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EFFECT OF BETA-LACTAMASE INHIBITORS ON BETA-LACTAMASES FROM ANAEROBIC BACTERIA

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SUMMARY

The effect of three clinically used beta-lactamase inhibitors - clavulanic acid, sulbactam and tazobactam - were investigated for their activity on beta-lactamases from *Bacteroides uniformis, Clostridium butyricum* and *Fusobacterium nucleatum.* Purification of the beta-lactamases was carried out by anion-exchange chromatography, gel filtration and FPLC-technique. The inactivation of beta-lactamase activity was determined spectrophotometrically with nitrocefin as substrate. The inhibitors in various concentrations were preincubated at 30°C together with the enzyme for different time periods before determination of the beta-lactamase activity. The three beta-lactamases tested were more susceptible to tazobactam than to clavulanic acid and sulbactam. Clavulanic acid and sulbactam were capable to reduce the enzyme activity of the *Bacteroides uniformis* beta-lactamase more effectively than the *Clostridium butyricum* and *Fusobacterium nucleatum* beta-lactamases.

ÖZET

Anaerop bakterilerin beta-laktamazları üzerine beta-laktamaz inhibitörlerinin etkisi.

Klinik olarak kullanılan üç beta-laktamaz inhibitörü olan klavulanik asit, sulbaktam ve tazobaktamın *Bacteroides uniformis*, *Clostridium butyricum* ve *Fusobacterium nucleatum*'un oluşturduğu beta-laktamazlara aktiviteleri araştırılmıştır. Beta-laktamazlar anyon-değiştiren kromatografi, gel filtrasyon ve FPLC-tekniği ile saflaştırılmıştır. Beta-laktamaz aktivitesinin inaktivasyonu substrat olarak nitrosefin kullanılarak spektrofotometrik olarak tayin edilmiştir. Beta-laktamaz aktivitesinin tayininden önce çeşitli konsantrasyonlardaki inhibitörler enzim ile 30°C'da değişik sürelerde preinkübe edilmiştir. Denenen üç beta-laktamaz tazobaktama, klavulanik asit ve sulbaktama olduğundan daha duyarlı bulunmuştur. Klavulanik asit ve sulbaktam *Bacteroides uniformis* beta-laktamazının enzim aktivitesini, *Clostridium butyricum* ve *Fusobacterium nucleatum* beta-laktamazlarına olduğundan daha etkili bir şekilde azaltmıştır.

INTRODUCTION

The mechanisms of beta-lactam resistance in anaerobic bacteria are production of beta-lactamases, alteration of penicillin-binding proteins, and blocked penetration of the beta-lactam agent (13). The most important mechanism is beta-lactamase production. Several species of *Bacteroides* produce beta-lactamases as well as a few species of *Clostridium* and *Fusobacterium*. The beta-lactam

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resistance in *Bacteroides fragilis* is commonly mediated by beta-lactamases that are mainly cephalosporinases in character. *Bacteroides fragilis* strains can also produce penicillinases and enzymes inactivating cefoxitin and imipenem. The non-*fragilis* species of *Bacteroides* produce beta-lactamases that are mainly penicillinases. Penicillinases have also been described in *Fusobacterium nucleatum* strains. Among the clostridia, *Clostridium butyricum, Clostridium clostridiiforme* and *Clostridium ramosum* have been shown to produce penicillinases (13).

There have been advances in two main areas to overcome the beta-lactamase problem, the synthesis of new beta-lactam antibiotics, and the development of inhibitors that bind to or inactivate beta-lactamases (5).

The aim of the present investigation was to compare the effect of three clinically used beta-lactamase inhibitor (clavulanic acid, sulbactam and tazobactam) on purified beta-lactamases from *Bacteroides uniformis, Clostridium butyricum* and *Fusobacterium nucleatum.*

MATERIALS AND METHODS

Bacterial strains. Three clinical strains isolated from human infections at Huddinge University Hospital and National Bacteriological Laboratory, Stockholm, Sweden were used: *Bacteroides uniformis* B371, *Clostridium butyricum* NBL 3 and *Fusobacterium nucleatum* F21. The strains were identified by Gram staining, biochemical tests, and gas-liquid chromatographic analysis according to Holdeman, Cato and Moore (9).

Production of beta-lactamases. *Bacteroides uniformis* B 371 beta-lactamase was produced in fermentors with a 3-liter working volume (FL 103, BioTec, Sweden). Temperature was controlled with an accuracy of ± 0.01°C, and pH with an accuracy of ± 0.05 pH unit. The impeller speed was 100 rpm (BioTec LP-300). The medium used was prereduced proteose peptone broth with 1 % (w/v) glucose (15). Cultivation was performed at 37°C and pH 7.0 under an atmosphere of 3 % H_2 and 5% CO_2 in N_2. The culture was harvested in the late log phase (19 h) by centrifugation (Sorvall SR 5, Du Pont, USA) at 10,000 x g for 30 min at 4°C and washed once in sodium phosphate buffer (0.01 M, pH 7.0) at 4°C. The cells were then disrupted by osmotic shock (2). The washed cells were suspended in 1/10 the original volume of 0.02 M Trishydrochloride buffer, pH 8.0, containing 20 % (w/v) sucrose. After 10 min the suspension was centrifuged at 10,000 x g for 30 min at 4°C and the supernatant fraction (20 % sucrose wash), after dialysis against sodium phosphate buffer (0.05 M, pH 7.0), was tested for beta-lactamase activity. The bacterial pellet was resuspended in distilled water (4°C) and centrifuged again at 10.000 x g for 30 min at 4°C. The supernatant fraction (cold water wash fraction) was assayed for beta-lactamase activity and the pellet was resuspended in 250 ml of the phosphate buffer containing 0.1 % Triton X-100. The suspension was centrifuged at 10,000 x g for 30 min at 4°C and the supernatant fraction (Triton X-100 extract) was assayed for enzyme activity. The cold water wash fraction contained the main part of the beta-lactamase and was used for the inhibition studies.

Clostridium butyricum NBL 3 beta-lactamase was produced in fermentors with a 3-liter working volume (FL 103, BioTec). Temperature was controlled with an accuracy of ±0.01°C, and the pH was controlled with an accuracy of ±0.05 pH unit. The impeller speed was 50 rpm (BioTec LP-300). The medium used was a

prereduced yeast and meat extract medium with 0.3 % starch (11). Cultivation was performed at 37°C and pH 7.0 under N_2 atmosphere. The culture was harvested in the late log phase (18 h) by centrifugation at 10,000 x g for 30 min at 4°C. The supernatant obtained was used for purification.

Fusobacterium nucleatum F 21 beta-lactamase was produced in bottle cultures in 6 liters of prereduced peptone yeast extract broth (16) and were harvested in the late log phase (20 h). The cells were centrifuged at 10,000 x g for 30 min at 4°C, washed twice in sodium phosphate buffer 0.01 M, pH 7.0 at 4°C, and suspended in 1/100 the original volume of the same buffer. The cells were then disrupted in an ultra sonicator for 4 min (Branson B15, Branson Sonic Power, Germany) in an ice bath. Cell debris was removed by centrifugation at 13,000 x g for 45 min at 4°C, and the supernatant obtained was used for purification.

Purification of beta-lactamases. The water wash fraction of *Bacteroides uniformis* B 371 was dialyzed against 0.02 M Trishydrochloride buffer, pH 8.0, containing 20 % (v/v) glycerol. The preparation of *Bacteroides uniformis* beta-lactamase had a specific activity of 38 U per mg protein.

The beta-lactamase from *Clostridium butyricum* NBL 3 was purified to homogeneity (3300 U per mg protein) according to the method described by Kesado et al. (11). The purification techniques used were mass anion exchange (QAE Zetaprep 250, step 1 and QAE Zetaprep 15, step 3), gel filtration (Sephacryl S-300, step 2), and anion-exchange chromatography (Mono Q [HR 5/5; FPLC], steps 4 and 5).

The beta-lactamase produced by *Fusobacterium nucleatum* F 21 was purified to homogeneity by gel filtration (Sephacryl S-300, step 1 and S-200, step 2), QAE Zetaprep 15 (step 3), and anion-exchange chromatography (Mono Q [HR 5/5; FPLC], step 4). The purified beta-lactamase of *Fusobacterium nucleatum* had a specific activity of 3500 U per mg protein. The enzymes produced by *Clostridium butyricum* and *Fusobacterium nucleatum* were both penicillinases (11, 16).

Determination of beta-lactamase activity. The beta-lactamase activity was assayed spectrophotometrically with nitrocefin (0.10 mM) in 50 mM sodium phosphate buffer (pH 7.0, 30°C) as described by O'Callaghan et al. (14).

Inhibition studies. The degree of inactivation of beta-lactamase activity by the three inhibitors was determined with nitrocefin as substrate in the spectrophotometric assay. Clavulanic acid, sulbactam and tazobactam at seven various concentrations, depending on the susceptibility of the enzyme to the inhibitor, were preincubated at 30°C for different time periods (0.5-120 min) before determination of the remaining beta-lactamase activity (6).

The degree of inactivation for beta-lactamase of *Clostridium butyricum* and *Fusobacterium nucleatum* was studied at three different enzyme concentrations (0.06 U/ml, 0.1 U/ml and 0.2 U/ml). For the beta-lactamase of *Clostridium butyricum*, 1.0 U/ml corresponded to 34 nM, and for the beta-lactamase of *Fusobacterium nucleatum* to 11 nM. The concentration used for the beta-lactamase of *Bacteroides uniformis* corresponded to an enzyme activity of 0.1 U/ml.

Percent inhibition was calculated as 100 x [(c-r)/c], where c is the activity in control samples incubated without inhibitor and r is the remaining activity in samples incubated with inhibitor. The concentration of inhibitor which caused 50 % inhibition (IC50), was calculated from a plot of percent inhibition versus the logarithm value of inhibitor concentration.

Chemicals. Clavulanic acid was provided by Astra, Sweden; nitrocefin from Glaxo Pharmaceuticals, UK; sulbactam from Pfizer, USA; and tazobactam from Lederle Laboratoires, USA.

RESULTS

Clavulanic acid

The inactivation of the three beta-lactamases by clavulanic acid (0.1 μM) occurred mainly during the first 20 min of incubation (Figure 1A). The beta-lactamase of *Bacteroides uniformis* weakly reactivated during the last part of the incubation period, whereas the other two beta-lactamases were continuously inactivated.

The inhibitory effect by different concentration of clavulanic acid is demonstrated in Figure 2A. For the beta-lactamase of *Bacteroides uniformis,* the calculated IC50 values were 34, 30 and 34 nM at incubation times of 20, 60 and 120 min, respectively.

For *Clostridium butyricum* beta-lactamase, the IC50 values decreased with time and increased with increasing enzyme concentration. At an enzyme concentration of 0.1 U/ml, the IC50 values were 100 nM (20 min), 70 nM (60 min) and 60 nM (120 min), respectively. When the enzyme concentration was twice as high (0.2 U/ml), the IC50 values increased to 400 nM (20 min) and 200 nM (60 and 120 min) (not shown in figure).

As for the beta-lactamase of *Clostridium butyricum,* the IC50 values of *Fusobacterium nucleatum* decreased with the length of incubation. The IC50 values were 6.8, 3.2 and 2.0 μM for 20, 60 and 120 min, respectively. The beta-lactamase of *Fusobacterium nucleatum* showed no significant differences in IC50 values at different enzyme concentrations.

Sulbactam

The beta-lactamase activity of *Bacteroides uniformis* was inactivated faster and to a higher degree by sulbactam (3.0 μM) than the two other enzymes studied (Figure 1B). After 60 min of incubation, the inactivating process stoped for the beta-lactamase of *Bacteroides uniformis*, whereas the inactivation of the other two enzymes continued during the whole incubation period.

The inhibitory effect by different concentrations of sulbactam is presented in Figure 2B and the calculated IC50 values for 20, 60 and 120 min are shown in Table 1. The IC50 values of sulbactam did not differ significantly when different enzyme concentrations of the three beta-lactamases were tested.

Tazobactam

The inhibition of beta-lactamase activity by tazobactam is shown in Figures 1C and 2C. The final level of inactivation of *Bacteroides uniformis* beta-lactamase by tazobactam (30 nM) was reached after 60 min of incubation. Also at the other concentrations of tazobactam investigated, the final level of inactivation was obtained after 60 min (not shown in figure). The time period had a very little influence on the IC50 value of tazobactam for *Bacteroides uniformis* beta-lactamase. After 20, 60 and 120 min of incubation, the IC50 value was 63, 50 and 47 nM, respectively.

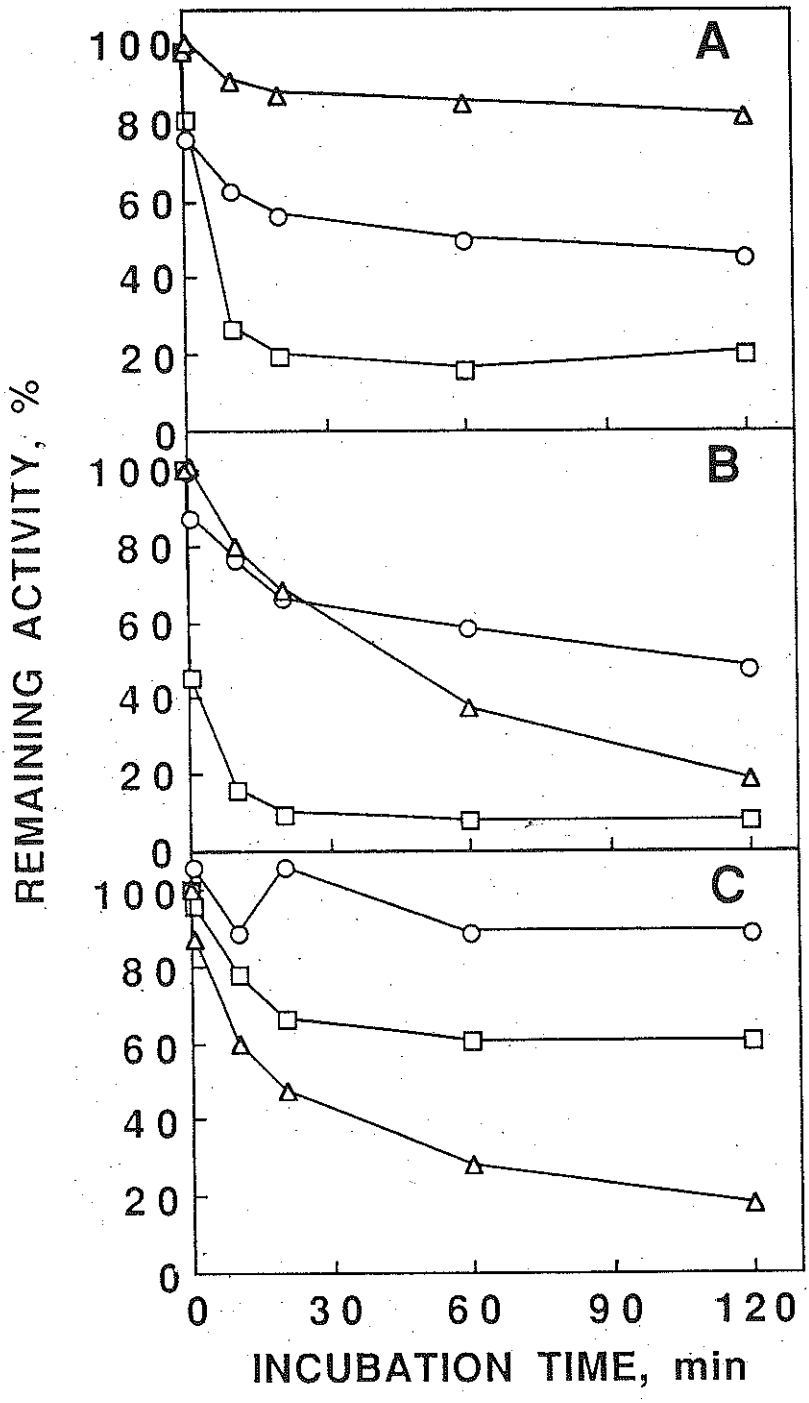

Figure 1. Effect of time on the inhibition of beta-lactamase activity by clavulanic acid (0.1 μM) (A), sulbactam (3 μM) (B), and tazobactam (30 nM) (C). The beta-lactamases (0.1 U/ml, B 371, □ ; NBL 3, O; and F 21, Δ) were incubated with the inhibitors for different time periods at pH 7.0 and 30°C before determination of the remaining activity.

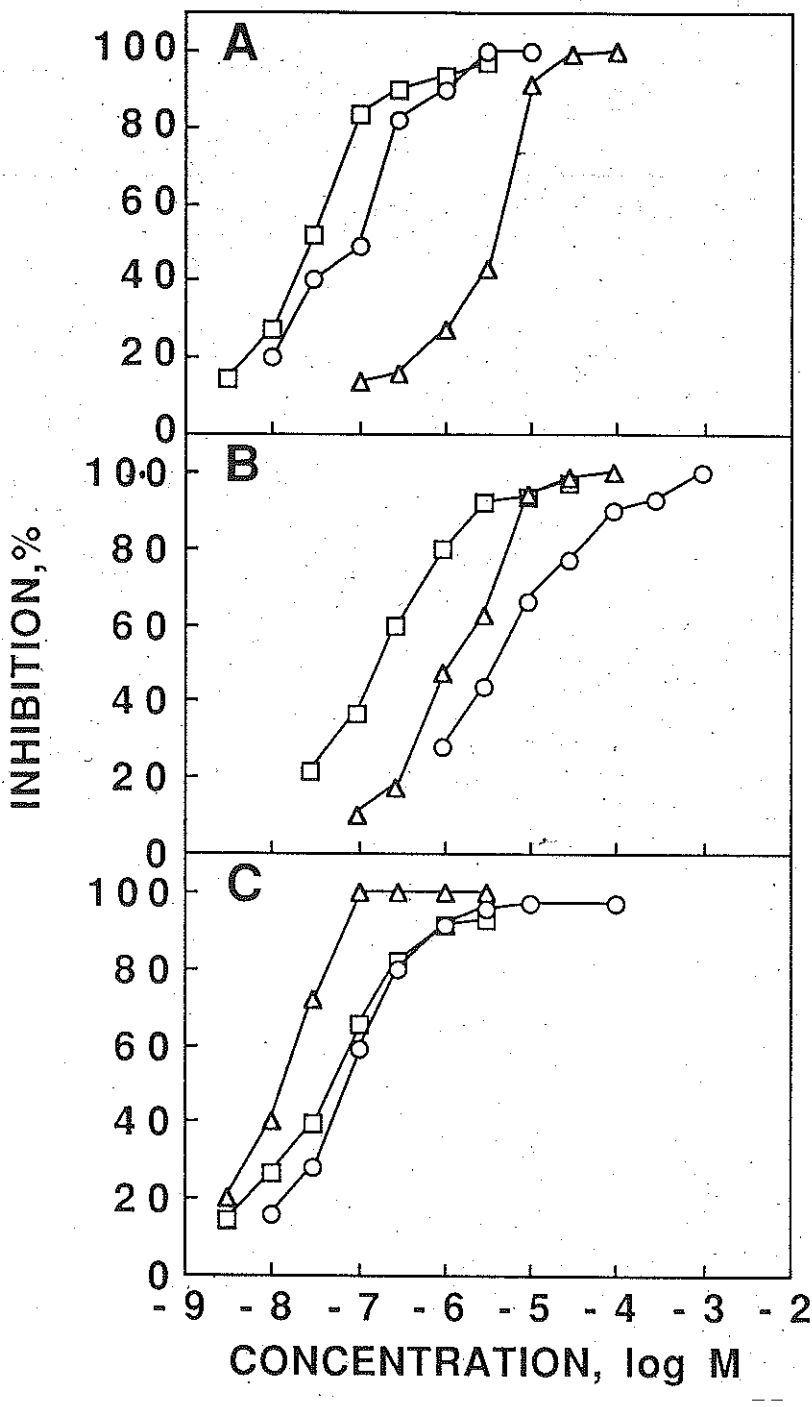

Figure 2. Effect of concentration of clavulanic acid (A), sulbactam (B), and tazobactam (C) on the inhibition of beta-lactamase activity. The beta-lactamases (0.1 U/ml, B 371, □ ; NBL 3, O; and F 21, Δ) were incubated together with the inhibitors for 60 min at 30°C before determination of the remaining activity. The activity in the control samples did not decrease during the incubation time.

Tablo 1. Concentration of sulbactam causing 50 % inhibition (IC_{50}) of the different beta-lactamases at different times of incubation.

Source of beta-lactamase	IC_{50} (μM)		
	Incubation time (min)		
	20	60	120
Bacteroides uniformis	0.3	0.2	0.2
Clostridium butyricum	5.7	3.2	1.3
Fusobacterium nucleatum	4.9	1.6	0.9

The beta-lactamase activity of the enzymes from *Clostridium butyricum* and *Fusobacterium nucleatum* decreased during the incubation period studied (Figure 1C). For inactivation of the beta-lactamase from *Clostridium butyricum* and *Fusobacterium nucleatum,* the IC50 values were dependent on the enzyme concentrations. At a *Clostridium butyricum* enzyme concentration of 0.06 U/ml, the calculated IC50 values were 100, 75 and 56 nM at 20, 60 and 120 min, respectively. The IC50 values obtained at an enzyme concentration of 2.0 U/ml were 2.9 times higher. For the beta-lactamase from *Fusobacterium nucleatum* (0.06 U/ml), the IC50 values were 30 (20 min), 15 (60 min) and 11 nM (120 min), respectively. Corresponding IC50 values were 45, 37 and 32 nM at an enzyme concentration of 2.0 U/ml.

DISCUSSION

The frequency of beta-lactamase production by various *Bacteroides* species is high. It is important to note that 75-100% of *Bacteroides fragilis* strains in the *Bacteroides fragilis* group are beta-lactamase producers (7). Other species within this group that have been studied (*Bacteroides ovatus, Bacteroides vulgatus, Bacteroides thetaiotaomicron, Bacteroides distasonis* and *Bacteroides uniformis*) have an incidence of beta-lactamase production that varies from 60 to 100%. The *Bacteroides fragilis* group is one of the most commonly encountered strains in clinical infections and is among the anaerobic bacteria most resistant to antimicrobial agents, primarily due to production of beta-lactamases. The resistance to beta-lactam agents in *Bacteroides* can be transferable. Among the fusobacteria, *Fusobacterium mortiferum* have been shown to produce beta-lactamase (77%) and other *Fusobacterium* species ranging between 21 to 50%. Appelbaum, Sprangler and Jacobs (1) have shown that clavulanic acid lowers the MICs for beta-lactamase producing fusobacteria when combined with amoxicillin. The reports of beta-lactam resistance in anaerobic Gram-positive bacteria are limited. The clostridia have been considered to be sensitive to beta-lactam antibiotics, but three clostridial species have been shown to produce beta-lactamases: *Clostridium butyricum, Clostridium clostridiiforme* and *Clostridium ramosum* (13). *Clostridium butyricum* and *Clostridium clostridiiforme* produce enzymes of penicillinase type, while *Clostridium ramosum* enzymes have a broad spectrum character. The beta-lactamase from *Clostridium butyricum* is chromosomally mediated (10). *Clostridium difficile* strains are resistant to cephalosporins and carbapenems, probably due to blocked penetration of beta-lactam antibiotics through the bacterial cell wall (13).

Almost as soon as a new beta-lactam antibiotic is introduced into clinical usage, some previously unrecognized beta-lactamases with capability of destroying beta-lactam agents are identified. There are several classes of beta-lactamases and a considerable variation in specificity and kinetic behaviour of the enzymes even within one class (8). Inhibitory characteristics can be as important as substrate profiles in distinguishing between different types of beta-lactamases.

The results in this study show that the three inhibitors had different inhibition patterns to the enzymes investigated. Clavulanic acid was most active against the *Bacteroides uniformis* beta-lactamase. Some beta-lactamases from the *Bacteroides fragilis* group have been reported to be inhibited by clavulanic acid (4), and other beta-lactamases are not inhibited (3, 4). The enzyme produced by *Clostridium butyricum* NBL 3 was inhibited by clavulanic acid. Beta-lactamases produced by *Clostridium butyricum* have previously been classified in group 4 (penicillin-hydrolyzing beta-lactamases not inhibited by clavulanic acid) (4). The beta-lactamase from *Fusobacterium nucleatum* F 21 belongs to group 2a in the Bush classification. In the present study, this enzyme was less inhibited by clavulanic acid than *Bacteroides uniformis* B 371 and *Clostridium butyricum* NBL 3 beta-lactamases, but still this inhibitor was considered to be active against the F 21 beta-lactamase. *Bacteroides uniformis* B 371 beta-lactamase was also the most susceptible enzyme to inhibiton by sulbactam, compared with the other two enzymes studied. Another *Bacteroides fragilis* beta-lactamase with a broad-spectrum activity has earlier been reported not to be inhibited by sulbactam (17). Tazobactam and clavulanic acid were superior to sulbactam in enhancing the therapeutic efficacy of piperacillin in mice infected with beta-lactamase producing *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Staphylococcus aureus* (12). The *Clostridium butyricum* NBL 3 and *Fusobacterium nucleatum* F 21 beta-lactamases were inhibited by sulbactam to the same degree during the first 20 min of preincubation. After that period, the F 21 enzyme was more inhibited than the *Clostridium butyricum* beta-lactamase. Tazobactam inhibited the *Fusobacterium nucleatum* beta-lactamase more rapidly than the *Bacteroides uniformis* and *Clostridium butyricum* beta-lactamases. Tazobactam reduced the enzyme activity of the three beta-lactamases more effective than clavulanic acid and sulbactam, which may have clinical implications.

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