Detection, Identification, and Comparison of \textit{Capnocytophaga}, \textit{Bacteroides ochraceus}, and DF-1

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Working independently, three laboratories had recognized considerable similarity among certain strains of dysgonic, fermentative, capnophilic, surface-translocating, gram-negative bacilli referred to as \textit{Capnocytophaga}, \textit{Bacteroides ochraceus}, and Center for Disease Control biogroup DF-1. To determine the relationship among these groups, 21 strains were exchanged and independently characterized by the three laboratories. Additionally, a fourth laboratory examined the deoxyribonucleic acid homologies of the same strains. Using methods common to dental microbiology, eight of the strains had been isolated from the gingival sulcus and periodontal lesions and identified as \textit{Capnocytophaga}. Three strains isolated from blood and transtracheal aspirate had been characterized by conventional anaerobic methods and recorded as \textit{B. ochraceus}. Ten strains isolated from sputum, blood, throat, spinal fluid, and tracheal aspirate had been identified as DF-1 with the methods of E. O. King and a buffered single-substrate technique. All strains were similar in respect to colonial and microscopic morphology, surface translocation, biochemical features, gas-liquid chromatograms of metabolic end products, and deoxyribonucleic acid composition. We conclude that these biogroups should be termed \textit{Capnocytophaga} species.


Presently available reports indicate that \textit{Capnocytophaga} species are among the normal flora of the oral cavity (3) and are potential etiological agents in juvenile periodontitis, a severe, progressive periodontal disease of adolescence (5, 10, 11). Recent work for Forlenza et al. (S. W. Forlenza, M. G. Newman, and U. Blachman, J. Dent. Res., Abstr. no. 1027, p. 1024, 1979) indicates that, like \textit{Eikenella corroden} and other oral bacteria, \textit{Capnocytophaga} species can cause systemic disease in the compromised host. Their data show that sepsis with these organisms occurs in patients with hematological or other malignancies and who are granulocytopenic, frequently as a complication of cancer chemotherapy. Perhaps significantly, all of their patients presented with mucosal ulcerations, and half of them had gingival bleeding which might have served as the portal of entry of the organisms into the blood stream. Although five of their six patients with \textit{Capnocytophaga} sepsis were of pediatric age, two of us (U. Blachman and M. J. Pickett, unpublished data) have received, within the past year, three blood culture isolates of \textit{Capnocytophaga} for identification, two of which had been obtained from middle-aged patients with hematological malignancies and one from a geriatric patient with Hodgkin's disease.

Organisms designated \textit{B. ochraceus} are rarely isolated in clinical laboratories and have been considered indigenous oral flora. The term DF-1 (dysgonic fermenter-1) is a designation of the Center for Disease Control for one group of dysgonic and fermentative gram-negative bacilli isolated mostly from respiratory specimens and less frequently from other human sites.

We report here (i) our examination of the possible synonymy of strains originally identified as \textit{Capnocytophaga}, \textit{B. ochraceus}, or DF-1, and (ii) our observations concerning growth, detection, and identification of these organisms.
MATERIALS AND METHODS

Isolates. Twenty-one strains representing each of the three generic or group designations were exchanged and independently characterized by Olive View Medical Center (OVMC), the Wadsworth Veterans Administration Hospital Anaerobic Laboratory (WVAH), the University of California–Los Angeles School of Dentistry Periodontal Microbiology Laboratory (UCLA), and the University of California–Los Angeles Department of Microbiology (UCLA-M). The strains and their sources are listed in Table 1.

Methods for characterization. (i) OVMC. Conventional methods of the Center for Disease Control (16) and the buffered single-substrate technique described by Blachman and Pickett (1) were used to identify the isolates.

(ii) UCLA. Either Trypticase soy broth, Todd-Hewitt broth, or thioglycolate broth (all from BBL Microbiology Systems) was used as primary growth medium. Each strain was maintained on Trypticase soy agar supplemented with 5% sheep blood (BBL). All inoculations were performed in air. Cultures were incubated in an anaerobic chamber (Coy) at 37°C in an atmosphere containing 80% nitrogen, 10% carbon dioxide, and 10% hydrogen. Biochemical tests and analysis of metabolic end products were performed using methods described by Sutter et al. (15), Holdeman et al. (4), Socransky et al. (13, 14), or Newman et al. (10, 11).

(iii) WVAH. The methods used were those described by Sutter et al. (15).

(iv) UCLA-M. The strains used for determination of moles percent guanine plus cytosine (mol% G+C) and relative binding of deoxyribonucleic acid (DNA) strands are given in Tables 3 and 4. DNA was extracted from cells grown for 24 h in heart infusion broth (Difco) with 0.5% glucose. The broth cultures were incubated at 35°C in an atmosphere containing 5% CO2. Extraction was by a modification of a procedure described by Marmur (9). Packed cells, 2 to 3 g (wet weight), were suspended in 25 ml of saline ethylenediaminetetraacetate (0.15 M NaCl plus 0.1 M ethylenediaminetetraacetate, pH 8). One milliliter of a 25% solution of sodium lauryl sulfate and 2 to 4 mg of pronase (nuclease free; Calbiochem) were added to the cell suspension, and the mixture was incubated overnight at 35°C to effect cell lysis (2). The isolated DNA was repeatedly deproteinized in chloroform-isooamyl alcohol (24:1, vol/vol) and treated with ribonuclease (Calbiochem) at least twice during extraction. The concentration of the extracted DNA was determined in a Beckman model 22 spectrophotometer. Isolated DNA was stored at 4°C over an excess of chloroform.

G+C content for each strain was determined by the method of Schildkraut et al. (12) using cesium chloride density gradient centrifugation in a Beckman model E ultracentrifuge. DNA from Micrococcus lysodeikticus (Calbiochem) was used as a reference.

Radioactive DNA was prepared by growing DF-1 strain OV-199 for 24 h in 500 ml of low-phosphate labeling medium with 1.5 mCi of 32PO4 (carrier free; ICN). The low-phosphate labeling medium was prepared from the broth described above, the pH was adjusted to 10 with 40% NaOH, and 1 ml of 1 M MgCl2 was added to each 150 ml of broth. The solution was stirred for 30 min at room temperature, and the resultant suspension was centrifuged at 13,000 x g for 20 min at 4°C. The supernatant was removed, adjusted to pH 7.3 with concentrated HCl, autoclaved, and inoculated. DNA labeled by this procedure was extracted and purified as outlined above.

DNA hybridization was performed on nitrocellulose filters in 50% formamide-2x SSC (1x SSC: 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) by the method of Kourilsky et al. (7). Microfilters (6-mm diameter) were punched out of Millipore HAWP filters, numbered with a soft lead pencil, and soaked overnight in 6x SSC. Each filter was then loaded individually with 1 µg of heat-denatured DNA (100°C for 10 min in dilute SSC) by a procedure we have recently developed (J. R. Greenwood and M. J. Pickett, submitted for publication). The DNA-coated filters were dried overnight and heated for 2 h at 80°C in a vacuum desiccator. Solutions of radioactively labeled DNA were sheared for 1 min at 40% power in a sonicator equipped with a microprobe (Ultrasonics Inc., Plainview, N.Y.) and denatured as above. For each hybridization experiment, one DNA-coated filter was added to a test tube (10 by 75 mm) containing 0.2 ml of 50% formamide-6x SSC. One microliter of radioactive DNA was added, and the tube was overlaid with a drop of mineral oil and incubated for 20 h at 35°C. Uncoated filters were also processed in an identical manner as a control on nonspecific binding of labeled DNA to filters. After incubation, filters were washed, dried at 100°C for 3 h, and counted in a Beckman model LS 3133 P liquid scintillation counter. Each hybridization experiment was done in triplicate.

Table 1. Sources of strains

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Designation</th>
<th>Blood</th>
<th>Cerebrospinal fluid</th>
<th>Oral</th>
<th>Perio-dental abscess</th>
<th>Perio-dental disease</th>
<th>Sputum</th>
<th>Throat</th>
<th>Tracheal</th>
<th>Transtracheal aspirate</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVMC</td>
<td>DF-1</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>UCLA</td>
<td>Capnocytophaga</td>
<td>2</td>
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<tr>
<td>WVAH</td>
<td>B. ochraceus</td>
<td>2</td>
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</tbody>
</table>

TABLE 1. Sources of strains
RESULTS

Morphology. By dark-field microscopy these bacteria are straight, fusiform rods of various lengths with granules in many cells. When grown with enhanced carbon dioxide either aerobically or anaerobically on Trypticase soy agar plates supplemented with 5% sheep blood, they display a characteristic surface translocation or gliding (Fig. 1). Scanning electron microscopy of the leading edge of the colony on the agar plates revealed a large mass of cells at the periphery of the expanding colony. The cells were devoid of flagella, fimbriae, and pilis (Fig. 2).

Biochemical features. Most features studied by the three laboratories were identical for all strains (Table 2). However, there was some strain variation in respect to hydrolysis of casein, esculin, gelatin, and starch, reduction of nitrate to nitrite, and acidification of carbohydrates. In their catabolism of glucose, all strains produced succinic and acetic acids and a trace of propionic acid.

G+C content and DNA hybridizations. The values for G+C and DNA hybridization determinations (Tables 3 and 4) indicate that these organisms are closely related. Relative binding ratios obtained under stringent conditions for hybridization were greater than 90% (90 to 106%). G+C values were 39.6 to 41.1 mol%.

DISCUSSION

The similar colonial and microscopic morphology, requirement for carbon dioxide for growth, biochemical features, moles percent G+C, and DNA-DNA hybridization values of our 21 strains indicate that all are representatives of a single group for which a generic designation, Capnocytophaga, has already been introduced. This designation is apt for all of these strains, and we concur with Socransky et al. (13) in recommending its adoption. Their studies suggest that this genus includes at least three distinct biogroups. Our data indicate that 10 of our 21 strains may be C. sputigena of Socransky et al. and another 4 may be their C. gingivalis.

The antibiograms of Capnocytophaga species, based on a study of 13 strains (S. W. Forlenza, M. G. Newman, A. K. Horikoshi, and U. Blachman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, C183, p. 340), indicate a remarkable similarity to those of obligately anaerobic gram-negative bacilli. All strains were resistant to the aminoglycosides tested but were sensitive to penicillin, ampicillin, carbenicillin, clindamycin, erythromycin, tetracycline, chloramphenicol, and metronidazole. Sensitivity to several cephalosporins was variable.

The infrequent occurrence of Capnocytophaga species in clinical specimens may be more apparent than real since the organism is relatively fastidious and slow growing and requires elevated (5 to 10%) CO₂ for growth. Even under optimal conditions for growth, colonies may not be apparent on a 24-h blood agar plate. However, after 48 to 72 h of incubation, colonies are 1 to 5 mm in diameter and, on appropriate media, the surface translocation noted above is always evident. It is likely that after prolonged incubation Capnocytophaga species may be overgrown by more rapidly growing bacteria or that the primary plate may have been discarded before growth was visible. However, once growth of Capnocytophaga species has been obtained, identification is not difficult. One may tentatively identify the isolate as Capnocytophaga species on the basis of filamentous, fusiform microscopic morphology, slow growth, requirement for elevated carbon dioxide concentration, yellow-orange colonies, and gliding on blood agar. Significantly, the latter feature may not be apparent on some media. In our laboratories we were able to demonstrate gliding on Trypticase soy agar supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md., or CalLabs, North Hollywood, Calif.). However, surface translocation was markedly inhibited or absent on blood agar plates obtained from several other sources and on chocolate agar prepared at OVMC. Presently, no information exists concerning the specific component(s) affecting either promotion or inhibition of gliding by Capnocytophaga.

Definitive identification, particularly to the species level as proposed by Socransky et al. (13), requires additional biochemical tests. It appears from data presented here that more than one method or basal medium may be used. Essentially equivalent results were obtained by conventional methods of the CDC and a buffered single-substrate technique employing aerobic incubation, and by methods commonly used in anaerobic bacteriology. Those instances in which results differed significantly among laboratories were mostly in tests for acidification of carbohydrates. OVMC and UCLA reported that all strains acidified glucose, lactose, maltose, and sucrose, whereas WVAH found that only 71 to 81% of the strains gave positive tests. The apparent discrepancy may be attributed to the limited growth obtained in the peptone-yeast medium used by WVAH as compared to either the supplemented Trypticase soy broth of UCLA or the heart infusion broth base used by OVMC, particularly when these media are incubated aerobically under elevated CO₂ (unpub-
Fig. 1. Diluted sample of subgingival dental plaque on 48-h Trypticase soy agar with 5% sheep blood (BBL). Gliding colonial morphology of Capnocytophaga (arrows) can be readily recognized. Bar, 1 cm.

Fig. 2. Scanning electron photomicrograph showing leading edge of Capnocytophaga colony on agar surface, UCLA strain CX-2. Note fusiform cellular morphology. Bar, 10 μm.
Catabolites of data). Published.

Hydrolysis of data. Published.

Catalase 0/21

Nitrite from nitrate 8/21

Growth in 20% bile

Growth on MacConkey agar

Acidification of Glucose

Acidification of Lactose

Acidification of Maltose

Acidification of Mannitol

Acidification of Sucrose

Acidification of Xylose

Hydrolysis of Casein (litmus milk)

Hydrolysis of Esculin

Hydrolysis of Gelatin

Hydrolysis of Starch

Catabolites of glucose: succinic (major), acetic (minor), and propionic (trace to minor) acids

differed was hydrolysis of esculin. The lower sensitivity of the method employed by OVMC is due to at least two factors: (i) the dysgonic growth on the test medium (Kligler iron agar with 0.002% esculin), and (ii) reading hydrolysis of esculin as disappearance of its fluorescence under ultraviolet light. This method is somewhat less sensitive than those that employ a ferric indicator to detect release of esculin.

In clinical laboratories both detection and identification of Capnocytophaga species may be difficult because of unfamiliarity with these bacteria. However, detection is greatly facilitated when gliding is present. Superficially, this radial extension of a Capnocytophaga colony bears some resemblance to the swarming of Proteus species. In its absence detection may be achieved by the yellow-orange pigmentation and fusiform microscopic morphology. Being fastidious, fermentative, and oxidase and catalase negative, these species may resemble other unusual gram-negative bacilli. Features that distinguish Capnocytophaga species from phenotypically similar bacteria are shown in Table 5.
### Table 5. Salient features of Capnocytophaga and biochemically similar fastidious gram-negative bacilli

<table>
<thead>
<tr>
<th>Feature</th>
<th>Capnocytophaga sp.</th>
<th>Actinobacillus actinomycetemcomitans</th>
<th>Cardiobacterium hominis</th>
<th>CDC HR-5</th>
<th>Haemophilus aphrophilus</th>
<th>Haemophilus vaginalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase</td>
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<td>Catalase</td>
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<tr>
<td>Cellular morphology</td>
<td>Fusiform</td>
<td>Short to coccooidal</td>
<td>Pleomorphic</td>
<td>Medium to coccooidal</td>
<td>Short to coccooidal</td>
<td>Pleomorphic</td>
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<tr>
<td>Gliding</td>
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<tr>
<td>Pigmented growth</td>
<td>Yellow-orange</td>
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<td>Growth</td>
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<td>In air</td>
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<td>In air + 5% CO₂</td>
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<tr>
<td>Kliger iron agar (slant)</td>
<td>K or A/N or A</td>
<td>K or A/A</td>
<td>K/A or NG</td>
<td>K/A</td>
<td>A/A or A, g</td>
<td>NG</td>
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<tr>
<td>Motility</td>
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<tr>
<td>Esculin hydrolysis</td>
<td>V</td>
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<td>Nitrate → nitrite</td>
<td>V</td>
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<td>Indole</td>
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<td>Beta-hemolysis, 5% human blood agar</td>
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<tr>
<td>Acid production from</td>
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<tr>
<td>Glucose</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>Lactose</td>
<td>V</td>
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<td>Malto1</td>
<td>+</td>
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<tr>
<td>Mannitol</td>
<td>V</td>
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<td>+</td>
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<tr>
<td>G+C (mol%)</td>
<td>38.6-41.1</td>
<td>43'</td>
<td>ND</td>
<td>42'</td>
<td>43'</td>
<td></td>
</tr>
</tbody>
</table>

*Adapted from data in reference 1. Abbreviations: A, acid; g, gas; K, alkaline; N, neutral; ND, no data; NG, no growth; V, variable; w, week.

*A occasional strain may be weakly positive.

*We have encountered two strains of *C. hominis* that were oxidase negative on 24-h chocolate agar medium but strongly oxidase positive on a 24-h blood agar plate.

*Adapted from data in reference 6.

*Data from reference 8.

*Data of J. R. Greenwood and M. J. Pickett (submitted for publication).

### ACKNOWLEDGMENTS

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### LITERATURE CITED


