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Characterization of the Chromosomal Cephalosporinases Produced by *Acinetobacter lwoffii* and *Acinetobacter baumannii* Clinical Isolates

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The β-lactamases produced by *Acinetobacter lwoffii* ULA-501, *Acinetobacter baumannii* ULA-187, and *A. baumannii* AC-14 strains were purified and characterized, and their kinetic interactions with several β-lactam molecules, including substrates and inhibitors, were studied in detail. The three enzymes appeared to be cephalosporinases with different acylation efficiencies (k_{cat}/K_m ratio values), and their hydrolytic activities were inhibited by benzylpenicillin, piperacillin, and cefotaxime, which did not behave as substrates. Carbenicillin was a substrate for the β-lactamase from *A. lwoffii* ULA-501, whereas it acted as a transient inactivator of the enzymes produced by the two *A. baumannii* strains. Clavulanic acid was unable to inactivate the three β-lactamases, whereas sulbactam behaved as an inactivator only at a high concentration (1 mM) which is difficult to achieve during antibiotic therapy. Analysis of the interaction with 6-β-iodopenicillanic acid also allowed us to better discriminate the three β-lactamases analyzed in the present study, which can be included in the group 1 functional class (5).

Members of the genus *Acinetobacter* are gram-negative, aerobic, nonfermenting bacteria responsible for a wide spectrum of infections in immunocompromised patients (1, 16). Albeit of low virulence, these microorganisms show a natural resistance toward a vast category of antibiotics, including the family of β-lactam drugs.

All of the mechanisms of resistance to these molecules, such as β-lactamase production, alteration of penicillin-binding proteins, and reduced levels of penetration across the outer membrane barrier, have been reported to be present in *Acinetobacter* spp. (12, 22). In particular, in a recent report Sato and Nakae (24) demonstrated the presence of a significant permeability barrier in *Acinetobacter calcoaceticus* strains. These bacteria showed very low levels of diffusion of β-lactam antibiotics (1% in the case of penicillins and cephalosporins and <2% for carbapenem antibiotics) compared with those of some strains of *Escherichia coli*. The same investigators concluded that this finding was more significant than that observed in *Pseudomonas aeruginosa* clinical isolates. On the other hand, β-lactamase production appears to contribute greatly to resistance phenomena in *Acinetobacter* spp. under conditions of low permeability of the outer membrane to β-lactam drugs. The β-lactamases produced by *Acinetobacter* strains can be either plasmid-derived enzymes, such as the well-characterized CARB-5 (23) and TEM-1 (15) enzymes, or chromosomally encoded enzymes (20), belonging to group 1 according to the classification of Bush et al. (5). The latter enzymes have already been studied in some *A. calcoaceticus* strains (2, 13, 20), but comprehensive knowledge of them is still lacking. In particular, production of these β-lactamases by other *Acinetobacter* spp. has not been investigated before, and the regulation of expression of these enzymes remains unclear.

In the present work, the β-lactamases produced by one strain of *Acinetobacter lwoffii* and two strains of *Acinetobacter baumannii*, isolated from immunocompromised patients and showing different patterns of resistance to β-lactam antibiotics, were purified, and their kinetic interactions with some β-lactam substrates and active-site serine β-lactamase inhibitors were studied in detail.

MATERIALS AND METHODS

Bacterial strains. *A. lwoffii* ULA-501, *A. baumannii* ULA-187, and *A. baumannii* AC-14 were all clinical isolates from immunocompromised patients and belonged to the collection of strains of the University of L'Aquila. The identification of the strains investigated in the present study was performed by standard criteria (27).

Antibiotics. Piperacillin was from Cyanamid (Catania, Italy), carbenicillin and clavulanic acid were from SmithKline Beecham Research Laboratories (Brentford, United Kingdom), cephaloridine and cephalothin were from Eli Lilly & Co. (Indianapolis, Ind.), cefotaxime and cefpirome were from Hoechst AG (Frankfurt, Germany), aztreonam was from the Squibb Institute for Medical Research (Princeton, N.J.), sulbactam and 6-β-iodopenicillanic acid (6-β-IP) were from Pfizer Central Research (Sandwich, United Kingdom), ceftazidime was from Glaxo (Verona, Italy), and imipenem was from Merck Sharp & Dohme Research Laboratories (Rahway, N.J.). All of these compounds were kindly given by the respective companies. Nitrocefin (Unipath, Milan, Italy) and benzylpenicillin (Sigma, Milan, Italy) were purchased from the respective companies.

MIC determinations. The determination of MICs for selected organisms was performed by the conventional macrodilution broth procedure, as recommended by a National Committee for Clinical Laboratory Standards reference manual (21).

Production and purification of β-lactamases. An overnight culture of each *Acinetobacter* strain in brain heart infusion broth (Oxoid, Milan, Italy) was diluted 10-fold with the same prewarmed medium (4 liters) and was grown for 16 h at 30°C with orbital shaking. The cells were harvested by centrifugation at 16,000 × *g* for 1 h at 4°C, washed twice with 50 mM Na^+-K^+ phosphate buffer (pH 7.4), and disrupted by cycles of ultrasonic treatment (four times for 1 min at 60 W each time). The cleared lysates were loaded onto a Sephadex G-75 column equilibrated with the same buffer. The fractions exhibiting β-lactamase activity were pooled, concentrated by ultrafiltration with an Amicon concentration apparatus (Amicon Corp., Beverly, Mass) with a YM10 membrane (cutoff, 10 kDa), and loaded onto an S-Sepharose fast-flow column (2.0 by 20 cm; Pharmacia-LKB Biotechnology, Uppsala, Sweden) equilibrated with 50 mM Na^+-K^+ phosphate

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TABLE 1. MICs of several β-lactams for the three *Acinetobacter* strains analyzed in the study

Antibiotic	MIC (μg/ml)		
	A. lwoffii ULA-501	*A. baumannii* ULA-187	*A. baumannii* AC-14
Ceftazidime	2	32	128
Cefotaxime	2	32	128
Cephaloridine	128	>256	>256
Cefpirome	2	32	64
Carbenicillin	2	64	>256
Piperacillin	32	256	>256
Imipenem	0.125	0.25	0.5
Aztreonam	8	128	>128

buffer (pH 7.4). The column was extensively washed to remove unbound proteins, and the β-lactamase was eluted in a linear gradient of NaCl (0 to 1 M) in the same buffer. The active fractions were pooled, concentrated by ultrafiltration, dialyzed overnight at 4°C against 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 8.0), and stored at −80°C for further investigations.

Determination of β-lactamase activity. The β-lactamase activity was spectrophotometrically determined by measuring the hydrolysis of 100 μM nitrocefin ($\lambda = 482$ nm; $\Delta\varepsilon_M^{482} = 15{,}000\ M^{-1}\ cm^{-1}$).

One unit represents the amount of enzyme that hydrolyzes 1 μmol of nitrocefin per min at 30°C in 10 mM HEPES buffer (pH 8.0) containing 0.2 M NaCl.

Protein content determination. The protein concentration was determined by the method of Bradford (3) by using bovine serum albumin as the standard.

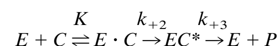
PAGE and M_r determination. Polyacrylamide gel electrophoresis (PAGE) was performed by following the method of Laemmli (18) on a 12.5% (wt/vol) polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS) by using a Mini-Protean II Apparatus (Bio-Rad Laboratories, Richmond, Calif.). The proteins (β-lactamase and standards) were stained with Coomassie blue R-250 for 90 min at room temperature and were destained with ethanol-acetic acid-water (30:7:63) solution.

M_r values were determined by measuring the relative mobilities of the proteins.

Isoelectric focusing analysis. Gel isoelectric focusing was performed in 5% polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5) by using a Multiphor II Apparatus (Pharmacia-LKB Biotechnology, Uppsala, Sweden). The gels were focused at 4°C and 25 W for 180 min. The pI value was determined by focusing 5 μg of each purified enzyme.

Determination of the kinetic parameters. Usually, the steady-state parameters (k_{cat}, K_m, and k_{cat}/K_m) were derived from the analysis of complete time courses of antibiotic hydrolysis, as reported previously by De Meester et al. (7). When the K_m value was very low, it was measured as K_i in the presence of nitrocefin as the reporter substrate. The k_{cat} value was then derived from initial rates at saturating substrate concentrations ($[S] \gg K_m$). When the conditions mentioned above were not fulfilled, K_m and k_{cat} values were measured under initial rate conditions by using Hanes linearization of the Michaelis-Menten equation (25). In all cases the reaction volume was 0.6 ml. The wavelengths and absorbance variations used were those reported previously (8, 19). Cells with path lengths of 0.2 to 1.0 cm were used, depending on the solution concentration.

Inactivation experiments. With compounds behaving as transient inactivators, a rather stable acyl-enzyme (EC^*) was found to accumulate, and the interactions were studied on the basis of the following model:



$$E + C \overset{K}{\rightleftharpoons} E \cdot C \xrightarrow{k_{+2}} EC^* \xrightarrow{k_{+3}} E + P$$

where E, C, $E \cdot C$, EC^*, and P are the enzyme, the substrate, the Henri-Michaelis complex, the stable inactive complex, and the hydrolysis product, respectively. k_{+2} and k_{+3} were the acylation and deacylation rate constants, respectively, while K is the dissociation constant of the Henri-Michaelis complex. The determination of the pseudo-first-order rate constant (k_i) characterizing the rate of EC^* accumulation was made by analyzing the hydrolysis of 150 μM nitrocefin, used as the reporter substrate, in the presence of increasing inactivator concentrations in 10 mM HEPES (pH 8.0) containing 0.2 M NaCl at 30°C. Less than 20% nitrocefin was hydrolyzed. The individual parameters k_{+2}, k_{+3}, and K were calculated with the help of the following equation (9):

$$k_i = k_{+3} + \frac{k_{+2} \cdot [C]}{[C] + K\dfrac{(K_m + [S])}{K_m}}$$

where K_m and $[S]$ are the K_m for and the concentration of the reported substrate, respectively. The linear variation in k_i with increasing inactivator concentration ($[C]$) indicated that the range of studied concentrations was much less than K. In this case, the equation reported above was simplified as follows:

$$k_i = k_{+3} + \frac{k_{+2} \cdot [C] \cdot K_m}{K \cdot (K_m + [S])}$$

The k_{+2}/K value was then obtained from the slope of the line, and k_{+3} was obtained from the extrapolation at $[C]$ equal to 0.

Interaction with p-CMB. A total of 25 μg of each β-lactamase was incubated at 30°C with 300 μM *p*-chloromercuribenzoate (*p*-CMB) dissolved in 10 mM HEPES buffer (pH 8.0) containing 0.2 M NaCl at 30°C. After 15 min, 10 μl of the reaction mixture was added to 600 μl of 100 μM nitrocefin; the initial rate of hydrolysis was compared with that obtained with the untreated enzyme in order to calculate the average residual activity.

RESULTS

MIC determination. One *A. lwoffii* and two *A. baumannii* clinical isolates were selected for the present investigation on the basis of their different patterns of susceptibility to β-lactam antibiotics. The MICs of several β-lactams for these strains are reported in Table 1.

Purification of the three β-lactamases. The enzymes produced by the three *Acinetobacter* strains were purified by two chromatographic steps, as reported in Table 2. The β-lactamases were obtained with a rather similar yield, ranging from 29 to 35% of the total units calculated. Each enzyme was estimated to be pure (>90%) by SDS-PAGE, and an M_r value of 38,000 was calculated (data not shown). Moreover, the pI values calculated in the present study were in all cases >9.0; these data were in good agreement with those reported by other investigators (2, 13, 14).

Kinetic parameter determination. Table 3 summarizes the kinetic parameters calculated for molecules behaving as substrates. Surprisingly, carbenicillin was a good substrate for the β-lactamase produced by *A. lwoffii* ULA-501, showing a phys-

TABLE 2. Purification of the β-lactamases from the three *Acinetobacter* strains

Strains	Purification step	Total protein (mg)	Sp act (U/mg)[a]	Total activity (U)	Purification factor	% Yield
A. lwoffii ULA-501	Crude extract	420	1.8	756	1	100
	Sephadex G-75 chromatography	38	16	608	9	80
	S-Sepharose fast flow	1.7	131	223	73	29
A. baumannii ULA-187	Crude extract	400	1.6	640	1	100
	Sephadex G-75 chromatography	37	11	407	7	63
	S-Sepharose fast flow	1.8	125	225	78	35
A. baumannii AC-14	Crude extract	300	2.1	630	1	100
	Sephadex G-75 chromatography	16	29	464	14	74
	S-Sepharose fast flow	1.2	157	188	75	30

[a] Nitrocefin (150 μM) was used as the substrate.

TABLE 3. Kinetic parameters determined with the three *Acinetobacter* β-lactamases[a]

Substrate	*A. lowffii* ULA-501			*A. baumannii* ULA-187			*A. baumannii* AC-14		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)
Carbenicillin	6.8 ± 0.3[b]	8.47	1.25×10^6	NH	NH	NH	NH	NH	NH
Benzylpenicillin	0.24 ± 0.02[b]	ND	ND	0.83 ± 0.03[b]	ND	ND	0.89 ± 0.02[b]	ND	ND
Piperacillin	0.031 ± 0.002[b]	ND	ND	0.047 ± 0.005[b]	ND	ND	2.27 ± 0.5[b]	ND	ND
Nitrocefin	20 ± 1	56	2.80×10^6	50 ± 3	190	3.80×10^6	50 ± 5	480	9.60×10^6
Cephaloridine	15 ± 1	7.30	4.87×10^5	400 ± 10	82	2.05×10^5	660 ± 15	178	2.70×10^5
Cephalothin	3.7 ± 0.2[b]	0.37	1.00×10^5	120 ± 5	150	1.25×10^6	75 ± 4	260	3.47×10^6
Cefotaxime	0.048 ± 0.003[b]	ND	ND	0.19 ± 0.04[b]	ND	ND	0.09 ± 0.01[b]	ND	ND
Cefpirome	NI	NI	NI	NI	NI	NI	NI	NI	NI

[a] All of the calculated values were the means of five different measurements. Standard deviations did not exceed 10%. Abbreviations: NH, no hydrolysis was found up to a concentration of 500 μM. ND, not determined. In this case we were unable to calculate k_{cat} and k_{cat}/K_m ratio values because these β-lactam molecules were not hydrolyzed by the three enzymes. NI, no interaction; hydrolysis and inhibition-inactivation were not found up to a concentration of 5 mM. For more details see the Materials and Methods section.

[b] The K_m value was determined as K_i in competitive experiments with 150 μM nitrocefin as the reporter substrate.

iologic efficiency (k_{cat}/K_m ratio) of the same order of magnitude as that calculated for nitrocefin. Carbenicillin also behaved as a transient inhibitor of the enzymes produced by the two *A. baumannii* strains.

Benzylpenicillin and piperacillin showed high degrees of affinity for the three β-lactamases, but these penicillins were not hydrolyzed at all.

Referring to the hydrolysis of nitrocefin, cephaloridine, and cephalothin, the most interesting result was the low K_m value found with the *A. lwoffii* enzyme with respect to that calculated for *A. baumannii* β-lactamases.

Effects of inhibitors on the β-lactamases. Although cefotaxime and cefpirome were not hydrolyzed, they interacted in two different ways when they were used with the three β-lactamases tested in the present study. Cefpirome, up to a concentration of 5 mM, did not affect the activities of the enzymes when nitrocefin was used as the reporter substrate. On the contrary, cefotaxime behaved as a competitive inhibitor of all β-lactamases, with similar K_i values, as reported in Table 3.

Carbenicillin, which behaved as a good substrate for the β-lactamase produced by *A. lwoffii* ULA-501, acted as a progressive inactivator of the enzymes from the two *A. baumannii* strains (Table 4). By analyzing the k_{+2}/K ratios calculated for these latter two β-lactamases, one could note that carbenicillin acylated the enzyme from *A. baumannii* AC-14 20-fold more efficiently than the protein from *A. baumannii* ULA-187, whereas the k_{+3} values calculated for each β-lactamase were rather similar.

Aztreonam behaved as a transient inhibitor of the three *Acinetobacter* β-lactamases. As shown in Table 4, this compound had a low acylation value (k_{+2}/K ratio) when it interacted with the enzyme from *A. lwoffii* ULA-501. This cephalosporinase appeared to be very different from the other enzymes with respect to its minimal acylation efficiency (its k_{+2}/K ratio was 10,000-fold lower).

When clavulanic acid was used as an inhibitor (up to a concentration of 5 mM) in the presence of nitrocefin as the reporter substrate, no significant decrease in the initial rate of substrate hydrolysis was observed. This indicated an extremely reduced affinity of the three β-lactamases assayed for this classical active-site serine β-lactamase inhibitor.

In contrast, sulbactam behaved as a progressive inactivator, with kinetic parameters varying between the three enzymes. In fact, K values (dissociation rate constant of the Henri-Michaelis complex) were determined with the enzymes produced by the two *A. baumannii* strains, which also showed efficiency-acylation values (k_{+2}/K) of the same order of magnitude (Table 4).

In the case of the β-lactamase from *A. lwoffii* ULA-501, we were able to calculate only k_{+3} and k_{+2}/K ratio values, because of the linear trend of the k_i value (pseudo-first-order inactivation rate constant) in the presence of increasing concentrations of sulbactam. Moreover, the k_{+2}/K ratio value calculated with this enzyme was 10-fold higher than those of the other two β-lactamases studied.

In the case of 6-β-IP, the enzymes from the two *A. baumannii* strains showed very similar k_{+3} and k_{+2}/K ratio values, although they were higher than those reported by De Meester et al. (6) for class C β-lactamases. The β-lactamase from *A. lwoffii* ULA-501 appeared to be more resistant to the action of 6-β-IP and had an acylation value similar to those calculated for other class C β-lactamases (Table 4).

TABLE 4. Kinetic parameters of inactivation of the *Acinetobacter* β-lactamases[a]

β-Lactam	*A. lwoffii* ULA-501				*A. baumannii* ULA-187				*A. baumannii* AC-14			
	K (μM)	k_{+3} (s^{-1})	k_{+2} (s^{-1})	k_{+2}/K ($M^{-1} s^{-1}$)	K (μM)	k_{+3} (s^{-1})	k_{+2} (s^{-1})	k_{+2}/K ($M^{-1} s^{-1}$)	K (μM)	k_{+3} (s^{-1})	k_{+2} (s^{-1})	k_{+2}/K ($M^{-1} s^{-1}$)
Carbenicillin	—	—	—	—	ND	4.89×10^{-3}	ND	7.60×10^5	ND	2.03×10^{-3}	ND	1.37×10^7
Aztreonam	ND	5.25×10^{-3}	ND	8.50×10^2	ND	1.03×10^{-2}	ND	3.80×10^7	ND	1.10×10^{-2}	ND	5.93×10^7
Sulbactam	ND	9.30×10^{-4}	ND	6.46×10^2	1,000	ND	5.15×10^{-2}	5.15×10^1	500 ± 20	ND	4.00×10^{-2}	8.20×10^1
Clavulanic acid	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
6-β-IP	480 ± 10	ND	3.97×10^{-2}	8.27×10^1	ND	1.85×10^{-3}	ND	6.90×10^3	ND	1.90×10^{-3}	ND	2.80×10^3

[a] ND, not determined. The plots of k_i versus $[C]$ were linear (k_{+2}/K) and were extrapolated to $[C] = 0$ (k_{+3}). —, In this case carbenicillin behaved as a good substrate, and only K_m, k_{cat}, and k_{cat}/K_m values were determined. NI, no interaction; hydrolysis and inactivation were not found up to a concentration of 5 mM. For more details see Materials and Methods. The calculated values were the means of five different measurements. The standard deviations did not exceed 10%.

Usually, cephalosporinases are reported to be insusceptible to the action of p-CMB. However, under our experimental conditions, a certain level of inhibition was observed, although the cysteine residue content was not determined. In particular, only the enzyme from *A. lwoffii* ULA-501 showed a residual activity of 57%, whereas the other two β-lactamases were inhibited at the same extent (about 70% inhibition).

DISCUSSION

Group 1 cephalosporin-hydrolyzing β-lactamases of gram-negative bacteria have been described as a rather homogeneous group of enzymes on the basis of their primary structures, molecular masses, alkaline pIs, and functional properties, including preferential activity against cephalosporins and poor inhibition by clavulanic acid (5). By analyzing the catalytic efficiencies of these enzymes, however, it has been shown that these β-lactamases are also able to efficiently inactivate penicillins (11).

We have purified and characterized three such enzymes, one from an *A. lwoffii* clinical isolate (ULA-501) and the others from two *A. baumannii* clinical isolates (ULA-187 and AC-14). The three enzymes appeared to be similar to each other and also to other group 1 enzymes, including a previously purified cephalosporinase from *A. calcoaceticus*, when the molecular masses and the pI values were considered (2, 13). Our data were also in good agreement with those reported previously for the chromosomal cephalosporinases produced by enteric bacteria and *Pseudomonas* spp. (5). The only relevant differences in physicochemical properties were observed with the data reported by Hood and Amyes (14) and could be related either to strain variation or to the technique used to purify the protein.

Notwithstanding the structural similarities, some differences with other group 1 β-lactamases and also among each other were evident by analyzing the kinetic parameters of substrate hydrolysis and the inactivation of these enzymes. The overall catalytic efficiencies (k_{cat}/K_m ratio value) of the three β-lactamases calculated with cephaloridine, cephalothin, and nitrocefin were lower than those usually reported for other group 1 cephalosporinases (10). The kinetic parameters of inactivation observed with aztreonam and 6-β-IP showed some relevant differences when they were compared with those reported previously for other group 1 enzymes (4). In particular, the *A. lwoffii* and *A. baumannii* β-lactamases appeared to be less or more susceptible, respectively, to aztreonam and showed similar or higher levels of susceptibility to inactivation by 6-β-IP (6). Carbenicillin allowed us to differentiate the β-lactamase from *A. lwoffii* ULA-501 from the enzymes produced by the two *A. baumannii* strains investigated in the present study. In fact, this carboxypenicillin was a good substrate for the former enzyme, while it behaved as a transient inactivator of the other two β-lactamases. Using this molecule with the cephalosporinases from *A. baumannii* strains, it was also possible to distinguish two different situations. The k_{+2}/K ratio and k_{+3} values showed a possible reactivation of the enzyme produced by *A. baumannii* ULA-187, whereas the β-lactamase purified from *A. baumannii* AC-14 was acylated and inactivated with a high degree of efficiency, as indicated by a k_{+3} value very close to zero under our experimental conditions.

The group 1 β-lactamases produced by *Acinetobacter* spp. therefore appear to be somewhat heterogeneous from a functional standpoint, and for this reason, further characterization of these enzymes at the level of their primary structures could be very interesting to improve the current knowledge of this group of enzymes.

Comparison of the susceptibility data with the kinetic parameters derived from the present study suggests that the *Acinetobacter* cephalosporinases could play a role in β-lactam resistance to some compounds which are efficiently hydrolyzed by these enzymes, whereas resistance to other molecules, such as piperacillin, cefotaxime, cefpirome, and aztreonam, is likely due mainly to a permeability barrier and/or to a reduced affinity of the penicillin-binding proteins present in the strains investigated. Sulbactam appeared to be able to inhibit the *Acinetobacter* β-lactamases only at concentrations which are difficult to achieve in vivo, and therefore, it is unlikely to be clinically useful as a β-lactamase inhibitor in these cases. This compound, however, has recently been reported to be effective in vitro against *Acinetobacter* strains (17, 26). Our data confirm the need to search for newer drugs that are able to overcome this apparent impermeability and to escape the actions of β-lactamases. In this respect, the study of new and efficient suicide inhibitors for chromosomal cephalosporinases remains of permanent importance. From the enzymology point of view, the cephalosporinases produced by this bacterial genus, although exhibiting differences in their kinetic behaviors, need to be further characterized in more detailed studies of their nucleotide sequences and the regulation of cephalosporinase expression.

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