Novel Carbapenem Derivative SF2103A: Studies on the Mode of \( \beta \)-Lactamase Inactivation

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A novel carbapenem, SF2103A, is a strong inhibitor of various types of \( \beta \)-lactamase. Equimolar concentrations of SF2103A completely inactivated the cephalosporinases of *Proteus vulgaris* and *Citrobacter freundii* and type Ib and type II penicillinas mediated by R plasmids in a progressive manner. The inactivation of the two penicillinas and *P. vulgaris* cephalosporinase was apparently irreversible; however, when the inactivated enzymes were separated from excess SF2103A by gel filtration, they showed very slow reactivation. The hydrolysis of SF2103A by these three \( \beta \)-lactamases was below the limit of detection. It is concluded that SF2103A acts as a tight-binding competitive inhibitor for the penicillinas and *P. vulgaris* cephalosporinase. In contrast, the inactivation of *C. freundii* cephalosporinase by SF2103A was evidently reversible. The rate constant of reactivation of the enzyme was compatible with the turnover rate of the enzyme in the steady state of SF2103A hydrolysis. Thus, SF2103A simply acts as a poor substrate for *C. freundii* cephalosporinase.

Recently, new classes of \( \beta \)-lactams having an altered nucleus have been found to be potent \( \beta \)-lactamase inhibitors (7). Among these compounds, suicide substrates such as clavulanic acid (10, 13, 16) and sulbactam (9, 12, 25) demonstrate irreversible inactivation by a pathway branching from the acyl enzyme intermediate. However, one or two orders of magnitude of inhibitor concentration over enzyme concentration are necessary for complete inactivation of enzyme activity because these inhibitors are hydrolyzed during the inactivation process (10). In contrast, most carbapenems including olivamic acid derivatives (3, 6, 15) show reversible inactivation which is due to the branching of transiently stable enzyme-inhibitor complexes from acyl intermediates (3). Richmond (17) reported that imipenem (\( N \)-formimidoyl thienamycin) irreversibly inactivated some \( \beta \)-lactamases, but our detailed kinetic study (21) has suggested that the inhibition of most of the enzyme activity by imipenem is also reversible.

SF2103A (Fig. 1) is a novel carbapenem derivative isolated from *Streptomyces sulfonofaciens* sp. nov. that shows very strong inhibition of a wide variety of \( \beta \)-lactamases (11). We have found that SF2103A acts as a tight-binding competitive inhibitor for \( \beta \)-lactamases. The scheme for enzyme inactivation does not include a branch from the normal process for enzymatic hydrolysis. In this paper, we describe kinetic studies on the inactivation by SF2103A of four types of \( \beta \)-lactamases: type Ib penicillinase (TEM-2 penicillinase) (14), type II penicillinase (OXA-2 penicillinase) (5), *Citrobacter freundii* cephalosporinase, and *Proteus vulgaris* cephalosporinase.

**MATERIALS AND METHODS**

**Bacterial strains.** The bacterial strains used in this study were selected from a set of gram-negative bacteria producing different \( \beta \)-lactamases (22). *Escherichia coli* ML1410 (RGN823) and *E. coli* ML1410 (RGN238) harbor R-plasmids that mediate the constitutive production of type Ib (20) and type II (24) penicillinas, respectively. *P. vulgaris* GN76/C-1 produces cephalosporinase constitutively and was derived from wild-type strain GN76. *C. freundii* GN346 is a clinical isolate which produces cephalosporinase semiconstitutively (19).

**B-Lactam antibiotics and related compounds.** \( \beta \)-Lactam antibiotics were kindly provided by the following pharmaceutical companies: benzylpenicillin and SF2103A, Meiji Seika Co., Tokyo, Japan; cephalothin, Torii Pharmaceutical Co., Tokyo, Japan.

**Preparation of \( \beta \)-lactamases.** Type Ib penicillinase was prepared from *E. coli* ML1410 (RGN823) and purified to homogeneity by adsorption and elution on a DEAE-Sephadex ion-exchange column equilibrated with 5 mM Tris-hydrochloride buffer (pH 7.4) and by gel filtration on a Sephadex G-75 column. Type II penicillinase and *C. freundii* and *P. vulgaris* cephalosporinases were prepared from *E. coli* ML1410 (RGN238), *C. freundii* GN346, and *P. vulgaris* GN76/C-1, respectively, and purified to homogeneity by the same method as above except for the use of a carboxymethyl-Sephadex column equilibrated with 10 mM phosphate buffer (pH 6.0 or 7.0 [type II]) instead of a DEAE-Sephadex column. The purity of these enzymes was confirmed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The specific activities of type Ib, type II, *C. freundii*, and *P. vulgaris* \( \beta \)-lactamases used in this study were 2200, 61, 130, and 832 U/mg of protein, respectively. We used benzylpenicillin and cephalothin as substrates for the assay of penicillinase and cephalosporinase activity. One unit was defined as the amount of enzyme which hydrolyzed 1 \( \mu \)mol of substrate per min at pH 7.0 and 30°C.

**Competitive inhibition.** Solutions (3 ml each) of various concentrations of substrate, i.e., benzylpenicillin for the penicillinas and cephalothin for the cephalosporinas, in 50 mM phosphate buffer (pH 7.0) were prewarmed to 30°C with SF2103A (initial concentrations of 0 to 20 \( \mu \)M for the penicillinas and 0 to 0.1 \( \mu \)M for the cephalosporinas). A 50-\( \mu \)l volume of enzyme solution was added to the mixture (final concentration of 6.7 nM type II penicillinase, 1.67 nM type Ib penicillinase, 1.25 nM *P. vulgaris* cephalosporinase, or 3.3 nM *C. freundii* cephalosporinase). The absorbance change at 232 nm for benzylpenicillin or at 265 nm for cephalothin was monitored during incubation at 30°C for several minutes. An absorbance change from enzymatic
hydrolysis of SF2103A was negligible under these conditions. The apparent $K_m$ value ($K_p$) in the presence of SF2103A was obtained from a Lineweaver-Burk plot of the initial rate of hydrolysis. The value of $K_p$ was calculated from $K_m$ and $K_p$ in the absence of SF2103A.

**Progressive inactivation.** A 1-$\mu$M solution of $\beta$-lactamase in 50 mM phosphate buffer (pH 7.0) was preincubated with the indicated concentration of SF2103A at 30°C. At different time intervals, 5 $\mu$L of the mixture was withdrawn and added to 3 ml of either 200 $\mu$M benzylpenicillin for the penicillinases or 100 $\mu$M cephalothin for the cephalosporinases unless otherwise stated. Enzyme activity was determined from the initial rate of substrate hydrolysis as measured spectrophotometrically. As a control, the enzyme incubated without SF2103A was added to the substrate solution containing SF2103A at the same final concentration. Competitive inhibition was virtually excluded by the dilution and addition of excess substrate under these conditions.

**Gel filtration and reactivation of the inactive enzyme.** P. vulgaris cephalosporinase (1.9 $\mu$M) was completely inactivated by incubation with 5 $\mu$M SF2103A for 60 min at 30°C and pH 7.0. This mixture (200 $\mu$L) was rapidly filtered through a Sephadex G-25 column at 4°C. The pooled enzyme fractions were incubated at 30°C. At different time intervals, 30 $\mu$L was withdrawn and added to 3 ml of the same buffer containing 100 $\mu$M cephalothin. Enzyme activity was assayed spectrophotometrically. Reactivation of type Ib penicillinase was measured by the same procedure except that 2.5 $\mu$M enzyme was inactivated by 100 $\mu$M SF2103A and enzyme activity was detected by using benzylpenicillin as the substrate.

**Values of $k_{\text{inact}}$ and $K_{\text{inact}}$.** Values of the rate constant of inactivation ($k_{\text{inact}}$) and the affinity of the inhibitor for the inactivation site ($K_{\text{inact}}$) were obtained from the double-reciprocal plot of the initial rate of inactivation versus the initial concentration of SF2103A. Fifty microliters of 0.04 $\mu$M P. vulgaris cephalosporinase was rapidly mixed with 2.95 ml of various concentrations of SF2103A in 50 mM phosphate buffer at pH 7.0. After 10 s of incubation at 30°C, 30 $\mu$L of 10 mM cephalothin was added. The residual active enzyme fraction was calculated from the initial rate of inactivation of cephalothin. For C. freundii cephalosporinase, the inactivation rate was measured in the same way except for an initial concentration of enzyme of 0.1 $\mu$M. The inactivation rates of the penicillinases were measured by incubation of 1 $\mu$M enzyme with various concentrations of SF2103A for 1 min followed by the addition of benzylpenicillin (final concentration of 200 $\mu$M).

**Hydrolysis of SF2103A.** The degradation of SF2103A was monitored by the absorbance change at 266 nm when 15 $\mu$M SF2103A was incubated with 2 $\mu$M C. freundii cephalosporinase in 2 ml of phosphate buffer at 30°C and pH 7.0.

**RESULTS**

**Inhibition of $\beta$-lactamase activity by SF2103A.** We chose four $\beta$-lactamases of gram-negative bacteria in this study. Type Ib penicillinase, identical with TEM-2 penicillinase (14), and type II penicillinase, identical with OXA-2 penicillinase (5), are penicillinases with broad and narrow substrate specificities, respectively. C. freundii and P. vulgaris $\beta$-lactamases are cephalosporinases with narrow and relatively broad substrate specificities, respectively. More detailed properties of these $\beta$-lactamases were reported previously (18).

When SF2103A was added simultaneously with the substrate to a $\beta$-lactamase solution, it competitively inhibited the activity of each of the $\beta$-lactamases tested at an early stage and then showed a progressive inhibition that was not immediately reversible. $K_i$ values obtained from early stages of the inactivation of cephalosporinases were much smaller than those of penicillinas (Table 1). The progressive nature of the inactivation was detected by the dilution method described above (Fig. 2). The activities of type II and type Ib penicillinases and P. vulgaris cephalosporinase were completely inactivated by equimolar concentrations of SF2103A after several minutes of preincubation. Inactivation of these $\beta$-lactamases was irreversible for 5 h, at least in this assay.

Initial rates of inactivation were measured as described above. $k_{\text{inact}}$ and $K_{\text{inact}}$ were determined from a double-reciprocal plot of the initial rate of inactivation of the enzyme at different initial inhibitor concentrations by the method of Charnas and Knowles (4) (Table 1). The value of $k_{\text{inact}}$ for type Ib penicillinase ($2.3 \times 10^{-2}$ s$^{-1}$) was similar to that of clavulanic acid for TEM-2 penicillinase ($3 \times 10^{-2}$ s$^{-1}$ [13] or $4 \times 10^{-2}$ s$^{-1}$ [4]), whereas it was much larger than that of sulbactam for TEM-2 penicillinase ($8 \times 10^{-4}$ s$^{-1}$ [12] or $2.8 \times 10^{-4}$ s$^{-1}$ [1]).

The difference between the values of $K_{\text{inact}}$ and $K_i$ for SF2103A for $\beta$-lactamases was less than one order of magnitude. This difference was significantly smaller than the 30-fold difference between the $K_{\text{m}}$ and $K_i$ values of PS-5 for P. vulgaris cephalosporinase (15). It seems unnecessary to assume separate sites for competitive and progressive inactivation of $\beta$-lactamases by SF2103A.

**TABLE 1. Kinetic constants of SF2103A against $\beta$-lactamases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$ ($\mu$M)</th>
<th>$K_{\text{inact}}$ ($\mu$M)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{inact}}$ (s$^{-1}$)</th>
<th>$K_{\text{m}}$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. freundii cephalosporinase</td>
<td>0.017</td>
<td>0.033</td>
<td>$1.1 \times 10^{-3}$</td>
<td>$6.3 \times 10^{-2}$</td>
<td>$3 \times 10^{-6}$</td>
</tr>
<tr>
<td>P. vulgaris cephalosporinase</td>
<td>0.020</td>
<td>0.005</td>
<td>$1.6 \times 10^{-4}$</td>
<td>$2.5 \times 10^{-6}$</td>
<td>$1.3 \times 10^{-5}$</td>
</tr>
<tr>
<td>Type Ib penicillinase</td>
<td>13.7</td>
<td>3.8</td>
<td>$1.0 \times 10^{-3}$</td>
<td>$2.3 \times 10^{-2}$</td>
<td>$9.3 \times 10^{-2}$</td>
</tr>
<tr>
<td>Type II penicillinase</td>
<td>5.2</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* This value was determined from the reactivation curve during prolonged incubation as shown in Fig. 3.

* This value was determined from reactivation after gel filtration as described in the text.
Therefore, described below is an example of type II penicillinase (A), type Ib penicillinase (B), *P. vulgaris* cephalosporinase (C), and *C. freundii* cephalosporinase (D) were measured as described in the text except that 20 μl of the preincubation mixture of type II penicillinase and SF2103A (A) was diluted into 3 ml of the assay solution. The enzyme concentration in the preincubation mixture was 1 μM. The initial SF2103A concentration was 0.2 μM (△), 0.5 μM (●), or 1 μM (○) except in (D) in which the concentration was 1 μM (△), 2 μM (●), or 5 μM (○). A 200-μM concentration of benzylpenicillin (A, B) or 100 μM cephalothin (C, D) was used as the substrate in the assay solution.

When type Ib or type II penicillinases or *P. vulgaris* cephalosporinase were incubated with less than an equimolar concentration of SF2103A, the residual activity reached a constant level which depended on the initial inhibitor-enzyme (i-e) ratio (Fig. 2A, B, and C). By extrapolating the plot of final residual activity against the initial i-e ratio, the number of inhibitor molecules for inactivating one molecule of enzyme could be obtained. These values were 0.85, 0.72, and 0.98 against type II, type Ib, and *P. vulgaris* β-lactamases, respectively. The finding of a number less than one might indicate that these preparations contained inactive enzyme although the preparations were physically pure as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This explanation seems to be more reasonable than is the alternative that one molecule of SF2103A could interact with more than one molecule of enzyme. Therefore, the results suggest that one molecule of SF2103A stoichiometrically inactivated one molecule of the enzyme.

**Recovery of *C. freundii* cephalosporinase activity.** Among the β-lactamases tested, only the inactivation of *C. freundii* cephalosporinase was readily reversible (Fig. 2D). When 1 μM *C. freundii* cephalosporinase was incubated with more than an equimolar concentration of SF2103A, complete loss of enzyme activity temporarily occurred. Then, after a lag time which depended on the inhibitor concentration, the enzyme started to regain activity. Within 100 min after starting reactivation, enzyme activity was completely restored. Semilogarithmic plots of the inactive enzyme fraction versus time in the reactivation phase were linear (Fig. 3). The first-order rate constant of reactivation (k_{react}) calculated from the slope was 1.0 × 10^{-3} s^{-1} and was independent of the initial i-e ratio. This result indicated that there was no conversion of an unstable enzyme-inhibitor complex into a more stable one during the lag time before reactivation. If this were the case, apparent k_{react} values should depend on

![FIG. 2. Progressive inactivation of β-lactamases by SF2103A. After the enzymes were preincubated with SF2103A, the residual activities of type II penicillinase (A), type Ib penicillinase (B), *P. vulgaris* cephalosporinase (C), and *C. freundii* cephalosporinase (D) were measured as described in the text except that 20 μl of the preincubation mixture of type II penicillinase and SF2103A (A) was diluted into 3 ml of the assay solution. The enzyme concentration in the preincubation mixture was 1 μM. The initial SF2103A concentration was 0.2 μM (△), 0.5 μM (●), or 1 μM (○) except in (D) in which the concentration was 1 μM (△), 2 μM (●), or 5 μM (○). A 200-μM concentration of benzylpenicillin (A, B) or 100 μM cephalothin (C, D) was used as the substrate in the assay solution.](https://example.com/figure-2)

![FIG. 3. Reactivation of *C. freundii* cephalosporinase. The logarithm of the inactive fraction of the enzyme at the phase of reactivation in Fig. 2D was plotted against the incubation time. The inactive fraction was corrected for the final recovery of enzyme activity. The enzyme concentration was 1 μM. The initial SF2103A concentration was 1 μM (△), 2 μM (●), or 5 μM (○).](https://example.com/figure-3)
The lag time. By extrapolation of the plot in Fig. 3, the length of the lag time was found to be 0, 14, and 70 min when the initial i-e ratios were 1, 2, and 5, respectively.

**Reactivation of inactivated β-lactamas after gel filtration.** When type Ib penicillinase or *P. vulgaris* cephalosporinase was completely inactivated by prolonged incubation with excess SF2103A and then inactivated enzyme was separated from the inhibitor by gel filtration, the pooled inactive enzyme fractions showed very slow reactivation during incubation at 30°C. A semilogarithmic plot of the inactive enzyme fraction against incubation time showed first-order kinetics, but we could not establish whether enzyme activity returned completely to its original level because the reactivation velocity was too slow. The first-order rate constants of reactivation are shown in Table 1. From these rate constants the half-lives of degradation of the enzyme-inhibitor complex were estimated to be 17 and 77 hr for type Ib penicillinase and *P. vulgaris* cephalosporinase, respectively. Therefore, the inactivation of these two β-lactamas by SF2103A was not strictly irreversible, although no reactivation was detected unless they were separated from the inhibitor by gel filtration.

**Hydrolysis of SF2103A by β-lactamas.** Hydrolysis of SF2103A by *C. freundii* cephalosporinase as measured by the decrease in absorbance at 266 nm (Fig. 4) showed a steady state after an initial burst of SF2103A consumption. The burst size was 0.8 of the initial enzyme concentration. The steady-state rate constant of hydrolysis (kcat) was 1.1 × 10^{-3} s^{-1}. This value was independent of the initial concentration of SF2103A which varied from 10 to 20 μM and was very close to the k_{react} value (1.0 × 10^{-3} s^{-1}).

The rates of SF2103A hydrolysis by type Ib and type II penicillinases and *P. vulgaris* cephalosporinase were slower than the limit of spectrophotometrical detection due to spontaneous degradation of SF2103A.

**DISCUSSION**

The structure of SF2103A closely resembles an olivic acid derivative, MM4550, except that the C-3 side chain of MM4550 is replaced by a sulfonate group in SF2103A (11). SF2103A was hardly hydrolyzed by TEM-type penicillinase which could, however, hydrolyze MM4550 (6). The inactive penicillinase after reaction with SF2103A was much more stable than that produced with MM4550 as reported by Easton and Knowles (6), although the inactivation by SF2103A was also reversible after gel filtration.

Among the β-lactamas tested in this study, only *C. freundii* cephalosporinase showed rapid reversibility of inactivation. Although the interaction of SF2103A with *C. freundii* cephalosporinase had similar features to that of MM4550 with TEM β-lactamase (6), e.g., the presence of a steady state of inhibitor hydrolysis after an initial burst, we propose a different minimum scheme in which SF2103A inhibits the enzyme as a poor substrate without any branching from the enzymatic pathway.

\[
E + I \xrightarrow{K_m} EI \xrightarrow{k_3} A \xrightarrow{k_4} E + P
\]

E is the free enzyme, I is the inhibitor (SF2103A), EI is the Michaelis complex of E and I, A is the tight-binding complex of enzyme and SF2103A, and P is the SF2103A-derived product from decomposition of the tight-binding complex. This scheme is based on the following observations: (i) the value of kcat in the steady state (1.1 × 10^{-3} s^{-1}) was in excellent agreement with the value of k_{react} (1.0 × 10^{-3} s^{-1}), indicating that both kcat and k_{react} represent the same process of k3 in the scheme; and (ii) the burst size in SF2103A hydrolysis was 0.8, whereas if a branched pathway were operative, the burst size usually would be more than 1.

Under the above scheme, the burst size (π[I]/[I]o) was calculated from the following equation (8)

\[
\pi/[I]_0 = ([k_2/(k_2 + k_3)])^2/(1 + K_m/[I]_0)
\]

According to the kinetic constants in Table 1 and an initial concentration of SF2103A of 15 μM, the burst size was calculated to be 0.98, which is in good agreement with the experimentally obtained value of 0.8.

The lag time of reactivation corresponds to the time required for the consumption of free SF2103A. During the lag time, the rate of SF2103A degradation is equal to k3 (k_{react} or kcat) in the above scheme. Thus, in consideration with the initial burst (ca. 1), the length of the lag time can be calculated as lag time = (([I]o/[I]o - 1))/k3. By using this equation and the value of k_{react} in Table 1, we obtained lag times of 0, 16.7, and 66.7 min when the initial i-e ratios were 1, 2, and 5, respectively. These values are entirely compatible with the experimentally obtained lag times of 0, 14, and 70 min in the case of the initial i-e ratios of 1, 2, and 5, respectively. This agreement confirms the nonbranched mechanism of the above scheme. In addition, it indicates that the purity of the *C. freundii* cephalosporinase preparation was close to 100% because, if the purity was less than 100%, the experimentally obtained lag time should be larger than the lag time calculated from the lag time equation above.

This scheme was also confirmed by computer-assisted simulation of the interaction of SF2103A with *C. freundii* cephalosporinase (Fig. 5). The progress curve of the reacting species in the above scheme could be calculated by numerical integration of the rate equations derived from the scheme by a modification of the method of Runge-Kutta-Gill (23). A program named YHS1 for numerical integration of the set of rate equations derived from the above scheme was developed in our laboratory by using HITAC M-180 computer. A copy of this program written in FORTRAN 77 can be obtained from the authors. K_m, k_3, and k_4 values for numeri-
FIG. 5. Progress curves of the reacting species for the scheme shown in the text for a hypothetical model system of C. freundii cephalosporinase inactivation by SF2103A. Curves were computed by a modification of the numerical method of Runge-Kutta-Gill (23). The values of $K_r$, $k_{act}$, and $k_{react}$ in Table 1 were used for the values of $K_m$, $k_2$, and $k_3$, respectively, for these computations. Open circles represent experimentally obtained values of residual enzyme activity. In these computations, the initial enzyme concentration was 1 $\mu$M and the initial SF2103A concentration was 1 $\mu$M (A), 2 $\mu$M (B), or 5 $\mu$M (C).

The scheme described above is similar to the mechanism for inactivation of Enterobacter cloacae P99 cephalosporinase by aztreonam (2) which acts as a tight-binding competitive inhibitor of this enzyme. However, inactivation of C. freundii cephalosporinase by aztreonam was not detectable. In contrast, SF2103A showed strong inhibition only against cephalosporinases. It was readily hydrolyzed by TEM-2 penicillinase (2). In contrast, SF2103A showed strong inhibition against penicillinases and also against the broad-spectrum cephalosporinase of P. vulgaris, whereas it showed only reversible inactivation against C. freundii cephalosporinase. In preliminary experiments, aztreonam did not show progressive inactivation of P. vulgaris cephalosporinase (unpublished data). This apparent complementarity between the inactivation spectrum of SF2103A and that of aztreonam is of interest in connection with their similarity in inactivation mechanisms.

SF2103A itself has only moderate antibacterial activity (11). Therefore, in practical use, it may be utilized as a $\beta$-lactamase inactivator in combination with other $\beta$-lactamase-sensitive $\beta$-lactams. Thus, a synergistic effect of SF2103A on the antibacterial activity of the combined $\beta$-lactams against $\beta$-lactamase-producing bacteria could be of practical importance. When SF2103A was combined with cefazolin, cefoperazone, cefotaxime, or cefotizoxime, synergy was expressed by the fractional inhibitory concentration index, the minimum fractional inhibitory concentration values for E. coli W3630 (RGN823), P. vulgaris GN76C-1, and C. freundii GN346 were 0.05 to 0.19, 0.05 to 0.50, 0.003 to 0.02, and 0.13 to 0.62, respectively (unpublished data). These strains are high producers of $\beta$-lactamases (22). Thus, as a $\beta$-lactamase inhibitor, SF2103A was most effective against the P. vulgaris strain. It appeared moderately effective against the E. coli strains producing penicillinase and weakly effective against the C. freundii strain. The lower synergism of SF2103A against the C. freundii strain may be attributed to the rapid reversibility of cephalosporinase inactivation. Moderate effectiveness against the penicillinase-producing strains may be due to the low affinity of SF2103A for the penicillinases although SF2103A is a strong progressive inactivator for these penicillinases.

Our observations indicate that SF2103A is an effective inhibitor against a wider variety of $\beta$-lactamases than is either clavulanic acid or sulbactam and that SF2103A has a different mechanism of $\beta$-lactamase inactivation.

LITERATURE CITED