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Cell-Wall-Defective Variants of *Fusobacterium*

CAROLINE C. JOHNSON,[1]† HANNAH M. WEXLER,[2]* SUSI BECKER,[3] MANUEL GARCIA,[3] AND SYDNEY M. FINEGOLD[1,2,4,5]

Medical[1] and Research[2] Services, Veterans Administration Wadsworth Medical Center, Los Angeles, California 90073; Animal Diseases Research Institute, Nepean, Ontario, Canada 82H 8P8[3]; and Departments of Medicine[4] and Microbiology and Immunology,[5] University of California at Los Angeles School of Medicine, Los Angeles, California 90024

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The activity of antimicrobial agents against *Fusobacterium* species has been reported as variable in the literature. For some strains, the inconsistency arises from difficulty in determining the endpoint of growth in agar dilution susceptibility tests. Certain strains persist as a subtle haze beyond the levels of antibiotic that permit conventional colonial growth. We have determined by light and electron microscopy that this haze represents the colonial growth of cell-wall-defective (CWD) variants of the parent *Fusobacterium*. The CWD forms could be propagated indefinitely in hypertonic medium containing the antibiotic inducing agent. However, when the antibiotic was eliminated, the organisms would revert to their native morphology. Formation of CWD variants was observed in the presence of cell-wall-active drugs (e.g., β-lactam agents) but not with drugs that work by a different mechanism (e.g., clindamycin or chloramphenicol). Fourteen of 22 *F. varium* strains, 8 of 11 *F. mortiferum* strains, 2 of 10 *F. gonidiaformans* strains, and 1 of 4 of *F. necrophorum* strains could be induced to a CWD form in vitro in the usual agar dilution susceptibility test. Although the clinical significance of CWD variants of *Fusobacterium* is unknown, they may be a source of confusion in interpreting agar dilution susceptibility tests.

Fusobacterium isolates are frequently encountered in the clinical microbiology laboratory. These organisms have been associated with a variety of human and veterinary infections, including septicemia, brain abscess, pleuropulmonary infection, intraabdominal abscess, and many others (2, 7). The species most commonly encountered in infection are *F. nucleatum*, *F. necrophorum*, *F. varium*, *F. mortiferum*, and *F. gonidiaformans*. Although typically found as part of a polymicrobial flora, fusobacteria may also be found in pure culture, which further verifies the pathogenicity of these organisms (6, 8).

The activity of antimicrobial agents against *Fusobacterium* species has been reported as variable in the literature (8, 13, 14, 17, 18). The inconsistency encountered between some reports primarily involves strains of *F. varium* and *F. mortiferum*. In our experience, the variability in susceptibility reporting is caused by difficulty in determining the endpoint of growth for certain strains in agar dilution MIC testing. At low antimicrobial agent concentrations, these strains display conventional colonial growth; however, at increasing drug concentrations conventional growth is replaced by a haze. This study was undertaken to define the nature and significance of the haze observed when some *Fusobacterium* strains are grown on agar containing high concentrations of β-lactam agents.

MATERIALS AND METHODS

Source of bacterial isolates. The bacterial strains used in this study were clinical isolates recovered from patients with anaerobic infections. Strains were identified according to routine methodology (9, 16) and stored in skim milk at −70°C. A total of 81 strains (Table 1) representing seven species, namely, *F. nucleatum*, *F. varium*, *F. mortiferum*, *F. gonidiaformans*, *F. necrophorum*, *F. naviforme*, and *F. russii*, were used in this investigation.

Agar dilution susceptibility tests. Antimicrobial agent powders were supplied by the manufacturers and diluted according to their specifications on the day of the experiment. Agar dilution testing was performed as described previously (16). The susceptibility testing procedure used in this laboratory is essentially identical to the National Committee for Clinical Laboratory Standards procedure, except that we use brucella agar supplemented with 5% laked sheep blood and vitamin K_1 (10 μg/ml), which we have found to be more supportive for anaerobic organisms than the Wilkins-Chalgren agar used in the National Committee for Clinical Laboratory Standards procedure. Two endpoints were determined for strains that exhibited a haze. The first was the drug concentration at which conventional colonial growth was no longer observed; the second was the concentration of the drug beyond which the haze was no longer evident.

Induction and propagation of CWD bacterial variants. The haze observed on the agar dilution susceptibility plates was transferred to a fresh medium by using an inoculating loop or by removing a 0.5-cm agar block and using it as an inoculating device. (Either the agar block was placed on top of a fresh agar plate with the colonies on the agar block in contact with the fresh agar, or the agar block was placed in broth medium and the tube was shaken gently to release the growth.) The medium designed for support of cell-wall-defective (CWD) bacteria consisted of mycoplasma broth base (Difco Laboratories, Detroit, Mich.), 1.0% glucose, 10% yeast extract (Flow Laboratories, McLean, Va.), and 20% mycoplasma-free horse serum (MSG) (Flow). Agar (1.5%) was added to make a solid medium (MSGA). Cefox-

* Corresponding author.
† Present address: Department of Medicine, Veterans Administration Medical Center, Philadelphia, PA 19104.

TABLE 1. Variability of susceptibility to cefoxitin for *Fusobacterium* species[a]

Species	No. of strains	Haze not considered growth		Haze considered growth	
		MIC range (μg/ml)	% Susceptible at breakpoint[b]	MIC range (μg/ml)	% Susceptible at breakpoint
F. varium	22	0.5–16	100	0.5–16,384	36
F. mortiferum	11	4.0–64	91	4.0–16,384	18
F. necrophorum	4	0.25–4.0	100	0.25–2,048	75
F. nucleatum	31	0.062–8.0	100	0.062–8.0	100
F. naviforme	2	0.125–0.5	100	0.125–0.5	100
F. russii	1	1.0	100	1.0	100
F. gonidiaformans	10	0.062–1.0	100	0.062–4,096	80

[a] Depending on whether or not haze is read as growth.
[b] Breakpoint for cefoxitin is 32 μg/ml.

itin (4,096 μg/ml) was added when maintenance of the CWD variant was desired. Inoculated media were incubated at 37°C in an anaerobic chamber. Broth and plate cultures were examined for evidence of growth every other day for 3 weeks. Any growth was subcultured to both MSG and MSGA with and without cefoxitin (4,096 μg/ml). CWD variants were also prepared by direct inoculation of the *Fusobacterium* strain into antibiotic-containing MSG or MSGA.

Visualization of CWD bacterial variants. Conventional methods were used to visualize CWD forms. Colonial growth on agar was examined at ×20 magnification after a cover slip containing dried Dienes stain (Difco Laboratories, Detroit, Mich.) was inverted on top of a suspected colony (5). Microscopic morphology was evaluated by dark-field examination of wet mounts and Giemsa staining of colonies fixed in Bouin solution (10).

Electron microscopy. Selected *Fusobacterium* strains were grown on L-form induction medium (3) containing 20% horse serum with and without 50 to 250 μg of penicillin per ml. Colonies were fixed in situ with 1.0% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. The colonies were scraped off the agar, washed three times with 0.1 M phosphate buffer–0.2 M sucrose, postfixed with 1% OsO_4 in 0.1 M phosphate buffer for 1 h at room temperature, and then washed in the same solution. Fixed cells were pelleted in capillary tubes, and then suspended in 2% Noble agar, dehydrated in a graded series of ethanol, and embedded in Jembed 812 resin. Ultrathin sections were double stained with uranyl acetate and lead citrate. These were examined in a Hitachi HU-12A electron microscope operating at 75 kV and photographed on Kodak electron microscope film 4489.

RESULTS

Marked differences in the susceptibility of *Fusobacterium* to cefoxitin were observed, depending on the interpretation of the endpoint (Table 1). When the MIC was read at the point where conventional colonial growth was no longer evident, only 1 of 81 strains (1%) was resistant to cefoxitin. However, when haze on the agar was considered to be growth, then 26 of 81 isolates (32%) were resistant. The discrepancy in susceptibility results was striking for certain species, particularly *F. varium* and *F. mortiferum*.

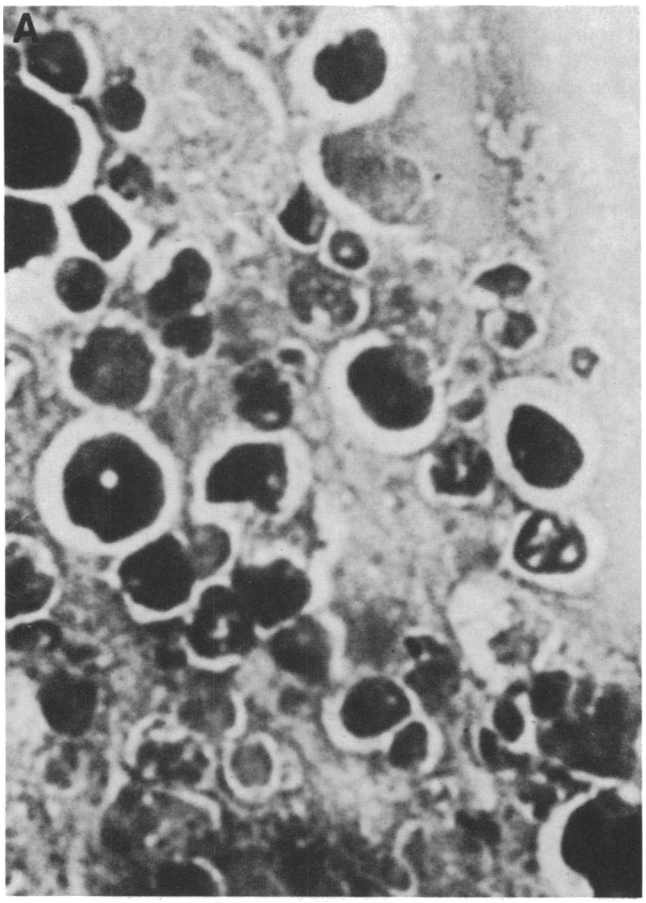
When the haze was examined microscopically at ×20 magnification, tiny, translucent colonies were observed. Individual colonies were stained intensely by the Dienes method. Attempts to subculture the haze onto brucella blood agar or fluid thioglycollate medium were unsuccessful. However, on transfer to MSGA containing 4,096 μg of cefoxitin per ml, growth in the form of fried-egg colonies appeared. These colonies, typical of those produced by L forms, appeared earliest at 5 days and reached a maximum diameter of 1.0 mm at 10 days. By dark-field microscopy, individual organisms appeared as spherical bodies 4 to 6 μm in diameter containing a coarse, granular interior. Giemsa stains of colonies growing in the presence of high concentrations of the antibiotic also revealed typical rounded forms of a CWD variant.

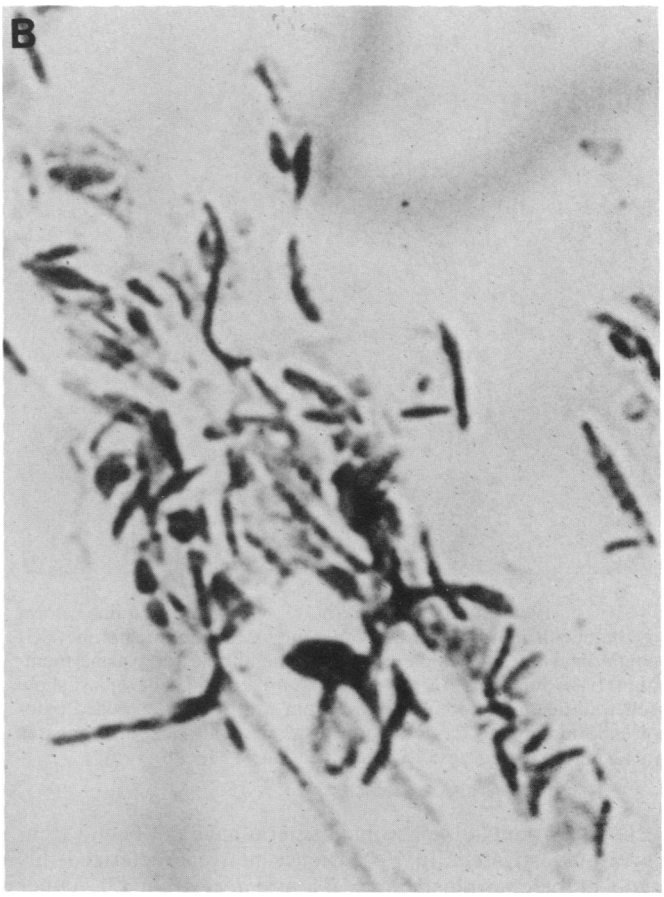
Spheroplast formation could also be induced by direct inoculation of certain *Fusobacterium* strains into hypertonic medium (MSG) containing high concentrations of cefoxitin. Only the 25 strains that had produced haze on agar dilution susceptibility plates yielded typical L-form colonies and microscopic spheroplasts by this method (Table 2). Production of CWD forms was most common for *F. varium* and *F. mortiferum* isolates. Those strains not producing spheroplasts were incapable of growing in the presence of the inducing agent, cefoxitin, at 4,096 μg/ml. CWD variants could be propagated indefinitely provided subcultures to the defined hypertonic medium containing the inducing agent were performed regularly (at least every other week). Upon subculture to antibiotic-free medium, reversion to the colonial and gram stain morphologies of the parent strain had taken place by the second serial passage (Fig. 1). The organism recovered after reversion was phenotypically identical to the initial strain.

Antibiotics which successfully induced CWD forms from *Fusobacterium* strains were ampicillin, cefoxitin, moxalactam, piperacillin, and imipenem. No induction of CWD bacterial variants was observed with chloramphenicol, clindamycin, erythromycin, polymyxin, or tetracycline.

TABLE 2. Prevalence of induction of CWD variants from different *Fusobacterium* species when exposed to cefoxitin (4,096 μg/ml)

Species	No. of strains	
	Total	Forming CWD variants (%)
F. varium	22	14 (64)
F. mortiferum	11	8 (73)
F. necrophorum	4	1 (25)
F. nucleatum	31	0 (0)
F. naviforme	2	0 (0)
F. russii	1	0 (0)
F. gonidiaformans	10	2 (20)





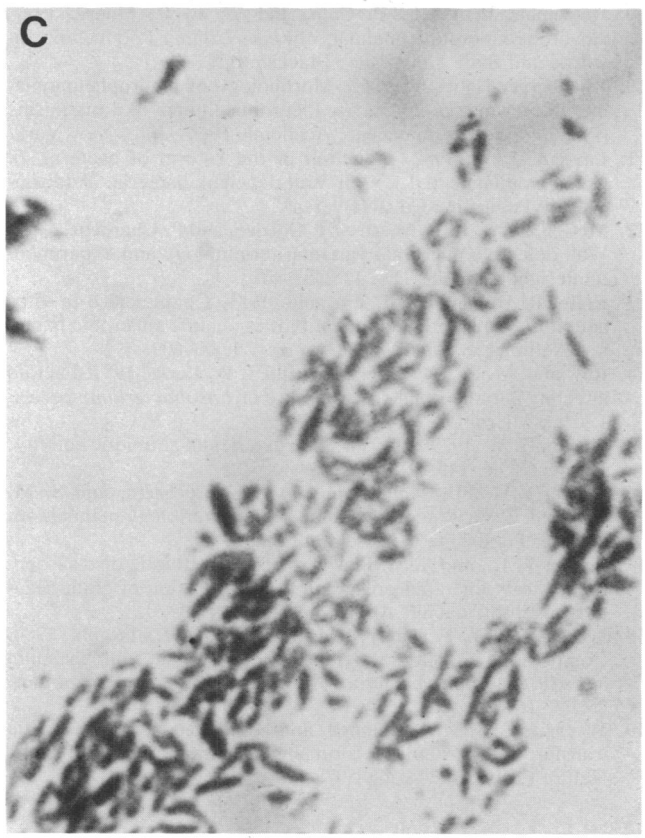


FIG. 1. (A) Gram stain of *F. varium* grown on media containing 4,096 μg of cefoxitin per ml. Note typical spheroplast formation. Magnification, ×1,000. (B) Gram stain of *F. varium* after one subculture on antibiotic free medium. Magnification, ×1,000. (C) Gram stain of *F. varium* after two subcultures on antibiotic-free medium. Organisms have reverted to parent morphology. Magnification, ×1,000.

Electron microscopy of a strain of *F. varium* grown in the presence of penicillin G demonstrated the nature of the morphologic alteration (Fig. 2). Fragmented loss of the peptidoglycan layer resulted in true spheroplast formation with partial loss of the gram-negative outer membrane. These morphologic changes were not observed in the absence of the antibiotic inducer.

DISCUSSION

The term CWD variant is used to refer to bacteria with altered morphology and cultural characteristics consistent with a damaged, deficient, or absent cell wall structure (12). The clinical significance of CWD bacteria is uncertain; however, their in vitro occurrence is well established (11, 14). In addition, they can be propagated in culture while maintaining the biochemical identity of the parent bacterium. Removal of the inducing agent or condition usually results in reversion to the parent organism. However, in some cases, after serial subcultures, genetically stable L forms may emerge. This transition from the unstable to the stable CWD state often reflects underlying genetic mutation (presumably loss of a DNA segment concerned with cell wall synthesis) (19).

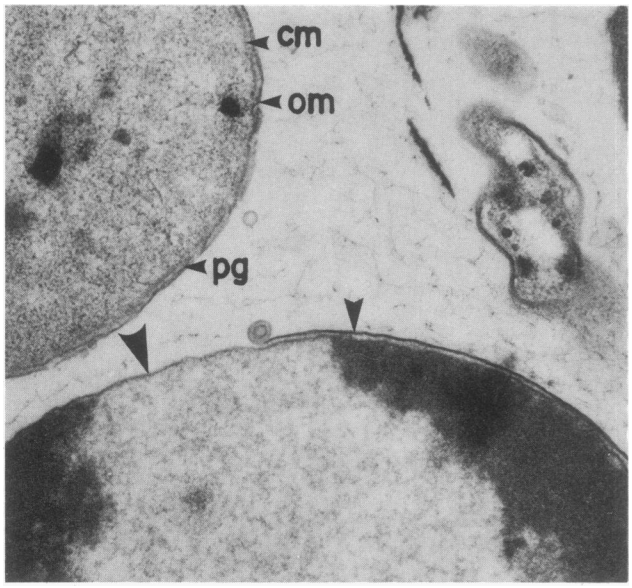


FIG. 2. Electron micrograph of *F. varium* grown in L-form medium with 25 U of penicillin G per ml. OM, Outer membrane; PG, peptidoglycan; and CM, cytoplasmic membrane (or plasma membrane). An adjacent intact *Fusobacterium* cell and spheroplast show cell wall peeling (medium arrow) from cell, leaving exposed cytoplasmic membrane (large arrow). The cell wall ribbon terminates into a multilayered vesicle. Magnification, ×33,750.

CWD variants of anaerobic bacteria have not been extensively investigated. In 1944 Dienes first characterized this type of pleomorphism in a *Bacteroides* strain (5). Other investigators later associated L forms with acute or recurrent thromboembolic disease (1). In two specific cases, the L-form pathogen was identified as a *Sphaerophorus* (*Fusobacterium*). The significance of this report is clouded by lack of technical details concerning methods of microbiologic isolation and identification and by uncertainty as to whether the organisms recovered were indeed related to the pathogenesis of the disease. Although the significance and frequency of inducing CWD forms from anaerobes are uncertain, the phenomenon in vitro is seen with some frequency in *Fusobacterium* species (14). A technique for protoplast formation and regeneration has also been described for a rumen isolate of *F. varium* (4).

Two major concerns are raised by the observations in this study. First, the aberrant growth pattern assumed by these strains may lead to inconsistent interpretation of agar dilution susceptibility endpoints. In turn, this could lead to variable reporting on the activity of cell-wall-active antimicrobial agents against *Fusobacterium* spp. To deal with the problem of formation of CWD variants during agar dilution susceptibility testing, we presently report the MIC for all *Fusobacterium* isolates as the lowest drug concentration permitting no conventional colonial growth or less than two colonies. The persistent haze produced by the CWD variants is ignored for purposes of MIC reporting on the assumption that the CWD form is not clinically significant. Second, the CWD variants that are clearly evident in vitro may also exist in vivo. Although there is no convincing evidence that L forms have a significant pathogenic role in any disease, the possibility should be considered for anaerobic infections that often are chronic, refractory to therapy, or recurrent (15). Whether these strains are able to remain viable in vivo when exposed to antibiotics and to revert back to the parent form when the agent is no longer present needs to be investigated.

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