepared with sterile horse serum according to the techniques of Mackie & McCartney (1948, p. 168), and inspissated at 85° for 2 hr.; they were streak inoculated and incubated at 25° for 14 days. The blood agar used was nutrient agar with 5% (v/v) sterile defibrinated horse blood added.

Resistance

Heat resistance. Two methods were used. (i) 5 ml. nutrient broth cultures grown for 2 days at 25° were well shaken, and then heated in a water bath at $56^{\circ} \pm 0.2^{\circ}$, and a loopful removed to blood agar plates after 5, 10, 15 and 30 min. To the broth which had been heated for 30 min. was added 10 ml. fresh broth, and after incubation for 4 days at 25°, it was plated out for sterility. Plates were incubated for 4 days at 25°.

(ii) Bampton (1913) used heat-resistance to classify his strains. His method was followed as closely as possible: five loopfuls of a 3-day culture grown on nutrient agar at 20° were emulsified in 50 ml. 0.85% (w/v) NaCl, and 5 ml. of this suspension were heated for 30 min. in a water bath, either at 42.5° or at $45^{\circ} \pm 0.2^{\circ}$. One millilitre was then added to pour plates of nutrient gelatin, which were incubated at 20° for 4 days and the colonies counted.

Phenol resistance. A sample (0.8 ml.) of a nutrient broth culture grown at 25° for 2 days and then well shaken was mixed with 0.2 ml. of 5% (w/v) aqueous phenol. It was incubated at 20° and after 5 and 10 min., 0.05 ml. samples were transferred to 5 ml. lots of nutrient broth, of which a loopful was plated on to blood agar. The plates and broths were incubated at 25° for 4 days, the broths being then plated for sterility.

Resistance to penicillin. Nutrient agar plates containing 100 units sodium benzylpenicillin/ml. (Glaxo Laboratories Ltd., Greenford, Middlesex) were inoculated by streaking one loopful of a 2-day broth culture grown at 25°; the plates were incubated at 25° for 2 days. The mesophils were also tested on similar plates incubated at 35° for 24 hr.

Metabolism and nutrition

Anaerobic growth. Nutrient agar plates were inoculated with a loopful of 48 hr. broth culture and incubated for 4 days at 25° in a McIntosh and Fildes's jar containing hydrogen.

Growth temperature. Since some strains, e.g. TV, will not grow on dry agar media near the upper limit of their temperature range, all tests were made by running a few drops of a 2-day broth culture down a nutrient agar slope. Slopes were incubated at 2, 10, 16, 20, 25, 30, 35 and 37° for 1 week, and those strains growing at 37° also tested at 44° for 4 days. The fluid as well as the agar was watched for growth.

pH value for growth. Tests were done upon nutrient agar adjusted to pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 ± 0.1 , by streaking a loopful of 24 hr. broth culture on to it and incubating for 4 days at 25°. Some tests were also done in nutrient broth adjusted to various pH values from 5.0 to 10.0 in steps of 0.2, similarly inoculated and incubated.

Utilization of citrate as sole source of carbon. The medium of Koser (1923) and that of Simmons (1926) were used. Koser's citrate was made from analytical grade reagents in doubly glass-distilled water in chromic + sulphuric-acid-cleaned glassware and inoculated by straight wire from a young broth culture. To Simmons's medium was added 0.01 % of yeast extract (Difco), and the slopes streaked with a loopful of broth culture. Both media were incubated at 25° for 7 days, and those tubes of Koser's medium which showed growth were subcultured with a straight wire into a second tube and similarly incubated.

Tolerance of sodium chloride. Nutrient agar and nutrient broth containing a total of 3 and 6.5 % (w/v) of sodium chloride were inoculated with a loopful of young broth culture and incubated at 25° for 7 days.

The catalase effect. Nutrient agar ditch plates were prepared, the ditch containing 1 µg. pure horse liver catalase/ml. Drops of tenfold dilutions (from 1/10 to 1/10⁶) of 24 hr. broth cultures were run across the plate and ditch, and the plates incubated for 2 days at 25°. Organisms showing the catalase effect give, with the more dilute inocula, colonies only upon the ditch.

Production of hydrogen cyanide. Nutrient or blood agar plates, heavily inoculated to give growth over most of the plate and incubated at 25° for 2 days, were tested by placing the end of an indicator paper inside the plate (but not touching the medium) and replacing the lid. The indicator paper was made as follows: benzidine acetate was dissolved in boiling water and the saturated solution cooled and filtered. To the filtrate was added one-tenth of its volume of 3 % cupric acetate solution. The tip of a strip of filter paper was dipped in the mixture, and this becomes blue in the presence of HCN. Other gases giving a positive test are chlorine, bromine and hydrogen chloride, while sulphur dioxide and hydrogen sulphide inhibit the reaction (Anonymous, 1938). The paper may turn brown after 10 min., apparently due to ammonia from the cultures.

Chemical proof that the gas is hydrogen cyanide was obtained as follows: a nutrient agar slope in a large flat bottle was inoculated with 10 ml. broth

culture, and incubated at the optimum temperature for 1 or 2 days, the neck of the bottle being closed by a bung with two delivery tubes which were clamped off. After incubation a gentle stream of air was blown into the bottle and the outcoming air bubbled through 1 ml. 10% (w/v) NaOH mixed with 0.1 ml. 1% (w/v) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. After 10 min. this solution was heated to boiling for a few seconds, and a drop of 1% (w/v) FeCl_3 added. Upon cautious acidification with 5M-HCl the colour of Prussian blue proved the presence of HCN in the culture.

Nature of attack upon glucose. Strains MK, LW and HB were grown in the glucose ammonium medium of Mandelstam (1954) with the lactate omitted, the first strain at 35° semi-anaerobically (in deep tube culture), the latter two at 25° aerobically (in shallow layers of medium) for 8 days. The pH values of all three cultures fell to about 5.2. After removing organisms by centrifugation, the supernatant fluid was divided into two parts. One part was evaporated to small volume and continuously extracted with ether for some hours; the ether extract was chromatographed on paper in butanol + acetic acid and in butanol + ammonia (sprayed with bromocresol green and bromthymol blue respectively). The other part was steam distilled after adding an equal volume of 2N-sulphuric acid, and the volatile acids in the distillate identified by gas-phase chromatography (James & Martin, 1952).

Pigment. Violacein was extracted with ethanol from nutrient agar cultures grown for a week at 25°. The pigmentation of *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI was also studied on 1% peptone water or peptone agar containing 1% glucose, sucrose or mannitol, incubated at 25°. Absorption curves were measured in a Unicam spectrophotometer, and are shown in Figs. 1 and 3 with the optical density plotted logarithmically, so that curves of different concentrations of the same substance are parallel.

Growth requirements. Some strains were tested by inoculating with a needle from young broth cultures into the medium of Mandelstam (1954) containing lactate but without glucose, with precautions against contamination with growth factors. When growth occurred the tubes were subcultured into a second tube of medium with a needle. Cultures were inoculated at 30° for mesophils and 20° for psychrophils.

Fermentation of carbohydrates

Fermentation reactions were studied in 1% peptone water containing 0.5% of sodium chloride, 1% of carbohydrate and 1% of Andrade's indicator (Mackie & McCartney, 1948, p. 159). Durham tubes were included to detect gas. These media were sterilized by steaming on 3 successive days. The nature of the attack on carbohydrates was studied in the medium of Hugh & Leifson (1953) with glucose, mannose and fructose. All tubes were incubated at 25° for 14 days before discarding.

Biochemical tests

Litmus milk. Prepared according to Mackie & McCartney (1948, p. 163). Cultures were incubated at 25° for 2 weeks.

Indole production. Peptone water cultures incubated for 5 days at 25° were shaken with ether and tested with Böhme reagents as given in *Topley & Wilson's Principles of Bacteriology* (1946, p. 367).

Ammonia, methyl red and Voges-Proskauer tests. These were done by the techniques given in *Topley & Wilson's Principles of Bacteriology* (1946, pp. 368-9) after 5 days of incubation at 25°.

H₂S production. Lead acetate paper was placed between the plug and the side of the tube of a nutrient broth culture and observed for 7 days at 25°.

Methylene blue reduction. Tested on a 5 ml. nutrient broth culture grown at 25° for 24 hr., by adding one drop of 1 % aqueous methylene blue and incubating for 1 hr. at 25°.

Reduction of nitrate to nitrite. Duplicate cultures in nutrient broth containing 0.1 % KNO₃ and 0.25 % agar were incubated at 25° and tested after 1 and 5 days by addition of Griess-Ilosvay reagents (using dimethyl α -naphthylamine instead of α -naphthylamine) as described in *Topley & Wilson's Principles of Bacteriology* (1946, p. 368). All negative reactions were checked by adding zinc dust to determine whether any nitrate remained (ZoBell, 1932).

Destruction of nitrite. Duplicate cultures in nutrient broth containing 0.0005 % NaNO₂ and 0.25 % agar were incubated at 25° and tested after 1 and 5 days in the same way as the preceding test. Absence of a pink colour showed disappearance of the nitrite.

Catalase. A loopful of growth on nutrient agar (grown for 2 days at 25°) was stirred into a drop of hydrogen peroxide solution (20 vol. %) and observed for bubbles.

Urease. Urea agar slopes (Christensen, 1946) were incubated at 25° for 4 days.

Phosphatase. The method of Bray & King (1948) was used and readings made after 2 days at 25°.

Hydrolysis of aesculin. Iron was used as the indicator of hydrolysis. The medium consisted of 1 % peptone, 0.1 % aesculin and 0.05 % ferric citrate (scales) in distilled water. Both this liquid medium in tubes, and plates of the medium solidified with 1.5 % of agar, were inoculated and observed at 25°, the tubes for 14 days and the plates for 4 days. The media were sterilized by autoclaving at 115° for 15 min.

Haemolysis. Growth on horse blood agar plates was examined for zones of haemolysis after incubation at 25°.

Hydrolysis of caseine, gelatin and starch. These were tested on cultures grown for 2 days at 25° upon skim-milk plates, and for 4 days on gelatin plates and starch plates by the methods of Smith, Gordon & Clark (1952, pp. 41, 43).

Egg-yolk reaction. Tested upon egg-yolk plates (Knight & Proom, 1950), consisting of nine parts of nutrient agar and one part of a Seitz-filtered mixture of one egg yolk in 250 ml. physiological saline. Plates were inoculated and incubated at 25° for 4 days.

Utilization of malonate and production of phenylpyruvic acid. Tested in the combined medium of Shaw & Clarke (1955) after growth at 25° for 2 days.

Gluconate test. Tested in the modified Hayne's medium of Shaw & Clarke (1955) in shallow layers (8 ml. in tubes of 22 mm. internal diameter). After growth for 4 days at 25° unshaken, 1 ml. of Benedict's Qualitative Reagent for glucose was added, and after standing for 10 min. at room temperature, the tubes were heated at 100° for 10 min.

Arylsulphatase. Tested in peptone water containing 0.001 M-potassium phenolphthalein disulphate (Whitehead, Morrison & Young, 1952) after growth for 7 days at 25°, by adding M-NaOH drop by drop.

RESULTS

Staining and morphology

Morphology. All the strains were rods with rounded ends. At 18 hr. of incubation the rods of the majority of the mesophils were small, often coccobacillary, averaging about $0.75 \times 2 \mu$. in dimensions, while the rods of most of the psychrophils were larger, averaging $1.0 \times 8.7 \mu$. However, strain AM had large rods and strain MB small ones. After 4 days growth the difference was less marked, the sizes in the two groups averaging 0.75×2.1 and $0.85 \times 2.5 \mu$. respectively. In most strains the organisms were single or in pairs, but a few, mainly psychrophils, showed chains of five to ten members. There was little pleomorphism but the longer organisms were frequently slightly curved.

Motility. All the strains of *Chromobacterium* examined were motile in broth cultures. The flagellation showed characteristic features: there was a single polar flagellum, and in addition one or more long subpolar or lateral flagella, which generally stained more readily and had a shorter wavelength than the short polar flagella. The lateral flagella were most abundant in young agar cultures, and were scanty in broth cultures, and they differed antigenically from the polar flagella (Sneath, 1956*a*). In young nutrient agar plate cultures (18 hr. at 25°) the peritrichous flagella were observed in all strains except FH, AM, MW, MH, MC and HF. The only strains in which it was difficult to demonstrate polar flagella were NT and PT. These differences in flagellation do not correlate with other features of the strains, and are not useful in dividing the group.

Staining. The mesophils generally showed bipolar staining with Löffler's methylene blue, with an oval pale central area; the psychrophils commonly showed barred staining, also with some oval pale areas. The difference may depend on the length of the rods. Four-day cultures showed occasional bizarre forms, but the bipolar and barred staining persisted.

All the strains of *Chromobacterium* examined were Gram-negative at 18 hr. and 4 days, except for HA, EC, MA and MW, which tended to be Gram-variable in young cultures. There were commonly Gram-positive granules (discussed below). None of the strains was acid-fast. A few acid-fast spore-like bodies were seen in strains EC, HB, CA, NT, GA and AM, but they were probably not spores. Many strains, chiefly the gelatinous ones, showed evidence of intercellular slime, but no clearly defined capsules were observed by Hiss staining. Fat globules were present in most organisms after 18 hr., being

larger and more abundant in the mesophils (except for strains AM, LW, SH and RT) than in the psychrophils. Strain HE, however, showed abundant fat. After 4 days both groups showed moderate amounts of fat. The globules were mainly subpolar, not central like the vacuoles. 'Metachromatic granules' were commonly seen at the poles, but their metachromasia was doubtful, since blue granules were not often observed in Neisser stained films, although brown granules occurred commonly. The only strain which showed unmistakable blue granules was EC, in which they were large and abundant. The polar granules may have been the structures which retained the Gram stain (often for several minutes decolorization with ethanol) and which were visible in more than half the strains of both mesophils and psychrophils; they were present in old as well as young cultures. Similar granules were seen in *Pseudomonas aeruginosa* NCTC 2000.

Spores. Reports of spores are found in many of the old descriptions, but none has been confirmed as heat-resistant. In my experience the spore-like bodies are not resistant to heating at 56° for 30 min., nor do broth or agar cultures survive this treatment.

Other strains. *Corynebacterium viscosum* and *Pseudomonas iodina* are very similar to one another. Both were rods (measuring about $0.8 \times 2 \mu$.) showing pleomorphism, with clubbed ends and arrangement in irregular or palisade-like clumps. Both were Gram-positive (including strain RE) in young cultures though they were Gram-variable after 4 days. They showed barred staining, were not acid-fast, were non-motile and non-sporing. *Corynebacterium viscosum* showed some visible fat, a definite capsule and well-marked metachromatic granules; *Pseudomonas iodina* showed none of these. Strain TI was polar flagellated (lophotrichous), Gram-negative and not acid-fast. It measured $0.9 \times 2.5 \mu$. after 18 hr., showed bipolar staining, moderate fat but no metachromatic granules, spores or capsules.

Cultural behaviour

Nutrient agar plates. The colonial appearances are very variable, since colony form depends in large measure upon the 'roughness' of the strain. The most smooth mesophils, which showed little auto-agglutination in saline suspensions and gave turbid growth in broth, produced round, low convex colonies with entire edge and a smooth shiny surface. The more rough mesophils (strains AM, MW, LW, SH, RT and RV) which auto-agglutinated readily and gave little turbidity in broth, produced raised or low conical colonies with a polygonal outline and undulate or erose edge; the surface was copper-beaten or rough, and dull and granular.

None of the psychrophils studied appeared very rough, and they gave round convex colonies, occasionally umbonate, with entire edge and smooth shiny or matt surface. A few strains threw off rougher variant colonies.

The structure of the colonies of the *Chromobacterium* strains examined was amorphous with finely granular centre, and they were semi-transparent. Colonies of gelatinous strains, only seen among psychrophils, were viscid and

jelly-like, and after 4 or 5 days they could be detached whole with a needle; they were difficult to emulsify. The non-gelatinous strains were butyrous and easily emulsified.

As a rule the colonies were 1–1.5 mm. in diameter after 2 days at 25°. Most of the mesophils produced colonies of this size in 24 hr. at 37°. Pigmentation started in the centre of colonies of the mesophils, was slight in 24 hr. at 37° and marked in 48 hr. At 25° pigmentation in both groups was delayed until the second or third day. Some psychrophils showed pigmentation first at the edge of the colonies, others at the centre. Pale violet and non-pigmented variants were seen in most strains.

Old cultures (4–7 days at 25°) showed colonies about 5 mm. in diameter; generally those of mesophils were dark violet and flat with lobate edge and those of psychrophils were low convex, sometimes showing concentric violet zones. The degree of pigmentation and of gelatinous consistency on nutrient agar (7 days at 25°) is shown in Table 2. Gelatinous strains may show non-gelatinous variants, as noted by Corpe (1953).

Corynebacterium viscosum and *Pseudomonas iodina* showed round greyish convex smooth colonies which were semi-opaque. Colonies of *C. viscosum* were non-pigmented on sugar-free media and were moderately viscid. *Pseudomonas iodina* sometimes developed iodine-like dark crystals of iodinin after some days at 20–25°. Strain TI produced colonies like those of smooth mesophils, but of a pale yellow colour and slightly viscid.

Smell. Aerobic plate cultures of mesophils gave an odour of hydrogen cyanide and ammonia. The psychrophils had a characteristic sour odour.

Gelatin plates (20°). The colonies of *Chromobacterium* varied from round, entire low convex shiny colonies to flat colonies with undulant surface and lobate or effuse edge. The colonies of most mesophils sank into cups of liquefied gelatin in a few days. Most psychrophils showed a little liquefaction in 5–7 days but rarely earlier. Deep colonies were lenticular or round, often with a mulberry-like internal structure. *Corynebacterium viscosum* and *Pseudomonas iodina* gave round convex entire smooth colonies, rather opaque. Those of the latter rapidly sank into the medium. Strain TI gave low convex round colonies, slowly sinking into the gelatin.

Nutrient agar slopes. Most cultures of *Chromobacterium* produced a smooth shiny violet growth, slightly raised with a finely lobate edge, but rough strains showed a dull or copper-beaten surface and irregular edge. Gelatinous growth and pigmentation were well developed after 7 days. *Corynebacterium viscosum* and *Pseudomonas iodina* gave semi-opaque shiny grey growth, and strain TI a pale yellow smooth growth.

Gelatin stab (20°). Most of the mesophils showed a filiform colourless growth along the stab, with a round pigmented button of surface growth. Liquefaction occurred in a few days; it was infundibuliform or napiform, never stratiform. After 14 days the top centimetre was usually entirely liquefied. The psychrophils gave a button of surface growth but scanty or no growth in the stab; liquefaction was very slow—generally none after 14 days, though strains DA, IN and EA showed slight crateriform liquefaction. The growth

Table 2. *Metabolic characteristics*

Table 2. <i>Mesophilic and acidophilic</i>						Alkalinity on Simmons's medium
Strain	Pigment (7 days)	Gelatinous growth (7 days)	Highest growth temperature	Lowest growth temperature	Anaerobic growth (4 days)	(2 days)
Mesophils						
FH	++	—	44°	10°	+	+
MK	+++	—	37°	10°	+	±
BH	+++	—	44°	16°	+	—
BN	+++	—	44°	16°	+	±
AM	++	—	37°	10°	+	+
MW	++	—	37°	10°	+	±
TV	+	—	37°	10°	+	+
LG	+++	—	44°	10°	+	—
SL	+++	—	37°	10°	+	±
RV	++	—	37°	10°	+	±
DK	+++	—	37°	16°	+	±
TA	++	—	37°	10°	+	+
TB	++	—	37°	10°	+	+
PT	+++	—	37°	10°	+	±
LW	++	—	37°	10°	±	±
SH	++	—	37°	16°	±	±
RT	+++	—	37°	16°	±	—
MH	+++	—	37°	10°	±	±
Psychrophils						
NT	+	+++	25°	2°	—	+
EA	+	+++	30°	4°	—	+
EB	+	+	30°	2°	—	+
EC	+	+	30°	2°	—	+
GA	+	—	35°	2°	±	+
DA	++	—	30°	2°	—	+
NC	++	+	30°	2°	—	+
MA	++	+	30°	2°	—	+
MB	+	++	30°	2°	—	+
MC	++	+	30°	2°	±	+
HA	++	++	30°	2°	—	+
HB	++	++	30°	2°	—	+
HC	+++	+++	25°	2°	—	+
HD	++	+	30°	2°	—	+
HE	+++	++	30°	2°	—	+
HF	+	++	30°	2°	±	+
IN	+	+++	35°	2°	±	+
CA	+++	+++	30°	2°	—	+
PB	++	—	30°	2°	±	+
RU	++	++	30°	2°	—	+
Other bacteria						
<i>C. viscosum</i> GR		+	37°	10°	±	—
		(very viscid)				
<i>P. iodina</i> TE		—	37°	10°	—	—
<i>C. ianthinum</i> TI		—	37°	10°	±	+
		(viscid)				

Symbols. Pigment = + to +++ = increasing degrees of violet pigmentation. Gelatinous growth = + to +++ = increasing degrees of jelly-like consistency. Anaerobic growth: — = no growth visible with a lens; ± = growth visible with a lens; + = growth easily visible. Alkalinity on Simmons's medium: — = no change (c. pH 6.9); ± = slight alkalinity (c. pH 7.2); + = marked alkalinity (c. pH 7.5).

formed a thick pellicle on the liquefied gelatin and was well pigmented. *Corynebacterium viscosum* and *Pseudomonas iodina* gave rapid stratiform liquefaction with scanty growth in the stab and scanty pellicle without pigment. Strain TI showed moderate napiform liquefaction with yellow pellicle and a filiform growth in the stab.

Nutrient broth. Almost all strains gave a violet pellicle when the medium was not disturbed. This pellicle was fragile in the mesophils and thick in the gelatinous psychrophils. When the pellicle sinks, a very characteristic violet ring of growth, which adheres to the glass, remains at the surface; it is thin and powdery, except for the gelatinous psychrophils, where it is thick, viscous and shows hanging fronds, even in those which were only slightly gelatinous on agar. The fluid was clear with auto-agglutinable or gelatinous strains, and turbid with the others. A deposit was present and had the consistency of the pellicle; it might remain violet or become pale. The fluid itself was stained slightly violet by the heavily pigmented strains. Being strict aerobes, the psychrophils grew poorly in broth. *Corynebacterium viscosum* and *Pseudomonas iodina* grew poorly without pellicle. Strain TI gave a yellow ring, with turbidity and deposit.

Blood agar plates. The colonial appearances were similar to those on nutrient agar, but growth was better and isolated colonies grew readily. Rapid haemolysis is characteristic of mesophils (see below).

Löffler's serum slopes. Growth was good, soft and shiny. The mesophils generally produced moderate digestion in 2 weeks, leading to collapse of the medium. The psychrophils usually gave little digestion in this time (see Table 4). Pigmentation was erratic. *Corynebacterium viscosum* and *Pseudomonas iodina* gave greyish growth without digestion. Strain TI caused rapid digestion.

Potato slopes. Growth on potato has been used as a differential test by Cruess-Callaghan & Gorman (1933). In my hands it was not useful because it was difficult to assess the amount of growth when the degree of pigmentation varied widely. All strains grew, giving smooth undulant growth with a diffuse edge, often spreading. In the drier parts, flat stellate colonies might form. Violet pigmentation was heavy with mesophils. Psychrophils usually showed less pigment, brownish violet colours being the rule. Strains EB, EC, GA, MB, MC and HF gave yellowish growth, while strains NT, MA, and HE gave deep violet growth after 2 weeks. The gelatinous consistency of strains was not well marked on potato. *Corynebacterium viscosum* produced a slightly viscous cream-coloured growth, not spreading, with a sky-blue pigment after 10 days, which diffused across the potato. *Pseudomonas iodina* gave a raised moist grey growth without pigment. Strain TI gave a smooth growth which was reddish violet (diffusing slightly) after 7 days and brownish after 14 days.

Resistance

Heat resistance. None of the organisms studied showed unusual resistance to heat; 5 ml. broth cultures were sterile after 30 min. at 56°. With *Chromobacterium* strains and strain TI loopfuls of broth seldom showed viable

organisms after 5 min. at 56° and never after 10 min. *Corynebacterium viscosum* and *Pseudomonas iodina* showed a few viable organisms in a loopful after 15 min. The difference reported by Bampton (1913) between *Bacillus violaceus* and *B. membranaceus amethystinus* was not confirmed; no strain was completely killed by 42·5° or 45° in 30 min. and those which showed fewest colonies after heating (strains MW, NT, TV, LW, RT, TA, TB, CA and RU) had no other distinctive features in common.

Phenol resistance. No strain of *Chromobacterium* nor strain TI showed viable organisms in 0·05 ml. after 10 min. in 1 % (w/v) phenol, though an occasional strain showed viable organisms after 5 min. *Corynebacterium viscosum*, *Pseudomonas iodina*, *P. aeruginosa* (2 strains) and 'Bacillus prodigiosus' (2 strains) showed many surviving organisms after 10 min.

Resistance to penicillin. All strains of *Chromobacterium* and strain TI were resistant to 100 units benzylpenicillin/ml., but *Corynebacterium viscosum* and *Pseudomonas iodina* were sensitive to this concentration.

Longevity of cultures. Agar cultures of *Chromobacterium* strains usually died out in a week or two at 20°. Broth cultures survived for several weeks at 20°, while at 2° the psychrophils lived longer but the mesophils seemed to die out more rapidly. The alkalinity of old cultures may be responsible, since sloppy agar cultures (which become strongly alkaline only at the surface) survived longer. For transport through the post, agar stab cultures have been best. It is known that dried cultures also do not survive well (Rhodes, 1950); my experience has been similar. Good results, up to 1 year of storage, have been obtained by storing broth cultures at -78°. In these respects the organisms resemble the vibrios.

Metabolism and nutrition

Anaerobic growth. All the mesophils grew, but most psychrophils did not grow in 4 days (Table 2). No pigment was formed.

Growth temperature. The results are shown in Table 2, which gives the lowest and the highest of the temperatures used at which growth occurred. No strain grew both at 2° and at 37°. The optimum temperature was nearer the upper limit than the lower. Some mesophils (strains AM, MW, TA, TV, LW and MH) grew poorly (or failed to grow on very dry slopes) at 37°, but these did not resemble the psychrophils in other respects.

pH Value for growth. The behaviour was more or less uniform with all strains: growth took place on nutrient agar at pH 6 and 9, but not at pH 5 or pH 10; the optimum value was between pH 7 and 8. Tests with a few strains in broth showed that slow growth sometimes occurred at pH 5·2, but this did not appear to be of value in classification.

Utilization of citrate as sole source of carbon. It is possible that discrepancies may arise between results obtained with the Koser medium and the Simmons medium, since the former should in theory be free from growth factors, while the latter may contain growth factors from the agar. Therefore Koser's solution was made up to be growth-factor free as far as possible, while yeast extract was added to the Simmons medium (this amount of yeast extract gave scarcely visible growth and no change in pH value when the citrate was omitted).

All strains of *Chromobacterium* and strain TI grew slowly in Koser's medium in both tubes, and grew on Simmons's medium, turning it alkaline. With the latter there was considerable strain variation, the psychrophils turning it alkaline in 2 days, while some mesophils took 5 or 6 days. The differences did not correlate well with other features of the strains. *Corynebacterium viscosum* was negative in both media after 1 week. *Pseudomonas iodina* grew very slowly in both.

Tolerance of sodium chloride. Almost all strains grew well on 3% (w/v) NaCl agar, and failed to grow on 6.5% NaCl agar. There was often slow growth in 6.5% NaCl broth. Strain TI and *Corynebacterium viscosum* grew slightly on 6.5% NaCl agar, while *Pseudomonas iodina* grew luxuriantly.

Catalase effect. *Chromobacterium* strains, like *Pasteurella pestis*, are very sensitive to traces of hydrogen peroxide, as shown by the inability of single organisms to grow aerobically on nutrient agar, and the small number of separated colonies obtained when strains are plated out on this medium. The inhibition is overcome by blood, haematin or catalase (Sneath, 1955). The strains reported on by Sneath (1955) were all mesophils; but six psychrophils have since been examined and all show the effect.

Production of hydrogen cyanide. Aerobic plate cultures of all the mesophils smell of HCN and this can be demonstrated by indicator paper, most easily in cultures grown at 35–37°. The amount of HCN produced is considerable (Sneath *et al.* 1953), being more than that produced by *Pseudomonas aeruginosa* (Patty, 1921; Lorek, 1948) which does not give a positive reaction with the test-paper. Clawson & Young (1918) first reported on production of HCN by *Chromobacterium* but did not mention the amount produced. By growing a typical mesophil (MK) in flat bottles the gas was identified by the Prussian blue reaction. Anaerobic cultures produce little HCN. The psychrophils, 'Bacillus prodigiosus', strain TI, *Corynebacterium viscosum* and *Pseudomonas iodina* gave negative reactions with the indicator paper. In flat bottles strain HB was negative (2 and 4 days growth at 25°). Despite the production of cyanide, growth is scanty in the medium of Møller (1954) with all strains in 8 days at 25°.

Products of attack upon glucose. The main products detected were as follows: strain MK, which ferments glucose anaerobically, produced acetic acid, *n*-butyric acid, probably some succinic and lactic acids and an unidentified non-volatile acid; strain LW (which oxidizes glucose) produced acetic and succinic acids; strain HB (which oxidizes glucose) produced a little acetic, succinic and lactic acids and several unidentified non-volatile acids. There was probably some gluconic acid in all these cultures.

Pigment formation. All the strains of *Chromobacterium* produced on nutrient agar a violet pigment which was non-diffusing (slight diffusion may occur in old cultures), soluble in ethanol, and which became green and then red-brown with caustic potash. Both mesophils and psychrophils appear to produce the same pigment, violacein. Pigments from two mesophils, MK and RV, and from two psychrophils, NT and HB, were examined with a range of common reagents and by spectrophotometry. They behaved identically and in general agreement with the reports in the literature.

On treating ethanolic solutions with equal volumes of the following reagents, the corresponding colour changes were noted: 10% KOH: green, turning reddish and then brown in a few minutes; 50% HNO₃: yellowish orange; 50% H₂SO₄: emerald green, stable for many hours; glacial acetic acid: blue, stable; 2M-HCl: blue-green, turbid, stable; H₂O₂ (10 vol. %): remains violet, stable at 20°, decolorized at 80°; bromine water: decolorized; 5M-NH₄OH: sapphire blue, fading to colourless; 0.1M-FeCl₃: pale yellow. A solution of the pigment reduced with 25% acetic acid and zinc dust slowly decolorized; with 0.5% NaNO₂ in 10% acetic acid it becomes pale brown and turbid; it did not give a red colour with the Böhme indole reagents.

Samples of violacein from strains RV and NT were purified by the method of Strong (1944) and recrystallized from pyridine + chloroform. On heating, the pigment decomposed without melting. The absorption spectrum in 96% ethanol in water is shown in Fig. 1. Both samples gave identical curves, and the wavelengths of the observed maxima and minima, with the Specific Extinction Coefficients (optical density of 1 cm. thickness of a solution containing 1 g./l.) were:

208 mμ.	247 mμ.	257.5 mμ.	339 mμ.	374 mμ.	431 mμ.	579 mμ.
110	51.3	54.9	13.6	17.8	7.4	65.4

The extinctions are somewhat higher than those found by Ehrismann & Noethling (1936). The purified pigments were also examined in 10% (v/v) H₂SO₄ (sp.gr. 1.84) in 96% ethanol; the green solution was stable for some hours. Both samples gave identical curves. The observed maxima and minima (see Fig. 1) and Specific Extinction Coefficients were:

235 mμ.	250 mμ.	269 mμ.	280 mμ.	295 mμ.	301 mμ.
40.4	52.2	36.8	41.6	29.1	29.5
337 mμ.	413 mμ.	502 mμ.	653 mμ.	659 mμ.	699 mμ.
6.2	21.3	8.5	73.4	73.0	102.7

The infrared absorption spectra of the two crystalline pigment samples (kindly determined by Dr R. K. Callow) were virtually identical, and the 'finger-print region' of the spectrum is shown in Fig. 2.

Crude pigments from several mesophils and psychrophils (extracted from the organisms with ethanol) contained impurities which distorted the absorption curves in the ultraviolet region, but the curves were otherwise similar to those of purified samples, and were in good agreement with the curve published by Gilman (1953) for *Chromobacterium violaceum*. An example is shown in Fig. 1.

The dry pigment was almost insoluble in water, chloroform or benzene; it was readily soluble in ethanol or acetone and in ethanol + water and acetone + water mixtures, giving violet solutions. In ether it was very slightly soluble, the solution having a more reddish tone than in ethanol. Violacein has probably a hydroxyindolyl-oxindolyl-pyrrolyl-methene structure (Beer, Jennings & Robertson, 1954) and the infrared absorption spectrum is compatible with such a structure. Non-pigmented variants often became tinged with yellow after some days; this second pigment was not investigated.

Fig. 1. Absorption spectra of violacein (in $\frac{1}{2}$ cm. cells). —, Ethanolic solution of crystalline violacein (14.0 mg./l.);, crystalline violacein, 14.0 mg./l. in 10 % (v/v) sulphuric acid in 96 % ethanol; - - - -, crude violacein (from strain HB) in ethanol.

Fig. 2. Infrared spectrogram of violacein (in KCl disks). Upper curve: violacein from strain RV; lower curve: violacein from strain NT. The concentration of pigment was slightly different in the two experiments.

Corynebacterium viscosum did not produce pigment on nutrient or peptone agar. Upon glucose, sucrose or mannitol peptone agar it produced after 1 day at 25° a small amount of a green diffusing pigment, and after several days a large amount of a diffusing blue-violet pigment. The blue-violet pigment from sucrose peptone agar (extracted with water) in 0.5M-potassium phosphate

Fig. 3. Absorption spectra of pigments of *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI. —, pigment of strain TI in acid chloroform; ----, pigment of strain TI in 0.1M-NaOH;, pigment of *C. viscosum* in buffer (pH 7.0); - · - · - ·, iodinin in chloroform (9.4 mg./l. in $\frac{1}{2}$ cm. cell).

buffer (pH 7.0) showed an absorption peak at 574 mμ. and maximal transmission at 472 mμ. (Fig. 3); in 0.5M-phosphate buffer (pH 4.2) these were at 566 and 468 mμ., respectively. At both pH values there were shoulders or small peaks at about 275 and 380 mμ. Gilman (1953) gave a curve similar to that found at pH 4.2. The pigment became reddish in 0.1M-HCl, and blue or blue-green in 0.1M-NaOH. Addition of saturated aqueous MgSO₄ turned solutions purple.

Pseudomonas iodina produced the characteristic iodine-like crystals described by Davis (1939); these were insoluble in water, but soluble in warm ethanol and in chloroform, giving reddish purple solutions. The pigment, iodinin, is 1:6-dihydroxy-phenazine 5:10-dioxide (International convention) melting point 236° dec. (Clemo & Daglish, 1950). The crystals were erratic in occurrence, only some batches of nutrient and blood agar being suitable; peptone agar was more reliable. Gilman (1953) however reported the pigment as brownish and water-soluble, but his absorption curve is almost identical with that of iodinin in chloroform. Iodinin from strain RE recrystallized twice from chloroform had melting point 235° (dec.). The absorption spectrum of a solution in chloroform is shown in Fig. 3. There are some differences from the curve given by Clemo & McIlwain (1938), but a sample of their pigment (kindly provided by Professor McIlwain) had an absorption spectrum identical with that of my sample. The Molecular Extinction Coefficients observed at the maxima and minima were:

255 m μ .	291 m μ .	317 m μ .	352 m μ .	427 m μ .	534 m μ .
7,270	140,000	3,900	11,000	1,350	10,100

Strain TI appeared to produce several pigments. Upon nutrient agar, peptone agar or glucose peptone agar it produced only a pale yellow, poorly diffusing pigment. Upon potato and on mannitol and sucrose peptone agar it produced a poorly diffusing red-violet pigment at 20° or 25° but little at 37°. In old peptone water + carbohydrate tubes a dark red pigment sometimes formed in the pellicle. This pigment was almost insoluble in water, ethanol, chloroform or benzene, but dissolved in 0.1 M-HCl, and turned yellow; it could then be extracted with chloroform. The substance turned red-violet and passed into the aqueous phase on shaking the chloroform with 0.1 M-NaOH. It is possible that the three pigments are closely related, since the yellow one became mauve with alkalis and the mauve one became yellow with acids. Gilman (1953) used an ethanolic extract of organisms grown on nutrient agar for his studies; his extract evidently contained some yellow pigment.

The mauve pigment from strain TI grown on mannitol peptone agar was extracted with chloroform after scraping the growth into 0.1 M-HCl, and the chloroform extracted with 0.1 M-NaOH. The alkaline solution was again acidified with HCl and the process repeated twice. This partly purified pigment was yellow when extracted with chloroform from acid, and was stable for some hours; the absorption spectrum showed absorption maxima at 277, 367 and 447 m μ . and minima at 307 and 406 m μ . When extracted into 0.1 M-NaOH, the unstable mauve solution showed maxima at 255, 286 and 536 m μ . and minima at 262 and 446 m μ . with shoulders at about 360 and 400 m μ . (Fig. 3).

Growth requirements. Eight mesophils and two psychrophils grew in ammonium + lactate medium and could be subcultured in this. Although growth was much increased by addition of casein hydrolysate, the results suggest that there is no absolute requirement for amino acids or growth factors, despite the report of Linardos & Cleverdon (1955) that all strains require organic nitrogen. The results with Koser's citrate also support this.

Carbohydrate fermentation

It was considered most useful to study fermentation reactions in peptone water for comparison with earlier records, and because of their wide use. Work with chemically-defined media will clearly be valuable. None of the strains of *Chromobacterium* produced gas. Most of the psychrophils showed only slight acid production, and there were therefore many doubtful reactions. No acid was produced by any strain from the following compounds: cellobiose, raffinose, melezitose, *m*-erythritol, dulcitol, inositol, adonitol, lactose, rhamnose, α -melibiose. Doubtful reactions were often seen with the following carbohydrates, but were not sufficiently constant to be useful: salicin, D(+)xylose, L(+)arabinose, mannitol, aesculin, inulin, galactose, sorbose, glycerol, sorbitol, maltose (the last four most often with the mesophils). The carbohydrates which showed definite acidity with some strains are shown in Table 3, in which are also shown the results of testing a few carbohydrates in the medium of Hugh & Leifson.

It is seen that the mesophils attacked glucose, fructose, mannose and trehalose promptly. Sucrose and the triad dextrin, glycogen and starch were attacked, often late, by some strains, and it is possible that this was due to the occurrence of mutants, since in strain BN a variant which fermented starch promptly was isolated by plating the culture on to starch plates. Most of the mesophils were also tested at 87°; the reactions were substantially the same, but acid production was more rapid and more marked. The psychrophils often produced slight acidity from some sugars. Trehalose (in either medium) was the most useful sugar for distinguishing the two groups. Aesculin also differentiated the groups, but since those strains which split it did not ferment glucose strongly, it must be used as described below. Unlike the mesophils the psychrophils did not ferment glucose anaerobically. The four mesophils which did not ferment glucose were old stock strains, and they may well be loss-variants: they also attacked mannose slowly or not at all. *Corynebacterium viscosum* and *Pseudomonas iodina* gave no definite acidity in peptone water + carbohydrates, while strain TI gave slight acidity only in glucose or galactose. *P. iodina* appeared not to attack glucose aerobically or anaerobically.

Results of biochemical tests

Litmus milk. The results were variable. Slight acidity was more common with mesophils, but moderate alkalinity sometimes occurred after a few days. Some strains produced initial alkalinity and late acidity. The mesophilic strains usually peptonized the casein after some days, and often gave a small clot. The psychrophils commonly produced a large 'rennin' clot and seldom showed marked peptonization. Some pigment was usually formed, as a violet ring of growth or in a pellicle which was sometimes thick with gelatinous strains. About half the psychrophils bleached the litmus. Peptonization is the most useful feature, and parallels the results on milk plates (Table 4). *Corynebacterium viscosum* and strain TI produced slight acidity and slight peptonization. *Pseudomonas iodina* produced alkalinity and a clot.

Table 3. *Fermentation of carbohydrates*

Strain	Reactions in peptone water carbohydrates								Reactions in Hugh & Leifson's medium		
	Glucose	Fruc- tose	Man- nose	Treha- lose*	Sucrose	Glyco- gen	Dextrin	Starch	Glucose	Fruc- tose	Man- nose
Mesophils											
FH	A	A	A	A	—	—	—	—	F	F	F
MK	A	A	A	A	—	—	—	—	F	F	F
BH	A	A	A	A	Al	Al	Al	al	F	F	F
BN	A	A	A	A	—	Al	Al	Al	F	F	F
AM	A	A	A	A	—	—	—	—	F	F	F
MW	A	A	A	A	—	—	—	—	F	F	F
TV	A	A	A	A	—	al	al	al	F	F	F
LG	A	A	A	A	A	Al	Al	Al	F	F	F
SL	A	A	A	A	A	—	—	—	F	F	F
RV	A	A	A	A	A	—	—	—	F	F	F
DK	A	A	A	A	—	—	—	—	F	F	F
TA	A	A	A	A	—	—	—	—	F	F	F
TB	A	A	A	A	—	—	—	—	F	F	F
PT	A	A	A	A	—	Al	Al	Al	F	F	F
LW	A	A	—	A	—	—	—	—	O	O	—
SH	A	A	—	A	—	—	—	—	O	O	—
RT	A	A	a	A	—	—	—	—	O	O	O
MH	A	A	a	a	a	—	—	—	O	O	Ol
Psychrophils											
NT	a	—	—	—	—	—	—	—	O	O	O
EA	A	A	al	—	—	—	—	—	O	O	O
EB	a	a	a	—	—	—	—	—	O	O	O
EC	—	—	—	—	—	—	—	—	O	O	O
GA	—	—	—	—	—	—	—	—	O	O	O
DA	a	—	—	—	—	—	—	—	O	O	O
NC	—	—	—	—	—	—	—	—	O	O	O
MA	a	al	—	—	—	—	—	—	O	O	O
MB	—	al	—	—	—	—	—	—	O	O	O
MC	—	—	—	—	—	—	—	—	O	O	O
HA	—	—	—	—	—	—	—	—	O	O	O
HB	—	—	—	—	—	—	—	—	O	O	O
HC	—	a	—	—	—	—	—	—	O	O	O
HD	—	—	—	—	—	—	—	—	O	O	O
HE	—	a	—	—	—	—	—	—	O	O	O
HF	—	—	—	—	—	—	—	—	O	O	O
IN	—	—	—	—	—	—	—	—	O	O	O
CA	—	—	—	—	—	—	—	—	O	O	O
PB	—	—	—	—	—	—	—	—	O	O	O
RU	—	—	—	—	—	—	—	—	O	O	O
Other bacteria											
C. viscosum	—	—	—	—	—	—	—	—	Fl	O	O
GR	—	—	—	—	—	—	—	—	—	—	—
P. iodina	—	—	—	—	—	—	—	—	—	—	—
TE	—	—	—	—	—	—	—	—	—	—	—
C. ianthinum	a	—	—	—	—	—	—	—	O	O	O
TI	—	—	—	—	—	—	—	—	—	—	—

A = marked acidity; a = slight acidity; l = late (in 7–14 days); F = fermentative attack (acidity anaerobically); O = oxidative attack (acidity only aerobically); — = no acidity or doubtful result.

* Trehalose in aerobic Hugh & Liefson medium also gave acidity with all mesophils but no acidity with any psychrophil.

Table 4. *Biochemical reactions*

Incubation temperature 25°.

Strain	Aesculin hydrolysis (4 days)	Haemolysis horse blood agar (2 days)	Egg-yolk reaction (2 days)	Reduction of nitrate		Gelatin hydrolysis (4 days)	Casein hydrolysis (2 days)	Liquefaction of serum (14 days)	Gluconate test (4 days)
				to nitrite (5 days)	Destruction of nitrite (5 days)				
Mesophils									
FH	-	+	+	-	-	+	++	++	-
MK	-	++	++	+	+	++	++	++	-
BH	-	++	++	+	+	++	++	++	-
BN	-	++	++	+	+	++	++	++	-
AM	-	-	++	+	±	++	+	++	-
MW	-	++	++	+	+	++	++	++	±
TV	-	++	++	+	++	++	++	++	-
LG	-	++	++	+	+	++	++	++	-
SL	-	++	++	+	-	++	++	++	-
RV	-	++	++	+	+	++	++	++	-
DK	-	++	++	+	+	++	++	++	-
TA	-	++	++	+	++	++	++	++	-
TB	-	++	++	+	+	++	++	++	-
PT	-	±	+	+	+	++	++	+	-
LW	-	±	+	+	±	+	++	++	-
SH	-	±	+	+	±	+	++	++	-
RT	-	±	++	+	-	+	+	++	-
MH	-	+	++	+	+	+	++	+	-
Psychrophils									
NT	+	-	-	-	-	-	±	±	±
EA	+	±	-	+	++	±	-	-	+++
EB	+	-	-	+	±	±	-	+	+++
EC	+	-	-	+	+	-	-	-	+
GA	+	-	-	+	±	-	-	-	-
DA	+	-	-	+	-	±	-	-	-
NC	+	-	-	+	-	-	-	±	-
MA	+	-	-	+	±	-	-	-	-
MB	+	-	-	-	+	±	±	±	-
MC	+	-	-	+	++	±	+	-	-
HA	+	-	-	+	+	±	±	-	+++
HB	+	-	-	+	+	-	-	±	+++
HC	+	-	-	+	+	-	-	-	++
HD	+	-	-	+	+	-	-	+	-
HE	+	-	-	+	+	-	±	-	++
HF	+	±	-	+	+	±	-	+	-
IN	+	-	-	+	+	-	-	-	-
CA	+	-	-	+	+	-	-	-	-
PB	+	-	-	+	+	-	-	-	-
RU	+	-	-	+	+	-	±	-	-
Other bacteria									
GR*	+	-	-	+	±	+	+	-	-
TE†	-	++	-	-	-	+	-	-	-
RE‡	.	.	.	+	-	.	.	.	.
TI§	+	-	+	-	-	+	+	+	+++

Symbols. Aesculin hydrolysis (plate test): - = no browning; + = wide zone of browning. Haemolysis, casein hydrolysis and gelatin hydrolysis: - = no clearing; ± = clearing only beneath the growth; + = zone of c. 2 mm. clearing; ++ = zone of c. 5 mm. clearing. Egg-yolk reaction: - = no opacity; + = opaque zone of c. 2 mm.; ++ = opaque zone of c. 5 mm. Reduction of nitrate: - = no colour; + = detectable pink colour. Destruction of nitrite: - = strong colour; ± = faint colour; + = no colour; ++ = no colour after only 24 hr. incubation. Liquefaction of serum (Löffler's slopes): - = no change; ± = partial clearing of medium; + = furrowing of medium; ++ = collapse of slope. Gluconate test: - = no reduction of Benedict's solution on heating; ± to +++ = increasing reduction on heating, from a green colour to a deep orange precipitate. Strain RE: . = not tested.

* *C. viscosum*.† *P. iodina*.‡ *C. iodinum*.§ *C. ianthinum*.

Indole, ammonia, methyl red (MR) and Voges-Proskauer (VP) tests. All strains of *Chromobacterium* were indole-negative. Most strains were strongly ammonia-positive, a few weakly positive, and were all MR-negative, except that a few mesophils gave a doubtful reaction. All strains were VP-negative, the few doubtful reactions being probably due to the effect of the alkali on the pigment, which turned green and then brown. *Corynebacterium viscosum* and *Pseudomonas iodina* and strain TI were indole-negative, MR-negative and VP-negative; the first two were NH_3 -negative, the last NH_3 -positive.

H₂S production and reduction of methylene blue. No strain produced abundant H₂S, though the mesophils sometimes produced a trace when incubated at 37°. Most of the mesophils reduced methylene blue, while most of the psychrophils did not, or gave a doubtful result. *Corynebacterium viscosum* was negative in both tests; *Pseudomonas iodina* and strain TI were H₂S-negative but reduced methylene blue weakly.

Reduction of nitrate and destruction of nitrite. The majority of strains of *Chromobacterium* gave positive reactions in both tests (Table 4). Several mesophilic strains, e.g. strain TV, destroyed nitrates and nitrites so actively, particularly at 37°, that the test for nitrite was negative on the fifth day. No bubbles of nitrogen gas were noted in the tubes. Nitrite was usually not completely destroyed after 1 day. The negative strains are probably loss-variants, which may occur in nature. In strain SH, a variant which was negative in both tests occurred spontaneously.

Catalase, urease and phosphatase. All strains of *Chromobacterium* were catalase-positive, though sometimes weakly so. The method of testing by pouring hydrogen peroxide on to a slope culture often gave doubtful results. All strains were urease-negative and moderately to strongly phosphatase-positive. *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI were also catalase- and phosphatase-positive but urease-negative.

Hydrolysis of aesculin. The psychrophilic strains hydrolysed aesculin, producing a dark brown colour, while the mesophils did not (Table 4). Concordant results were obtained with the liquid and solid media, but the latter gave more rapid results, showing a brown zone in 24 hr. *Corynebacterium viscosum* was positive on plates but doubtful in the liquid medium.

Hydrolysis of casein, gelatin and starch. Zones of clearing due to hydrolysis of casein were produced in 2 days by most of the mesophilic strains but not by most of the psychrophils (Table 4). After longer periods many psychrophils were positive, and generally showed clearing under the growth. In the gelatin plates after 4 days the mesophils usually showed a wide zone of clearing, while the psychrophils produced none or only clearing beneath the growth (Table 4). The results run almost parallel to those for liquefaction in gelatin stabs. None of the strains studied here completely hydrolysed the starch in 4 days, but strains PT and LG gave partial hydrolysis beneath the growth.

Haemolysis on horse blood agar and egg-yolk reaction. The mesophils generally gave a wide zone of haemolysis in 2 days (Table 4), which was slight or absent with the psychrophils, though these sometimes showed a little haemolysis after 4 days of growth. The haemolysis is not true β -haemolysis, being

a partial clearing with a diffuse edge; probably much of it is due to the lecithinase.

Upon egg-yolk plates all the mesophils gave positive reactions (presumably due to lecithinase), usually in 2 days and well marked in 4 days, while the psychrophils remained negative (Table 4).

Malonate, phenylpyruvate, gluconate and arylsulphatase tests. None of the strains of *Chromobacterium* studied gave positive reactions for phenylpyruvic acid or arylsulphatase. Malonate was utilized only by strain CA in 2 days. The gluconate test gave unexpected results. A few strains (see Table 4) produced a substance which reduced Benedict's solution, but unlike the *Pseudomonas* cultures, or 2-keto-D-gluconic acid, which only reduce the reagent on heating, the *Chromobacterium* cultures reduced the reagent in the cold in a few minutes. Some preliminary tests on these cultures showed that the reducing substance immediately reduced phenol blue ($E'_0 = +0.224$ V.) but not methylene blue ($E'_0 = +0.011$ V.) at pH 7.0; this suggests a structure similar to ascorbic acid. *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI also gave negative reactions for phenylpyruvate arylsulphatase and malonate utilization.

DISCUSSION

The name *Chromobacterium* is used here in a narrow sense. The name was used by Topley & Wilson (1929) as one of convenience, to include red and yellow chromogens such as 'Bacillus prodigiosus' (*Serratia marcescens*); this organism appears to be a typical member of the Enterobacteriaceae, and if unpigmented, many strains could scarcely be distinguished from some organisms of the coli-aerogenes group. *Pseudomonas* also seems to be generically distinct. I agree with Gilman (1953) that *Corynebacterium viscosum* and *Pseudomonas iodina* cannot be retained in the genus. They are both Gram-positive non-motile rods, in morphology similar to diphtheroids, and they do not produce violacein: they would not be placed in *Chromobacterium* if they did not produce pigment. Strain TI is not the *C. ianthinum* of older authors. It does not produce violacein, and appears to be a pseudomonad and it should be excluded from the genus.

The results reported here show that the genus *Chromobacterium* can be divided into two main groups. Features which, apart from the violet pigment, violacein, are unusual in bacteria are the mixed type of flagellation, the poor survival of cultures on storage and the sensitivity to inhibition by peroxide. Despite the considerable differences in other characteristics (e.g. method of attack upon carbohydrates) and the absence of strains which are clearly intermediate between the two groups these unusual features support the thesis that they all belong to one genus. The systematic position of this genus is uncertain; it possesses many characteristics of the Pseudomonadaceae and some characteristics of the Enterobacteriaceae. It may be closely allied to *Vibrio*. In practice it is the production of violacein which is the main distinguishing feature of *Chromobacterium*. This is unsatisfactory, since unpigmented strains which may be common in nature are probably never assigned to the genus; there may be non-chromogenic species which should be placed in the genus.

The two groups distinguished within the genus have a large number of correlated characteristics, and may be regarded as two species or biotypes. One can distinguish a subgroup in the mesophils, represented by the four strains which do not ferment glucose anaerobically. Since, however, most of the other aberrant features of these four strains (e.g. anaerobic growth, attack on other carbohydrates, storage of fat) may perhaps depend on one or two blocks in the carbohydrate metabolism of these strains, and since all my examples are old stock cultures which may have lost some characteristics, it would seem wise not to regard them as a distinct group without further studies. Such loss

Table 5. *Differential characteristics of mesophilic strains (18) and psychrophilic strains (20) of Chromobacterium*

Characteristic	Mesophils	Psychrophils
Growth at 37°	Occurs	Absent
Growth at 4°	Absent	Occurs
Relative size of organisms	Usually small	Usually large
Fat in organisms	Usually abundant	Usually scanty
Proteolysis	Marked	Slight
Haemolysis	Marked	Slight
Anaerobic growth	Usually good	Scanty or absent
Utilization of citrate	Often slow	Rapid
Production of HCN	Abundant	Not detected
Egg-yolk reaction	Positive	Negative
Attack on glucose	Usually fermentative	Oxidative
Acid from trehalose	Positive	Negative
Hydrolysis of aesculin	Negative	Positive

is not unknown in other genera, e.g. *Pfeifferella pseudomallei* (see *Topley and Wilson's Principles*, 1946, p. 487). The features closely correlated with growth temperature are shown in Table 5. Of these, a positive egg-yolk reaction, aesculin hydrolysis, HCN production and acid from trehalose show almost absolute correlation. The following features show some correlation: some mesophils are naturally pathogenic for mammals, they do not give gelatinous growth, give good pigment on potato and are usually methylene blue positive; the psychrophils are not naturally pathogenic, are often gelatinous, often pigment poorly on potato and are usually methylene blue negative. The following characteristics are either not correlated with growth temperature (or with each other), or insufficient evidence is yet available: source from soil or water, country of source, type of flagellation, degree of pigmentation, degree of growth on potato, reaction in litmus milk, acid from sucrose and starch, gluconate reaction, attack on nitrates and nitrites, heat resistance at 45°, colonial form and presence of metachromatic granules: none of these seems suitable as a differential characteristic.

Nomenclature

The nomenclature of the genus is confused, and will require action by the International Committee on Bacteriological Nomenclature to make it legitimate. Proposals have been submitted to the Committee for the conservation

of the generic name *Chromobacterium*, with the designation of a type species and type strain; these proposals have been published (Sneath, 1956*b*). The position will be fully dealt with there, and only a summary is given below.

Bergonzini (1879) used the name *Cromobacterium* for those members of the genus *Bacterium* which produced pigment. He recognized four species, three of which are now classified as *Pseudomonas* spp. while the fourth is unrecognizable. Later he described a new species which he named *Cromobacterium violaceum* (Bergonzini, 1880). Buchanan (1918) revived the name (with emended spelling) as that of a genus, and defined it in terms which virtually restricted it to the group of bacteria studied in this paper. Buchanan designated the type species as *Chromobacterium violaceum* Bergonzini. This designation of the type is illegitimate under Rule 9 (2) c2 of the revised International Bacteriological Code of Nomenclature (Editorial Board, 1953 (p. 42); International Committee, 1953 (Minute 6, p. 158)). In addition, it is not possible to recognize the species of Bergonzini's *C. violaceum* from his description, although it was almost certainly a member of the genus as defined here. It would be confusing and inconvenient to create a new generic name, and it would be better to conserve the name, basing it on a new type culture.

There are, in my opinion, two species within the genus, corresponding to the mesophilic and psychrophilic groups described in this paper. Many of the early descriptions of micro-organisms of this genus are unrecognizable as to species, since almost the only features described by early authors which allow recognition of the species are the growth temperature, the rate of liquefaction of gelatin and the degree of anaerobic growth (in the depths of stab cultures). The first validly-named bacterium recognizable with reasonable certainty as a mesophil is *Bacillus violaceus* Eisenberg 1888; the first recognizable psychrophil is *Bacillus janthinus* Plagge & Proskauer 1887 (syn. *Bacillus lividus* Eisenberg 1891, *Chromobacterium lividum* Holland 1920). Even if further work shows that the two species should be further divided it is essential to decide the valid names and to fix their types by designating cultures.

There seems no cogent reason why the type species should be either the mesophilic or the psychrophilic species, but I have proposed that the type be a mesophil and that it retain the specific epithet 'violaceum' on the following grounds: (1) this epithet was the one most generally used by early authors for mesophilic strains; (2) mesophilic strains appear on the whole to have been the more widely studied, and comprise the majority of the oldest strains available (for instance strains in the American Type Culture Collection (see Table 1)). The valid name for the psychrophilic species would then appear to be *Chromobacterium lividum* (Eisenberg 1891) Holland 1920. The epithet 'lividum' seems to be the earliest which is not illegitimate under Rule 24 (4) (Editorial Board, 1953, p. 52; International Committee, 1953, p. 158).

Type strains have been selected and deposited in the National Collection of Type Cultures and the American Type Culture Collection. The strains already in these collections have features which suggest they show less variation and therefore newer and typical isolates have been chosen. The type strain of the mesophilic species is strain MK (NCTC 9757, ATCC 12472). The type strain of

the psychrophilic species is strain HB (NCTC 9796, ATCC 12473). They have been proposed as neotypes.

A short definition of the genus and the two species under the proposed names is given below.

Genus *Chromobacterium* Bergonzini 1879, p. 38, *emend.* Buchanan 1918, p. 52, *nomen conservandum propositum*

Bacteria belonging to the same genus as the proposed neotype species generally have the characteristics given below when tested by the techniques described in this paper. They are rod-shaped Gram-negative non-sporing bacteria which grow on ordinary media and are aerobic; some are also facultatively anaerobic. They are motile by both polar and peritrichate (lateral) flagella, show bipolar or barred staining, and produce a violet pigment (violacein) which is insoluble in water and in chloroform, but is soluble in ethanol, in which solvent it has a maximum optical density near 580 m μ . and minimum near 430 m μ .

They show the usual resistance to heat, die rapidly in culture and are not obligate halophils; they utilize citrate for growth but not malonate, and produce acid without gas from glucose. They show the 'catalase' effect, reduce nitrates and destroy nitrites, and they are indole-negative, MR-variable, VP-negative, catalase-positive, NH₃-positive and urease-negative. The type species is *Chromobacterium violaceum*.

Species 1. *Chromobacterium violaceum* Bergonzini 1880, p. 153, *nomen conservandum propositum*.

Bacteria belonging to the same species as the proposed neotype strain possess the features of the genus and generally also those listed below.

They grow at 37° but not at 4°; the rods in young cultures average about 0.7 μ . broad and 2 μ . long, and contain abundant fat. They are markedly proteolytic and haemolytic, give a positive egg-yolk reaction and produce hydrogen cyanide; they utilize citrate slowly: are facultative anaerobes and produce acid from glucose anaerobically. They produce acid in trehalose and do not hydrolyse aesculin.

The proposed neotype strain is strain MK (NCTC 9757, ATCC 12472), whose reactions are described in detail in the tables and text.

Species 2. *Chromobacterium lividum* (Eisenberg 1891, p. 81), Holland 1920, p. 219 (syn. *Bacillus janthinus* Plagge & Proskauer 1887, pp. 463, 464).

Bacteria belonging to the same species as the proposed neotype strain possess the features of the genus and generally also those listed below.

They grow at 4° but not at 37°; the rods in young cultures average about 1.0 μ . broad and 3.7 μ . long, and contain little fat. They are feebly proteolytic and feebly haemolytic, do not give a positive egg-yolk reaction and do not produce detectable amounts of hydrogen cyanide. They utilize citrate rapidly, are strictly aerobic and produce acid from glucose but only aerobically.

They do not produce acid from trehalose peptone water but they hydrolyse aesculin.

The type strain is strain HB (NCTC 9796, ATCC 12473) whose reactions are described in detail in the tables and text.

The help is gratefully acknowledged of Brigadier F. E. Buckland, R.A.M.C., Microbiological Research Department, Porton, Wiltshire. Dr A. T. James kindly performed the analysis of volatile fatty acids, and Miss Joan Webb identified acids by chromatography. The following workers kindly provided strains for study: Dr J. P. F. Whelan (Institute for Medical Research, Kuala Lumpur, Malaya); Professor Brooks (Botany School, University of Cambridge (BSC)); Dr N. P. Burman (Metropolitan Water Board (MWB) London); the National Type Culture Collection (NCTC) and the American Type Culture Collection (ATCC); Miss M. B. Morris (Hankey Culture Collection (HCC), Colonial Microbiological Research Institute, Port of Spain, Trinidad); Dr W. L. Sippel (Georgia Coastal Plain Experiment Station, Tifton, Georgia); Dr Darrasse (Institut Pasteur de l'Afrique Occidentale Française, Dakar); the Director, Department of Bacteriology, University of Michigan, Ann Arbor, Michigan (UM); Professor Ethel Eltinge (Mount Holyoke College, South Hadley, Massachusetts); R. J. Hans (Michigan State University, East Lansing, Michigan); Dr Ellen Garvie (National Institute for Research in Dairying, Reading; NIRD); Miss V. G. Collins (Freshwater Biological Association, Ambleside, Westmorland; FBA). Potassium phenolphthalein disulphate was kindly provided by Professor L. Young (St Thomas's Hospital Medical School, London). Dr S. T. Cowan and Professor R. E. Buchanan gave much assistance with the nomenclature.

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(Received 4 January 1956)