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Effect of Clavulanic Acid on Anaerobic Bacteria Resistant to Beta-Lactam Antibiotics

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The minimum inhibitory concentrations of penicillin and cephalothin necessary to inhibit growth of most anaerobic bacteria were reduced to a susceptible level by 1 to 5 μg of clavulanic acid per ml. In most cases, minimum inhibitory concentrations of cefoxitin were not affected.

Clavulanic acid is a new beta-lactamase inhibitor produced by *Streptomyces clavuligerus* ATCC 27064. It resembles the nucleus of penicillin but differs in several ways. It has no acylamino side chain, sulfur is replaced by oxygen, and it contains a beta-hydroxyethylidine substituent in the oxazolidine ring. Clavulanic acid irreversibly inhibits beta-lactamase enzymes produced by some *Enterobacteriaceae* (mainly R factor-mediated beta-lactamases) and staphylococci (11). Used in conjunction with a beta-lactam antibiotic, it may prove to be effective in treating infections caused by beta-lactamase-producing bacteria. Its usefulness in the chemotherapy of human infections is currently being evaluated.

Although there have been several reports that resistance of anaerobic bacteria to beta-lactam antibiotics is at least partially caused by beta-lactamase (1, 4, 9, 10, 13, 14), little is known about the effect of clavulanic acid on the susceptibility of these bacteria (P. A. Hunter and C. Reading, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., Abstr. no. 211, 1976). We have investigated the effect of clavulanic acid on the minimum inhibitory concentrations (MICs) of penicillin, amoxycillin, cephalothin, and cefoxitin for a total of 55 resistant strains (MIC of penicillin ≥4 μg/ml) from the following *Bacteroides* species: *B. fragilis* (two deoxyribonucleic acid homology groups), *B. melaninogenicus, B. oralis, B. bivius, B. disiens,* and *B. splanchnicus* (5, 15). We have also tested one resistant strain each of *Clostridium clostridiiforme* (formerly *B. clostridiiformis* [3]) and *C. ramosum*. All organisms used in this study were obtained from the Virginia Polytechnic Institute Anaerobe Laboratory culture collection.

Due to the small amount of clavulanic acid available (kindly supplied by Beecham Laboratories, Bristol, Tenn.), we used the microdilution technique for MIC determinations described by Rotilie et al. (12) instead of an agar dilution technique. Schaedler broth (8) was prepared anaerobically (6), and cystine was replaced by cysteine (0.5 mg/ml). The Schaedler broth was dispensed with and without clavulanic acid into the microtiter plates. Twofold dilutions of the antibiotics were prepared (50 μl per well). Before inoculation, the plates were transferred to an anaerobic glove box filled with an atmosphere of 85% N_2, 5% CO_2, and 10% H_2 and allowed to equilibrate for 2 to 3 h. All wells were inoculated in the glove box with 50 μl of inoculum, resulting in a final concentration of the antibiotics from 128 to 0.125 μg/ml and 1 or 5 μg/ml of clavulanic acid. The inoculum was prepared anaerobically by diluting an overnight culture to half the turbidity of a McFarland no. 1 standard and making a 1:100 dilution thereof in Schaedler broth. Since the *B. melaninogenicus* and *B. oralis* strains did not grow sufficiently in Schaedler broth, the clear supernatant fluid of chopped meat broth (6) was used instead. The plates were sealed with pressure-sensitive plastic film (Falcon Plastics, Oxnard, Calif.) and incubated at 37°C for 48 h in the anaerobic chamber. MICs were read as the lowest concentrations of antibiotic that inhibited visible growth. All tests were performed in duplicate on different days. Usually, tests did not differ by more than one dilution. In the few instances of larger discrepancies, the tests were repeated. In evaluating the data, the higher value was used if tests differed by one dilution.

Clavulanic acid alone showed weak antibacterial activity. MICs against the *B. fragilis* group were 16 to 256 μg/ml (geometric mean, 68 μg/ml), those against *B. melaninogenicus* and *B. oralis* were 2 to 64 μg/ml (mean, 14 μg/ml), and those against the other *Bacteroides*

species were 16 to 128 μg/ml (mean, 39 μg/ml).

The results are summarized in Tables 1 and 2. All strains except the strains of the *B. fragilis* homology group II were inhibited by far lower concentrations of penicillin and cephalothin in the presence of low amounts of clavulanic acid. The *B. fragilis* strains can be differentiated into two closely related but distinct groups by deoxyribonucleic acid homology determinations (7; J. L. Johnson, personal communication); homology group I is much more common than II among clinical isolates. In general, MICs of penicillin and cephalothin against homology group II were lower than those against homology group I. A possible explanation for the lack of susceptibility of homology group II to clavulanic acid may be that homology group II has only an intrinsic resistance to beta-lactam antibiotics and produces no beta-lactamase. The lack of difference between cephalothin and cefoxitin MICs also supports this explanation. Another possibility might be a permeability barrier preventing the uptake of clavulanic acid by strains of homology group II. Further studies are needed.

The MICs of cefoxitin, a cephamycin relatively resistant to beta-lactamases (4), were not influenced markedly by clavulanic acid except with *B. bivius* and *B. disiens*. With these strains, the MIC values decreased in the presence of clavulanic acid; this suggests that these strains may produce a beta-lactamase that can degrade cefoxitin. The MICs of *C. clostridiiforme* VPI 8035TA (MIC of penicillin >256 μg/ml; of cephalothin, 64 μg/ml; and of cefoxitin, 16 μg/ml) and *C. ramosum* VPI 2263 (all MICs >256 μg/ml) were not influenced even by 20 μg of clavulanic acid per ml, although these strains are known to produce beta-lactamase (14). Possibly the clostridial beta-lactamases are not susceptible to clavulanic acid in the concentrations tested.

With amoxycillin, similar results to the ones obtained with penicillin were found.

Since the bacteria (with the exception of the relatively rare *B. fragilis* homology group II and two clostridia) became essentially susceptible to the beta-lactam antibiotics tested in the presence of clavulanic acid, the main mechanism of resistance in these strains seems to

TABLE 1. *Effect of clavulanic acid on MIC values of penicillin*

Organism	No. of strains tested	Concn of clavulanic acid (μg/ml)	No. of strains inhibited at various penicillin concn (μg/ml)								
			≤0.125	0.25	0.5	1	2	4	8	16	>16
B. fragilis homology group I[a]	14	0						6	7		1
		1	2	2	4	4	1				1
		5	3	1	7	3					
B. fragilis homology group II[a]	6	0						5	1		
		1					2	3	1		
		5					2	3	1		
		20					3	2	1		
B. fragilis group (other)[b]	18	0						5	6	7	
		1	2		1	7	6	1		1	
		5	2	1	1	10	3	1			
B. melaninogenicus and *B. oralis*[c]	6	0						1		4	1
		1	4				2				
		5	1		2						
Bacteroides species (other)[d]	11	0						2	4	5	
		1	2			5	3	1			
		5	4		3	3	1				

[a] Deoxyribonucleic acid homology groups of *B. fragilis* according to J. L. Johnson (personal communication).

[b] Four *B. distasonis*, four *B. ovatus*, eight *B. thetaiotaomicron*, two *B. vulgatus*.

[c] Two *B. melaninogenicus* subsp. *asaccharolyticus*, one *B. melaninogenicus* subsp. *melaninogenicus*, one *B. melaninogenicus* subsp. *intermedius*, two *B. oralis*; three strains were inhibited by 4 μg of clavulanic acid per ml.

[d] Six *B. bivius*, three *B. disiens*, two *B. splanchnicus*.

Table 2. *Effect of clavulanic acid on MIC values of cephalothin and cefoxitin*

Organism	No. of Strains Tested	Concentration Clavulanic Acid (μg/ml)	Number of strains inhibited at various concentrations (μg/ml)																	
			Cephalothin									Cefoxitin								
			≤0.125	0.25	0.5	1	2	4	8	16	>16	≤0.125	0.25	0.5	1	2	4	8	16	>16
Bacteroides fragilis Homology Group I[a]	14	0						3	2	4	5			1	1	1	2	6	2	1
		1		1		5	3	4			1			1	1	2	3	6	1	
		5		1	4	2	6	1				1	1			2	5	4	1	
Bacteroides fragilis Homology Group II[a]	6	0							2	4									1	5
		1							3	3									3	3
		5							4	2									4	2
		20						1	4	1							1		4	1
Bacteroides fragilis Group (other)[b]	18	0						1	2	6	9				1	2	3	2	2	7	1
		1			1	1	2	6	6	2					1	2	3	2	2	7	1
		5			2	1	3	9	3						1	2	3	3	1	7	1
Bacteroides melaninogenicus and *B. oralis*[c]	6	0							1		5					1	3	2			
		1	2	1	1		1		1			1			3		1	1			
		5	1		1	1									2	1					
Bacteroides Species (other)[d]	11	0						1	4	4	2					3	5	3			
		1			2	3	5		1						1	5	3	1	1		
		5	2	1	4	3	1					1			5	3		1	1		

[a] Deoxyribonucleic acid homology groups of *B. fragilis* according to J. L. Johnson (personal communication).

[b] Four *B. distasonis*, four *B. ovatus*, eight *B. thetaiotaomicron*, two *B. vulgatus*.

[c] Two *B. melaninogenicus* subsp. *asaccharolyticus*, one *B. melaninogenicus* subsp. *melaninogenicus*, one *B. melaninogenicus* subsp. *intermedius*, two B. oralis; three strains were inhibited by 4 μg of clavulanic acid per ml.

[d] Six *B. bivius*, three *B. disiens*, two *B. splanchnicus*.

be formation of beta-lactamase. However, it is sometimes difficult to demonstrate beta-lactamase production in anaerobic bacteria, especially in the *B. fragilis* group, and amounts of enzyme found are low (1, 4, 9, 10, 13, 14). The difficulties encountered in assaying beta-lactamases may be caused by technical problems such as inhibited release of the enzyme from the cell or instability of the enzyme.

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