

Biochemical and Chemical Characterization of Pink-Pigmented Oxidative Bacteria

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The biochemical and chemical characteristics were determined for 156 clinical isolates of pink-pigmented bacteria that are similar to but distinct from *Methylobacterium extorquens* (synonymous with *Pseudomonas mesophilica*). These isolates were gram-negative, nonfermentative, usually nonvacuolated, coccoid rods; all grew at 35°C and were catalase and urease positive; the majority grew on MacConkey agar and were variable for oxidase production and motility. On the basis of oxidation of xylose and mannitol and hydrolysis of esculin, these 156 strains were subdivided into four groups that were designated "pink coccoid" groups I, II, III, and IV. Groups I, II, and III are similar to an unnamed taxon described by Gilardi and Faur in 1984; only strains of group IV hydrolyze esculin. The cellular fatty acid compositions of strains of groups I, II, and III were essentially identical and differed from strains of group IV by the absence of 3-OH-C_{14:0} and the presence of C_{19:0Δ} and 2-OH-C_{19:0Δ}. The fatty acid composition of group IV strains was most similar to that of *M. extorquens* but differed by the presence of small amounts of two C_{17:1} acids, 3-OH-C_{16:0}, and 2-OH-C_{18:1}.

Over the past 24 years, the Special Bacteriology Reference Laboratory of the Centers for Disease Control (CDC) has received for identification many strains of pink-pigmented bacteria that are gram-negative, nonfermentative, usually nonvacuolated coccoid rods that grow at 35°C and were arbitrarily designated CDC group "pink coccoid." These cultures have been isolated from a variety of clinical sources such as blood, genitourinary sites, wounds, and an occasional environmental source.

Descriptions of similar bacteria isolated from clinical specimens have been reported. In 1984, Gilardi and Faur described an unnamed taxon of pink-pigmented, oxidative, gram-negative, short, plump, rod-shaped bacteria that were isolated from blood, cerebrospinal fluid, and sputum (5). A similar isolate from a blood culture was reported by Odugbemi et al. (14) in 1988. In this case, a 9-year-old boy with a fever and cough was admitted to an emergency room in Ilorim, Nigeria. Epiglottitis was diagnosed, but no pathogens were isolated from nose and throat cultures. However, a pink-pigmented, oxidative, gram-negative coccus with a few rods was isolated from a blood culture.

In 1989, Korvick et al. (11) described two cases in compromised hosts of opportunistic infection caused by a pink-pigmented, oxidative bacterium. One case involved the infection of a 40-year-old woman with acute myelogenous leukemia. Three weeks after receiving chemotherapy, she returned to the hospital with a 1-day history of fever, chills, and diaphoresis. One aerobic bottle of two blood cultures obtained on the day of admission was positive after 5 days of incubation. A pink-pigmented gram-negative rod was isolated on subculture. The other case involved a 60-year-old man who was admitted to the hospital for drainage of a pancreatic abscess. He developed a cutaneous intestinal fistula postoperatively and additional complications that extended over 3 months. A positive blood culture that grew a slow-growing, pink-pigmented, gram-negative, oxidative bacterium was reported 24 h after the patient's death.

The diagnostic problems associated with the pink-pigmented bacteria indicate the need for greater awareness and further characterization of these organisms. In this study, 156 pink coccoid strains referred to CDC were examined by using conventional biochemical tests and fatty acid and isoprenoid quinone analysis and compared with a similar pink-pigmented bacterium, *Methylobacterium extorquens*. The strain described by Odugbemi et al. (14) and four additional strains from the unnamed taxon described by Gilardi and Faur (5) were included in the study.

MATERIALS AND METHODS

Strains. Ten strains of *M. extorquens* (American Type Culture Collection [ATCC] 43645, synonymous with Japan Collection of Microorganisms [JCM] 2802, type strain; seven reference strains, ATCC 14718 [JCM 2805], ATCC 21438 [JCM 2827], ATCC 21611 [JCM 2807], ATCC 14821 [JCM 2811], ATCC 27329 [JCM 2830], JCM 2808 [ATCC 21612], and JCM 2816; and two clinical isolates, CDC 87-013826 and CDC 87-039959) and 156 clinical isolates of pink coccoid bacteria were identified by using the conventional culture and biochemical tests described previously (3) and shown in Table 1. Strain (CDC 84-060300) described by Odugbemi et al. (14) was included. The sources of the 156 pink coccoid bacteria are listed in Table 2. The four strains received from G. L. Gilardi were 1, 9, 12, and 13 as designated in his previous study (5). All of the reference strains are listed under *M. extorquens* in the Japan Collection of Microorganisms *Catalogue of Strains* (2, 6, 7, 10a), but only two strains, ATCC 43645 and ATCC 14718, are listed as such in the ATCC catalog. Some question exists as to the appropriate nomenclatural status of *M. extorquens*. Clinical microbiologists are most familiar with the synonym *Pseudomonas mesophilica* described by Austin and Goodfellow in 1979 (1). However, in 1984, a new genus and species, *Protomonas extorquens* was proposed to include this group of methylotropic bacteria (15). Then, in 1985, Bousfield and Green proposed that bacteria of the genus *Protomonas* be reclassified in the genus *Methylobacterium* (2). Since the majority

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TABLE 1. Biochemical characteristics of *M. extorquens* and CDC pink coccoid groups I, II, III, and IV^a

Characteristic	% Positive at 48 h (7 days)				
	<i>M. extorquens</i> (10) ^b	Pink coccoid groups ^c			
		I (56)	II (15)	III (79)	IV (6)
Hemolysis	0	5 ly	7 ly	13 ly	16 ly
Oxidase	91	93	73	51	100
Growth on MacConkey agar	0 (73) ^d	73 (91)	93	60 (82) ^d	67 (100)
Growth on Thayer-Martin agar	100 ^e	100	100	100	100
Catalase	100	96	100	100	100
Acid from (OF base):					
Glucose	18 ^d	4 (8)	0	39 (89) ^d	16 ^d
Fructose	0 (91)	55 (73)	80 (87)	98 (99)	100
Xylose	18 (100) ^d	73 (100) ^d	0	46 (74) ^d	83 (100) ^d
Mannitol	0	0	0	53 (100) ^d	0
Methanol (1%)	0 (100)	0	0	0	0
Growth pigment	pk-coral	Pale pk-pk	Pale pk-pk	Pale pk-pk	Pale pk-pk
	100	100	100	100	100
Nitrate reduction	18	18	7	23	100 ^f
Urea hydrolysis	9 (100)	70 (93)	73 (93)	59 (98)	100
Esculin hydrolysis	0	0	0	0	100
Starch hydrolysis ^g	100	88	87	97	100
Simmons citrate	0 (18)	45 (52)	33 (40)	87 (93)	33 (66)
Sodium acetate ^h	0 (100)	55 (60)	67 (87)	48 (76)	100
Nutrient broth, 6% NaCl	0	12 (14)	20	1	17
Growth at:					
25°C	100	96	87	89	100
35°C	82	100	100	100	100
42°C	0	86	53	59	100
Motility 1 to 2 p flagella	100	38	20 (27)	11	100
Vacuolated rods	100	0	0	0	40

^a Abbreviations: ly, lysis; pk, pink; p, polar. None of the strains formed acid from lactose, sucrose, or maltose in oxidation-fermentation base. None of the strains grew on salmonella-shigella agar or cetrinide, produced indole, H₂S, or acid in triple sugar-iron agar, hydrolyzed gelatin, or peptonized litmus milk. All strains, except one strain of group I, grew in nutrient broth without added NaCl.

^b The number of isolates is given within parentheses in headings.

^c Cells predominantly coccoid and usually in chains like pop beads except group IV (coccobacilli and rods). Growth frequently runny and mucoid.

^d Reaction often weak.

^e Barely visible pinpoint colonies.

^f Gas formation with two strains (33%).

^g Hydrolysis detected on Mueller-Hinton agar at 7 days.

^h Growth on sodium acetate medium detected for some strains, but no hydrolysis detected.

of strains we tested were received as *M. extorquens*, this name is used throughout the text, although nomenclatural difficulties have yet to be resolved.

Preparation and GLC analysis of fatty acids. For fatty acid analysis, the pink coccoid strains were grown for 24 h at 35°C on heart infusion agar supplemented with 5% rabbit blood, and the *M. extorquens* strains were grown on nutrient agar for 48 to 72 h at 30°C. Different media and growth conditions were required for *M. extorquens* strains because the growth of most strains is inhibited by blood and a temperature of 35°C. The cells from one plate were harvested and saponified, and the liberated fatty acids were methylated as previously described (13). The resulting fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) with the HP5898A Microbial Identification System (Hewlett-Packard Co., Avondale, Pa.) described in an earlier report (17). Tentative identifications of the fatty acid methyl esters were confirmed by computer-calculated equivalent carbon-chain-length values, hydrogenation, acetylation, and combined GLC-mass spectrometry.

Isoprenoid quinone analysis. Four strains from each biochemical group (four pink coccoid groups and *M. extorquens*) were inoculated onto 10 to 20 plates and incubated as described above. Cells from 10 plates of each of the pink coccoid strains and 20 plates of each of the *M. extorquens* strains were extracted for quinones as previously described

(13). The quinones were dissolved in methanol and analyzed by reversed-phase high-performance liquid chromatography as described previously (4). The quinones were tentatively identified by retention time comparison with known standards and confirmed by mass spectrometry analysis of collected fractions (12).

RESULTS

The cultural and biochemical characteristics for the pink coccoid bacteria and *M. extorquens* are shown in Table 1. Using the guidelines established by the Special Bacteriology Reference Laboratory, a bacterial group or species was considered negative for a test if 10% or less of the strains were positive at 7 days, positive for a test if 90% or more of the strains were positive at 7 days, and variable if 11 to 89% of the strains were positive at 7 days. All pink coccoid strains exhibited a pale-pink growth pigment when grown on heart infusion agar, and the growth was frequently runny and mucoid. *M. extorquens*, which is inhibited by blood, produced a pink-coral growth pigment and dry colonies when grown on nutrient agar. The 156 pink coccoid strains were nonfermentative, usually catalase and urease (sometimes delayed) positive, and grew on Thayer-Martin agar and usually MacConkey agar; the oxidase and motility tests were variable. The 156 strains were placed into four pink coccoid

TABLE 2. Sources of 156 strains of pink coccoid groups

Source	No. of strains in pink coccoid group:			
	I	II	III	IV
Blood	13	3	42	2
Genitourinary sites	15 ^a	2	1	0
Wound	5	0	6 ^b	2 ^c
Eye	2	2	7	0
Exudate	3 ^d	0	0	0
Abscess	0	0	3 ^e	0
Sputum	2	0	0	1
Ear	2	1	2	0
Peritoneal fluid (dialysis)	2	0	1	0
Cerebrospinal fluid	2	0	2	0
Pleural fluid	1	1	1	0
Breast exudate	0	1	0	1
Endotracheal or tracheal	0	1	1	0
Aspirate or rectal swab	1	1	1	0
Miscellaneous	9 ^f	3 ^g	14 ^h	0
Total	56	15	79	6 ⁱ

^a Ten cervix or vagina, two genital, one urogenital, one urine, and one urethra.
^b Foot, toe, hip prosthesis, scalp, and two unknown.
^c Knee and hand.
^d Bone, laceration, and unknown.
^e Liver and two lung.
^f Nasopharyngeal, intra-abdominal fluid, pelvis, lymph node, aspirate around heart pacemaker, pus palm hand, saline contaminant, plastic ice balls, and unknown.
^g Stomach tissue, kidney transplant, and unknown.
^h Neck mass, furuncle, aortic valve, infected pacemaker site, intracerebral clot, gland exudate, elbow bursa, bone marrow, pericardial biopsy, mediastinum, pancreatic fluid, joint fluid, and unknown.
ⁱ Warmer geographical areas (two from Hawaii, two from Puerto Rico, one from Louisiana, and 1 from Florida).

groups on the basis of oxidation of D-xylose and D-mannitol and hydrolysis of esculin. Group I consisted of 56 strains that were xylose positive, mannitol negative, and esculin negative; group II consisted of 15 strains that were xylose negative, mannitol negative, and esculin negative; group III consisted of 79 strains that were xylose variable, mannitol positive, and esculin negative; and group IV consisted of 6 strains that were xylose positive, mannitol negative, and esculin positive. All strains were gram-negative coccoid rods. However, the cells of pink coccoid groups I, II, and III are predominantly coccoid and usually occur in chains like pop beads; the cells of pink coccoid group IV are coccobacilli and rods that occur singly and in pairs. All *M. extorquens* strains, but none of the pink coccoid strains, slowly produced acid from methanol.

The cellular fatty acid compositions of *M. extorquens* and the four pink coccoid groups are shown in Table 3. The fatty acid profiles of groups I, II, and III were essentially identical. Each group contained moderate to large amounts of C_{18:1ω7c} and small to large amounts of C_{19:0Δ} (depending on how much C_{18:1ω7c} had been converted to C_{19:0Δ}). In addition, most strains also contained small amounts of 3-OH-C_{16:0}, 2-OH-C_{18:1}, and C_{16:1ω7c} and moderate amounts of C_{16:0}. These three groups were readily distinguished from *M. extorquens* and pink coccoid group IV by the absence of 3-OH-C_{14:0} and the presence of C_{19:0Δ}. The fatty acid composition of pink coccoid group IV was most similar to that of *M. extorquens*, but could be distinguished by the presence of small amounts of two C_{17:1} acids, 3-OH-C_{16:0}, and 2-OH-C_{18:1}. Two strains of *M. extorquens* that tolerated growth on 5% rabbit blood at 35°C showed essentially the same fatty acid composition as that obtained

TABLE 3. Cellular fatty acid composition of *M. extorquens* and CDC pink coccoid groups I, II, III, and IV

Organism (no. of strains)	Fatty acid ^a composition														
	3-OH-C _{14:0}	C _{16:1ω7c}	C _{16:1ω5}	C _{16:0}	C _{17:1B}	C _{17:1C}	3-OH-C _{16:0}	C _{18:2}	C _{18:1ω6c}	C _{18:1ω7c}	C _{18:1B}	C _{18:0}	C _{19:0Δ11,12}	2-OH-C _{18:1}	2-OH-C _{19:0Δ11,12}
<i>M. extorquens</i> ^b (10)	4 (tr-8)	3 (0-9)	—	4 (2-9)	—	—	—	2 (0-7)	1 (0-5)	80 (62-92)	—	5 (2-10)	—	—	—
Pink coccoid group:															
I (56)	—	3 (tr-7)	1 (0-4)	15 (9-21)	0 (0-tr)	1 (0-5)	1 (0-3)	1 (tr-5)	tr (0-3)	52 (28-68)	tr (0-2)	2 (tr-5)	11 (0-34)	4 (0-14)	8 (tr-22)
II (15)	—	1 (0-4)	tr (0-2)	17 (12-26)	—	tr (0-2)	1 (0-3)	1 (tr-2)	0 (0-tr)	50 (21-66)	0 (0-tr)	2 (1-3)	14 (tr-45)	4 (tr-9)	9 (tr-30)
III (79)	—	1 (0-3)	0 (0-tr)	17 (9-31)	—	—	1 (0-3)	1 (tr-4)	tr (0-4)	42 (14-65)	tr (0-2)	2 (0-5)	20 (1-48)	3 (0-15)	12 (1-38)
IV (6)	6 (4-14)	13 (10-16)	tr (tr-1)	7 (5-9)	tr (tr-1)	1 (1-2)	1 (tr-1)	1 (1-2)	1 (1-1)	63 (52-69)	—	tr (tr-1)	—	4 (3-6)	tr (0-1)

^a The number before the colon is the number of carbon atoms, and the number after the colon is the number of double bonds. 2-OH or 3-OH indicate a hydroxyl group at the 2 or 3 carbon, respectively. B or C indicates the position of the double bond is not known. Values are percentages of total fatty acids and are arithmetic means; tr, less than 0.7%; 0, mean less than 0.1%; —, not detected. Values in parentheses indicate the range observed for each acid.
^b *M. extorquens* also contains 3-OH-C_{18:0} (mean, 1%; range, 0 to 1%).

from cells grown on nutrient agar at 30°C for 48 h. Only small quantitative differences in the unsaturated acids were observed.

Pink coccoid groups I, II, and III also contained an unidentified acid that we had not detected previously in other bacteria. The fatty acid methyl ester of this acid eluted from the SE-54 column after eicosanoic acid (C_{20:0}) at approximately 17.52 min and had an equivalent carbon-chain-length value of 20.188. This fatty acid methyl ester was not affected by hydrogenation but reacted with trifluoroacetic anhydride, indicating a saturated (or cyclopropane) acid with a reactive hydroxyl group. Mass spectrometry of the fatty acid methyl ester showed a molecular ion (M⁺) of 326 and an *m/e* of 90, which is consistent with a saturated 19-carbon cyclopropane fatty acid methyl ester with the hydroxyl group at the α -carbon (or 2-carbon) from the carboxyl group (10). The position of the cyclopropane ring was determined with the picolinyl ester derivative prepared as described by Harvey (8) and analyzed by GLC-mass spectrometry. The resulting mass spectrum showed an M⁺ of 403 and an abundant odd electron ion at an *m/e* of 291 resulting from cleavage through the ring at the 11,12 position. Confirmatory evidence of the 11,12 position was the increased abundance of the ion *m/e* of 332 containing three more carbon atoms than the ion at an *m/e* of 291 (8). Thus, the identity of this acid was clearly established as 2-hydroxy-11,12-methyleneoctadecanoic acid (2-OH-C_{19:0 Δ 11,12}). The mass spectra of the picolinyl ester derivation of C_{19:0 Δ 11,12} showed an M⁺ of 387 and an abundant odd electron ion at an *m/e* of 275, which is consistent with ring cleavage at the 11,12 position, confirming the identity as 11,12-methyleneoctadecanoic (lactobacillic) acid. The relative amounts of 2-OH-C_{19:0 Δ 11,12} in group I, II, and III strains ranged from trace to 38% of the total fatty acids and from trace to 48% of C_{19:0 Δ 11,12} (Table 3).

M. extorquens contained ubiquinones with 10 isoprene units (Q10) as its major isoprenolog and minor amounts of Q9, which is consistent with the results from earlier studies (15, 16). However, we did not detect Q11 as a minor component as reported earlier (15, 16), which may be due to fewer cells and/or differences in the extraction method. The four pink coccoid groups contained Q10 as the major isoprenolog and Q8 and Q9 as minor isoprenologs. The identities of the ubiquinones were confirmed by mass spectrometry, which gave typical fragmentation patterns including a base peak ion at an *m/e* of 235 and molecular ions at 726, 794, and 862 for Q8, Q9, and Q10, respectively (9).

DISCUSSION

Based on biochemical characteristics, cellular morphology, and cellular fatty acid composition, we feel that none of the isolates from the four pink coccoid groups is *M. extorquens*. Group I, II, and III isolates are biochemically similar to the strains designated "unnamed pink-pigmented taxon" by Gilardi and Faur in 1984 (5). Of the four strains received from Gilardi, two strains, 1 and 13, were identified as CDC pink coccoid group I, and the other two, 9 and 12, were identified as group III. The strain described by Odugbemi et al. (14) was identified as CDC pink coccoid group I. Since the cellular fatty acid compositions were essentially identical for pink coccoid group I, II, and III strains and separation of each group is based on only one or two carbohydrate tests, we believe that these three groups represent different species or biotypes within a single genus.

Although distinguishable, group IV strains were most similar to *M. extorquens* based on cellular fatty acids and cellular morphology but readily distinguished from *M. extorquens* and strains of the other three pink coccoid groups by its hydrolysis of esculin. Since this group only contains six isolates, biochemical and chemical studies on additional isolates will be required to further characterize this group.

The limited clinical histories obtained on patients yielding pink coccoid bacteria suggest that these strains are primarily opportunistic pathogens. Many of our isolates were from patients who had an underlying clinical illness, such as renal disease, diabetes, leukemia, or traumatic injury. The increased reporting of the isolation of these bacteria indicates the need for characterization and identification of these strains to determine their clinical significance. This study provides the microbiologist with additional information to identify and group these bacteria. DNA-DNA studies will be required to determine their taxonomic status.

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