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Relative Morphological Effects Induced by Cefoxitin and Other Beta-Lactam Antibiotics In Vitro

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Cefoxitin, a new semisynthetic cephamycin antibiotic, induced filament formation at subinhibitory concentrations with a β-lactamaseless strain of *Enterobacter cloacae* (HSC 18410 M66). The extent of filament induction by cefoxitin was similar to that seen with cephalothin, cefazolin, and benzylpenicillin. Filament induction by cefoxitin was markedly less than that seen with cephalexin, carbenicillin, ticarcillin, cephradine, and cephapirin. Antibiotics which failed to induce filaments at any level tested included cephaloridine, cephacetrile, cephalosporin C, the cephamycins, 6-aminopenicillanic acid, 7-aminocephalosporanic acid, A16884, A16886, and FL-1060. Those antimicrobial agents tested which lacked an aromatic substituent in the 7-position (for cephems) or in the 6-position (for penams) did not induce filaments. These observations suggest a possible relationship between filament induction of the test organism and the molecular nature of constituents in the 7- or 6-position of β-lactams.

Certain β-lactam antibiotics induce bacterial filament formation at subinhibitory concentrations, i.e., at levels below those which induce spheroplasts. The evidence indicates that such filament induction is a widespread, but not universal, occurrence among both penicillins and cephalosporins (4, 10, 11, 12, 15, 16, 18).

Cefoxitin (Fig. 1) is a new semisynthetic analogue of the naturally occurring cephamycin group of antibiotics (8). Cefoxitin, which is highly resistant to inactivation by β-lactamase (13, 14), has a mode of action similar to that of related β-lactam antibiotics, i.e., inhibition of cell wall biosynthesis (14).

In the present report, cefoxitin was compared with several other β-lactam antibiotics to determine its capacity to induce morphological aberrations, particularly with respect to the extent of filament induction at subinhibitory concentrations. To avoid interference from the effects of β-lactamase activity, we chose for the study a naturally occurring mutant of a clinical strain of *Enterobacter cloacae* that had lost the ability of the parent to produce β-lactamase (3).

Results indicated that cefoxitin induced filaments to an extent approximately equal to that seen with cephalothin, cefazolin, or benzylpenicillin, but to a lesser degree than that seen with cephalexin, carbenicillin, ticarcillin, or cephaloglycine. Filament induction by cefoxitin was greater than that observed with cephaloridine or cephacetrile. In the course of the study, a possible relationship was seen between induced morphological effects and the nature of the substituent in the 7-position (for cephalosporins) or in the 6-position (for penicillins). (This paper was presented in part at the 75th Annual Meeting of the American Society for Microbiology, 27 April to 2 May 1975, New York.)

MATERIALS AND METHODS

Organism. The test strain, *E. cloacae* HSC 18410-M66, was obtained from M. Goldner of the Research Institute of the Hospital for Sick Children, Toronto, Canada (3). The organism was deposited in the Merck Culture Collection and is there designated as MB-2647. Stock cultures were maintained at 2 to 5 C on nutrient agar slants for up to 3 weeks before use.

Test mixtures. Test mixtures for morphological studies were prepared using modified procedures of Lederberg (9) as follows: using a nutrient agar slant as the starter culture, MB-2647 was grown overnight in antibiotic medium no. 3 (Difco) (AM3) at 37 C on a rotary shaker (New Brunswick Scientific Co.) at 220 rpm. Antibiotics were dissolved in AM3 supplemented with 20% sucrose + 0.2% $MgSO_4 \cdot 7H_2O$. For the purpose of determining visible growth inhibition, parallel test mixtures were prepared in AM3 lacking sucrose and $MgSO_4 \cdot 7H_2O$, thus removing osmotic stabilization and permitting lysis of osmotically fragile forms.

Three milliliters of overnight culture consisting of approximately 2×10^9 cells/ml was added to each 10 ml of antibiotic solution. Test mixtures of 2 ml each were incubated in culture tubes (12 by 75 mm) on the shaker for 5 h at 37 C, immediately followed by preparation of wet mounts which were examined microscopically for cellular morphology at $\times 1000$ magnification by a phase contrast microscope. A range of antibiotic concentrations was examined which included (i) inhibitory levels, i.e., those in

FIG. 1. *Structure of cefoxitin.*

which the entire population of rods was converted to spherical forms; (ii) subinhibitory concentrations at which morphological distortions such as elongation and/or swelling could be seen; and (iii) noninhibitory concentrations in which cells appeared virtually identical to those of untreated controls.

Photomicrographs. Photomicrographs of rods and filaments were made from gentian violet-stained test mixtures. Photomicrographs of spherical forms were made directly from wet mounts.

Scanning electron micrographs (SEM) were prepared in the following manner: specimens were fixed overnight in phosphate-buffered (pH 7.3) 3% glutaraldehyde, rinsed in buffer, and postfixed in phosphate-buffered (pH 7.3) 1% osmic acid for 1 h. Washed specimens were dehydrated in graded ethanol and immersed overnight in amyl acetate. After critical-point drying in a Denton DCP-1 critical-point dryer, specimens were mounted on an aluminum stub and coated with ~20.0 nm of gold in a Denton DV515 evaporator. Photographs were taken on an AMR-900 scanning electron microscope operated at 10 kV. For SEM, one test mixture of each antibiotic was chosen from each dilution series at a subinhibitory concentration containing the greatest percentage and length of filaments visible in wet mounts. Samples chosen for SEM were immediately chilled until fixation, which was done within 5 h after the end of the incubation period.

Transmission electron micrographs (TEM) were prepared using the same fixation and dehydration methods described above for SEM. After dehydration, TEM specimens were embedded in Epon 812 and sections approximately 80.0 nm thick were cut with a diamond knife. Sections were stained with uranyl magnesium acetate and lead citrate and viewed with a Zeiss 9S2 electron microscope. Test mixtures chosen for TEM were selected and treated in the manner described above for SEM samples.

Antibiotics. Antibiotics were dissolved in AM3, with or without added sucrose and $MgSO_4 \cdot 7H_2O$. Solutions were stored at 2 to 5 C for up to 1 week before use.

Cefoxitin (sodium salt), the cephamycins, 6-aminopenicillanic acid, 7-aminocephalosporanic acid, and cephalosporin C (Na^+ salt) were prepared at Merck Sharp and Dohme Research Laboratories, Rahway, N.J. Other antibiotics were obtained from the following sources: cephalothin (Na^+ salt, Keflin), cephaloridine (Loridine), antibiotics A16884 and A16886, cephaloglycine (Kafocin), cephalexin (Keflex), and cefamandole from Eli Lilly and Co., Indianapolis, Ind.; cefazolin (Cefamezin) from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; benzylpenicillin (penicillin G, Na^+) and carbenicillin from Pfizer, Inc., New York, N.Y.; ticarcillin from Beecham, Inc., Piscataway, N.J.; cephradine from E. R. Squibb and Sons, Princeton, N.J.; and FL-1060 from Leo Pharmaceutical Products, Ballerup, Denmark.

RESULTS

Figure 2 shows gentian violet stains of rod forms (Fig. 2a), filaments (Fig. 2b), and phase contrast micrographs of wet mounts of spheres (Fig. 2c) as observed in the light microscope. The bar represents 5 nm. At inhibitory concentrations, all antibiotics tested induced spheroplast formation. At levels just below inhibitory concentrations, several antibiotics induced filament formation to varying degrees. At still lower concentrations, treated cells assumed rod shapes similar to untreated controls. Figure 2 corresponds to the appearance of the test organism in the presence of a noninhibitory concentration of cefoxitin, i.e., 0.25 μg/ml (Fig. 2a); at subinhibitory levels in which typical filaments were seen at 1, 2, or 4 μg of cefoxitin/ml (Fig. 2b); and at inhibitory concentrations (8 to 16 μg/ml) in which all cells were converted to spheroplasts (Fig. 2c). Similar modifications were observed with the same microorganism treated with corresponding ranges of cephalothin or with benzylpenicillin.

Gentian violet staining tended to reduce the size of cells when compared to the same sample observed with wet mounts. Wet mounts of filaments on the other hand were not suitable for photomicrographs because it was impossible to focus on the entire length of many of the filaments. We therefore used SEM to demonstrate the extent of filament formation.

Figure 3 shows the filament-inducing group of antibiotics tested, along with the untreated control (Fig. 3f). Each antibiotic is shown at the subinhibitory concentration in which the greatest percentage and longest filaments had been observed in corresponding wet mounts viewed through a phase contrast microscope at ×1000. It is apparent from Fig. 3 that cefoxitin (3a), (3b), cephalothin, cephalexin (3c), cefazolin (3d) and carbenicillin (3e) induce filaments of great length, some of which are ≧30 nm, as compared to cells of the untreated control (3f), which range from ~1 to 2 nm in length.

SEMs of cells grown in the presence of subinhibitory levels of those antibiotics which failed to induce filaments demonstrated morphologies similar to that of untreated controls; i.e., rods became slightly swollen and/or elongated (see Fig. 6). Substances in this group included 6-aminopenicillanic acid, 7-aminocephalosporanic acid, cephalosporin C, cephamycin A, and antibiotic A16884. One was hard pressed to find even a slightly elongated rod, although some

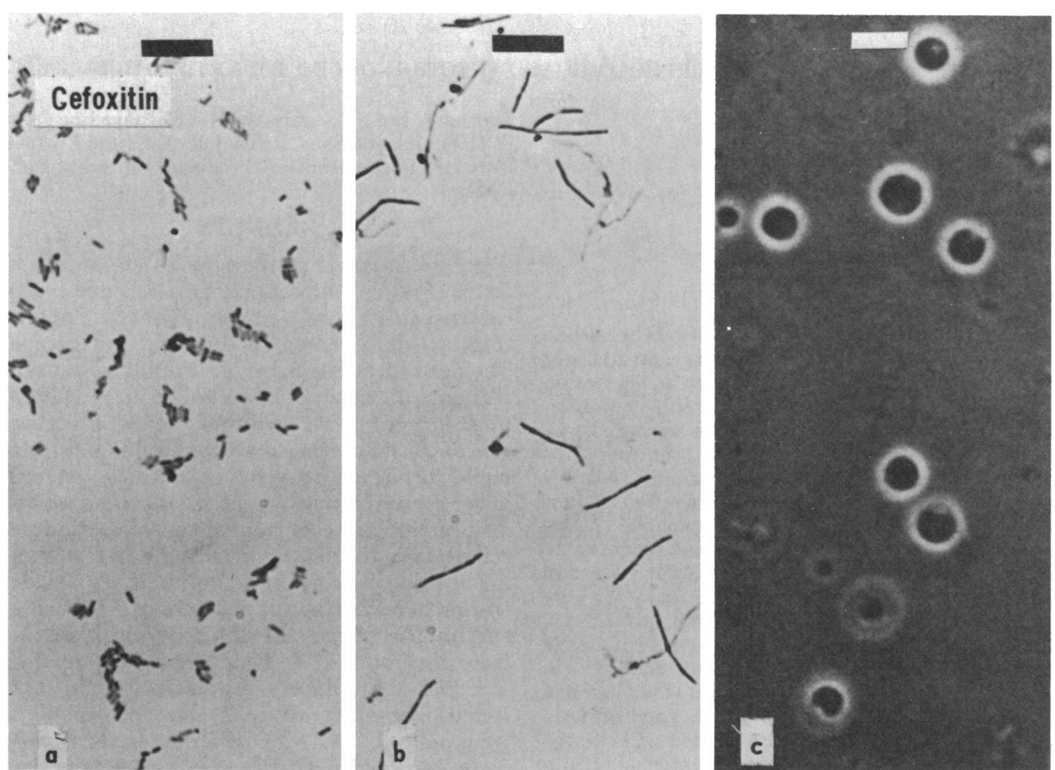

FIG. 2. *Bars = 5 nm. (a) Gentian violet stain of cells of E. cloacae HSC 18410-61 grown in the presence of 0.25 μg of cefoxitin/ml, a noninhibitory level. (b) Gentian violet stain of typical filaments of E. cloacae grown in the presence of subinhibitory concentrations of cefoxitin (1, 2, or 4 μg/ml). (c) Phase contrast photomicrograph of wet mount of spheres of E. cloacae induced in the presence of inhibitory concentrations of cefoxitin (8 to 16 μg/ml) Similar results were seen with corresponding levels of cephalothin and benzylpenicillin.*

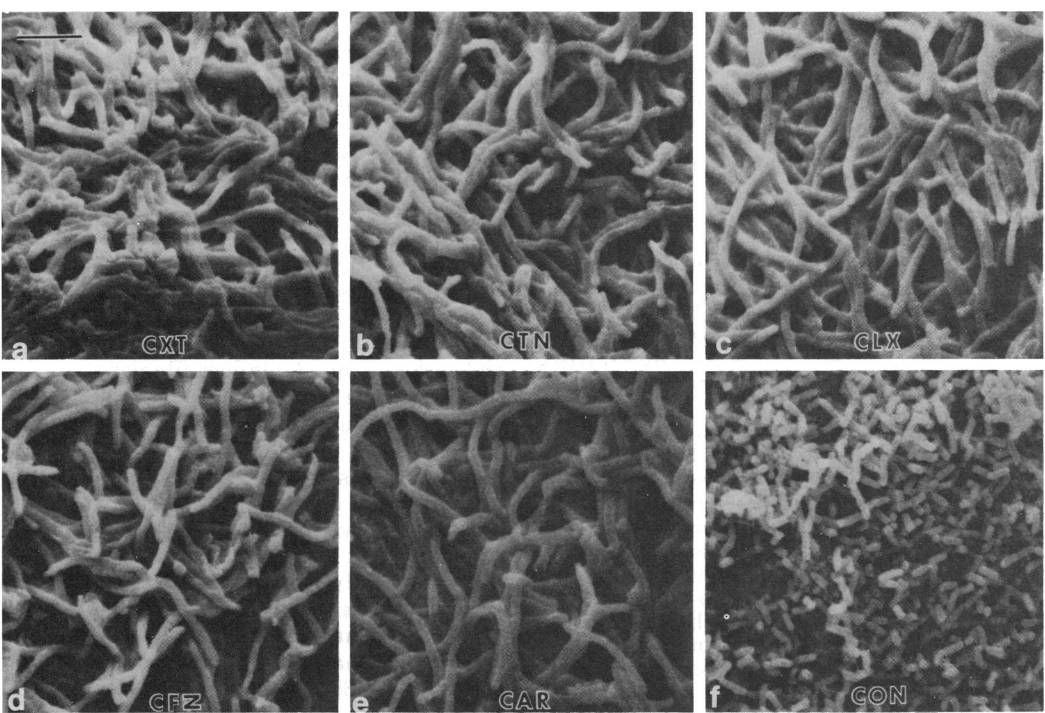

FIG. 3. *SEM (bar = 5 nm) of cells of E. cloacae grown in the presence of subinhibitory levels of filament-inducing antibiotics. (a) Cefoxitin (CXT); (b) cephalothin (CTN); (c) cephalexin (CLX); (d) cefazolin (CFZ); (e) carbenicillin (CAR); (f) untreated control (CON).*

swelling and outpouching were seen, particularly with cephamycin A.

Cephacetrile (Fig. 4) induced more elongation and swelling than did the preceding group, but was a markedly poor filament inducer when compared to the cefoxitin-cephalothin group (Fig. 3).

Figure 5a (untreated control) shows a TEM of normal cell division and accompanying septum formation with the test organism in the absence of antibiotic. Filaments induced in the presence of subinhibitory levels of cefoxitin (Fig. 5b), cephalexin (Fig. 5c), carbenicillin (Fig. 5d), and cephalothin (Fig. 5e) all demonstrate similar multinucleate, nonseptate filaments. Representing the non-filament-inducing group is antibiotic A16884 (Fig. 6a) in the presence of which are seen slightly elongated and swollen rods in varying stages of septum formation. The morphology of A16884-treated cells is typical of that induced by the other members of this group, e.g., cephalosporin C, the cephamycins, antibiotic A16886, 7-aminocephalosporanic acid, and 6-aminopenicillanic acid. The TEM of cephacetrile (Fig. 6b) demonstrated greater elongation than the preceding group but did not approach the extent of filamentation seen in the cefoxitin-cephalothin group. In addition, the TEM of cephacetrile demonstrated septum formation which is absent in the filament-inducing group.

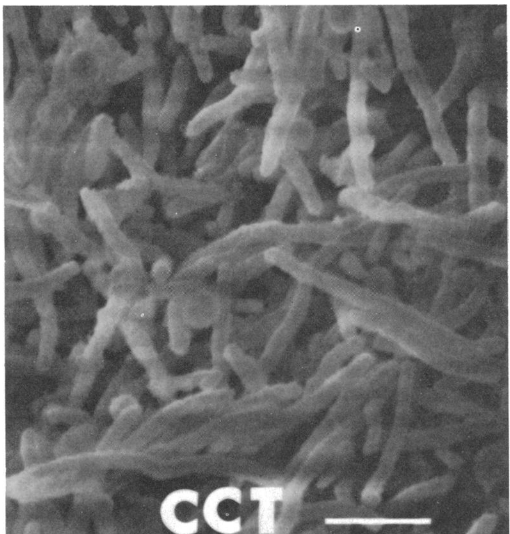

FIG. 4. *SEM of cells of E. cloacae grown in the presence of subinhibitory levels of cephacetrile. Bar = 5 nm. Cells are intermediate in length between those grown in the presence of similar levels of filament-inducing antibiotics (Fig. 5) and non-filament-inducing antibiotics (Fig. 6).*

Assuming an arbitrary definition of a filament as a rod-shaped cell of ≧15 nm in length, representing approximately 10-fold greater length than that of untreated control cells, a survey was made of the filament-inducing capacity of subinhibitory levels of the antibiotics tested. Tables 1 and 2 show the number of serial twofold dilutions of each antibiotic which induced formation of filaments ≧15 nm with the majority of the cell population, observed in a phase contrast microscope at ×1000 magnification. The results for both cephalosporins (Table 1) and penicillins (Table 2) suggest a possible relationship between filamentation and the nature of the substituent in the 7- or 6-position for cephalosporins or penicillins, respectively: all antibiotics tested which had nonaromatic groups in the 7-position did not demonstrate induction of filaments (as defined above) at any concentration tested. Such antibiotics included the cephamycins and A16884, which differ from each other only in the substituent in the 3-position, cephalosporin C and A16886, which also differ only in the three position, cephacetrile, and 7-aminocephalosporanic acid. Cephacetrile induced the longest forms in this group but did not meet the ≧15-nm criterion. On the other hand, cefoxitin, cephalothin, cefazolin, and especially cephalexin were excellent filament inducers. Cephalexin-induced filaments not only covered a wider range of serial twofold dilutions (six) but also produced filaments of much greater length than did the other antibiotics in the filament-inducing groups. Other cephalosporins tested, but not shown in Table 1, which induced filaments to an extent equal to or greater than those shown in Table 1 included: cephapirin, cephamandole, cephaloglycine, and cephradine. The only non-filament-inducing cephalosporin tested which had an aromatic substituent in the 7-position was cephaloridine.

DISCUSSION

The results show that at subinhibitory concentrations, against a β-lactamaseless strain of *E. cloacae,* cefoxitin, a new semisynthetic cephamycin antibiotic, is one of a group of filament-inducing β-lactam antibiotics. This group includes cephalothin, cephalexin, cefazolin, cephapirin, cephradine, cefamandole, cephaloglycine, carbenicillin, ticarcillin, and benzylpenicillin. With a second group of β-lactams, little or no filament induction was obtained. This group included the cephamycins, cephalosporin C, antibiotics A16884 and A16886, cephacetrile, 7-aminocephalosporanic acid, cephaloridine, FL-1060, and 6-aminopenicillanic acid. It is ap-

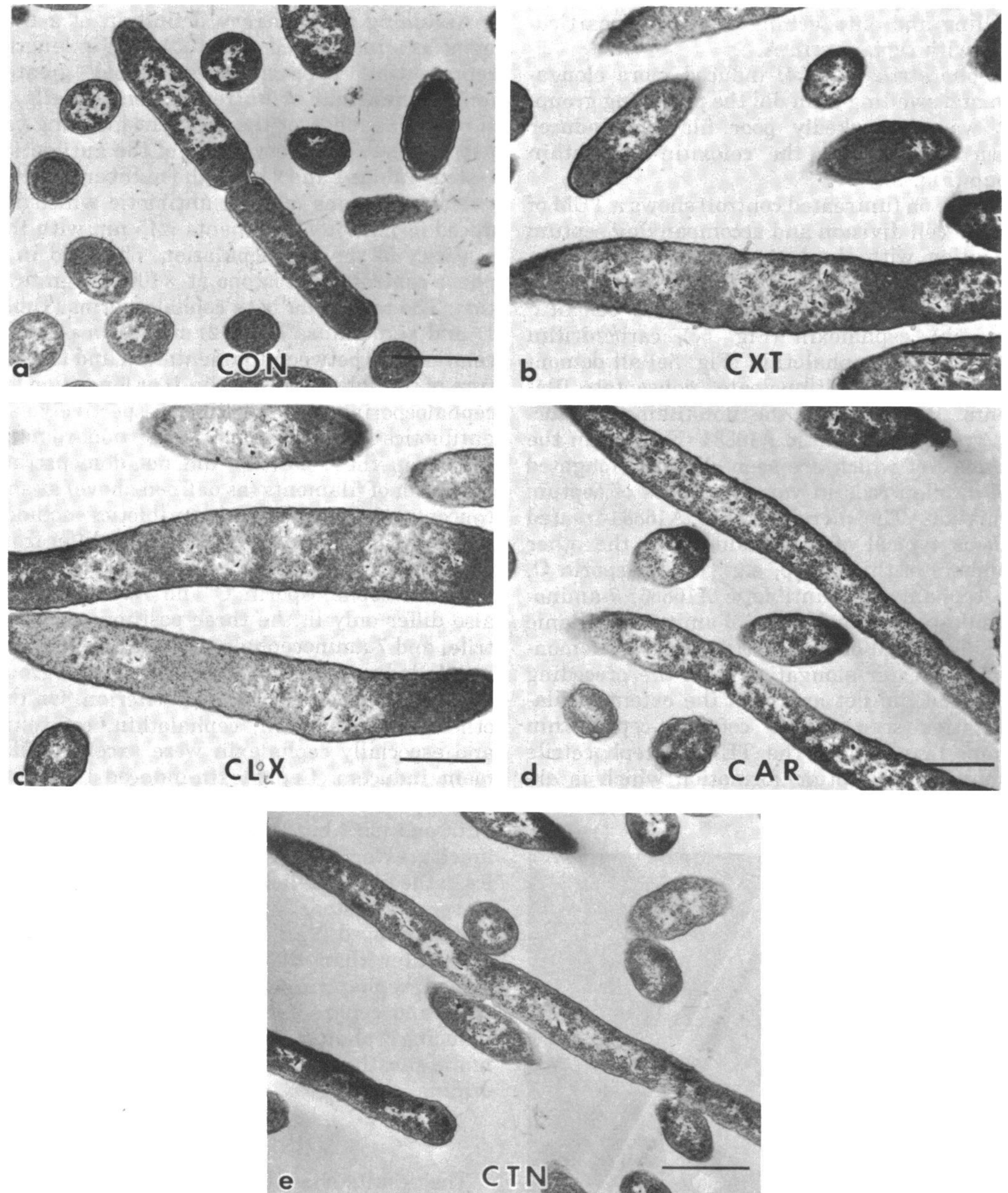

FIG. 5. *TEM of cells of E. cloacae grown in the presence of filament-inducing antibiotics. Bar = 5 nm. (a) Cells of untreated controls (CON) showing normal division and septation. (b), (c), (d), and (e) depict filamentous cells grown in the presence of subinhibitory concentrations of antibiotic. (b) Cefoxitin (CXT); (c) cephalexin (CLX); (d) carbenicillin (CAR); (e) cephalothin (CTN). Note the multinucleate, septumless filaments which are typically induced by this group of antibiotics.*

parent that the filament-inducing group includes many therapeutically proven β-lactam antibiotics. Thus, the possible reversion of β-lactam-induced filaments and other morphological variants to normal rods after antibiotic removal (12, 17) has not prevented the successful use of such antibiotics.

The results shown in Tables 1 and 2 suggest a possible relationship between filament induction and structure. All antibiotics tested which contained a nonaromatic substituent in the 7-position for cephems or in the 6-position for penams did not demonstrate filament induction. On the other hand, with the one exception

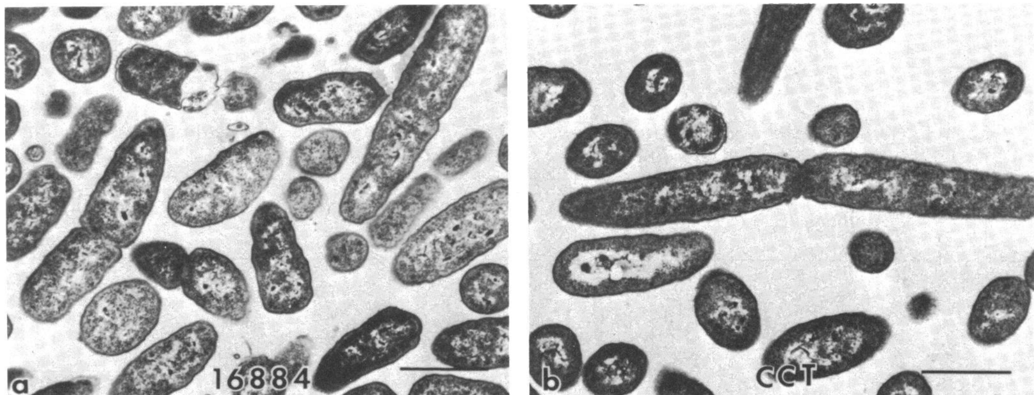

FIG. 6. *TEM of cells of E. cloacae grown in the presence of non-filament-inducing antibiotics. Bar = 5 nm. (a) Cells grown in the presence of subinhibitory levels of antibiotic A16884, showing the absence of filaments, and a degree of septum formation intermediate between that of untreated controls (Fig. 5a) and filament-inducing antibiotics (Fig. 5b, c, d, and e). Cells in this group are more swollen than untreated controls. (b) Typical cells grown in the presence of subinhibitory levels of cephacetrile (CCT), showing rods of intermediate length between that of untreated controls (Fig. 5a) and filamentous cells (Fig. 5b, c, d, e). The degree of septation in the presence of CCT is similar to that of A16884, i.e., intermediate between that of filaments and untreated controls.*

of cephaloridine, all β-lactams tested which contained an aromatic substituent in the 6- or 7-position demonstrated marked filament induction with the tester strain.

Our observations of extensive filamentation at subinhibitory levels agree with those of other reports, e.g., with cephalexin (4, 7, 11), benzylpenicillin (2, 4, 5, 10), cephalothin (4, 15), carbenicillin (10, 16), and ticarcillin (16). Agreement with other reports was also seen with respect to some of the relatively poor filament-inducing β-lactams, namely 6-aminopenicillanic acid (10), 7-aminocephalosporanic acid (10), cephaloridine (4, 10, 15), cephalosporin C (5), and cephacetrile (18). However, some filament induction with *Escherichia coli* and *Proteus vulgaris* has been reported with cephaloridine and cephalosporin C (5), both of which were negative in the present report; in addition, no filament induction of *P. vulgaris* was reported with cephaloglycine and cephalothin (10), both of which were excellent filament inducers in our study. These discrepancies may be attributable to intrinsic differences between tester strains, to differences in methodology, or to the effects of β-lactamase production by the test organism.

The apparent relationship seen between molecular structure and morphological effects on *E. cloacae* suggests certain explanations concerning mode of action. One such explanation is based on the assumption of the existence of an enzyme system separately concerned with biosynthesis and deposition of cross-wall (septum) peptidoglycan, as opposed to enzyme systems involved with side wall biosynthesis (1, 5, 6, 7, 19). In support of this assumption, Fleck and Mock (2) have found that the peptidoglycan of the side walls of benzylpenicillin-induced septumless filaments show no significant chemical or morphological differences when compared to normal cells, indicating that the side wall biosynthesis is not affected at filament-inducing concentrations of antibiotic. Conceivably, the configurations represented by the non-filament-inducing group may act as poor competitors for binding sites on the postulated septation enzyme(s). The reverse may also be true, i.e., the β-lactams comprising the filament-inducing group may present molecular configurations which can bind cross-wall enzyme(s), thereby competing with the "normal" substrate.

The fact that cephaloridine did not induce filaments despite the presence of an aromatic group in the 7-position suggests the possible influence on filament induction of net charge of the molecule. Cephaloridine contains an unusual positively charged pyridinium hydroxide inner salt at the 3-position which undoubtedly affects the overall charge of the molecule. Such a net charge could render cephaloridine a poor substrate for the septation enzyme despite the existence of a suitable molecular configuration. In addition to molecular structure and net charge, one cannot rule out the possible effect of permeability of the antibiotic in influencing morphological changes in the test organism.

TABLE 1. *Comparative filament-inducing capacity of subinhibitory levels of cephalosporin antibiotics versus Enterobacter cloacae HSC-18410-M66*[a]

Antibiotic	R_1 structure[a]	No. of twofold dilutions containing filaments[b]
Cefoxitin	(thiophene ring, S)–CH_2–C(=O)–NH– (OCH_3)	3
Cephalothin	(thiophene ring, S)–CH_2–C(=O)–NH–	3
Cefazolin	(tetrazole ring, N=N, N=)N–CH_2–C(=O)–NH–	3
Cephalexin	(benzene ring)–CH(NH_2)–C(=O)–NH–	6
Cephamycins, A16884	COOH–CH(NH_2)–$(CH_2)_3$–C(=O)–NH– (OCH_3)	0
Cephalosporin C, A16886	COOH–CH(NH_2)–$(CH_2)_3$–C(=O)–NH–	0
Cephacetrile	N≡C–CH_2–C(=O)–NH–	0
7-Aminocephalosporanic acid	NH_2–	0

[a] Structure: R_1, S, N, O, CH_2–R_2, COOH

[b] Designated numbers of dilutions refer to test mixtures in which the majority of the cell population consisted of filaments of ≧15 nm in length. Untreated control cells were ~1.5 nm in length.

TABLE 2. *Comparative filament-inducing capacity of subinhibitory levels of penicillin antibiotics versus Enterobacter cloacae HSC-18410-M66*[a]

Antibiotic	R structure[a]	No. of twofold dilutions containing filaments[b]
Benzylpenicillin	$C_6H_5-CH_2-C(=O)-NH\diagdown$	3
Carbenicillin	$C_6H_5-CH(COOH)-C(=O)-NH\diagdown$	5
FL-1060	$(CH_2)_6N-CH=N\diagdown$ (hexamethyleneimino ring)	0
6-Aminopenicillanic acid	$NH_2\diagdown$	0

[a] R-(penam nucleus: β-lactam ring fused to thiazolidine ring bearing S, CH_3, CH_3, COOH; O on β-lactam carbonyl)

[b] Designated numbers of dilutions refer to test mixtures in which the majority of the cell population consisted of filaments of ≧15 nm in length. Untreated control cells were ~1.5 nm in length.

Indeed any combination of the three factors (structure, net charge, and permeability) could contribute to the observed effects.

In summary, the results indicate that cefoxitin is one of a group of β-lactam antibiotics that induce filament formation at subinhibitory levels with a β-lactamaseless strain of *E. cloacae*. Since this group of β-lactams includes such therapeutically proven antibiotics as cephalothin, cefazolin, cephalexin, carbenicillin, benzylpenicillin, etc., cefoxitin is unlikely to pose any unusual problem because of its capacity for filament induction.

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