A comparison of aztreonam and imipenem induction of Class I \(\beta\)-lactamase in *Enterobacter cloacae* ATCC 13047

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Aztreonam and imipenem were shown to induce Class I \(\beta\)-lactamase in *Enterobacter cloacae* ATCC 13047 to a similar extent. Quantitatively, however, aztreonam was far less efficient as an inducer than imipenem. Optimum induction by aztreonam required a concentration of 200 mg/l, which was 800-fold greater than the concentration of 0.25 mg/l of imipenem which resulted in the optimum induction. The differences in the concentrations of aztreonam and imipenem that gave optimum induction were related to the inherent antibacterial activities of the antibiotics when these were determined under the conditions of broth culture. The \(\beta\)-lactamase activity of sonicated cell samples following induction was inhibited by the presence of aztreonam but not by imipenem. The inhibition was overcome by first washing the cell samples from induced cultures and then incubating the sonicates for a prolonged period at 4°C. It is proposed that the phenomena of an optimum inducing concentration and the interference with the assay of \(\beta\)-lactamase by the presence of residual antibiotic demonstrated in this study with aztreonam and imipenem would be of relevance when applied more broadly to studies of \(\beta\)-lactamase induction. In particular these would have a profound effect on the results of studies which attempt to compare the efficacy of \(\beta\)-lactams as inducers of Class I \(\beta\)-lactamase.

Introduction

Sykes & Bonner (1985) and Then (1987) were unable to demonstrate that aztreonam induced Class I \(\beta\)-lactamase in *Enterobacter cloacae*. Farmer & Reading (1987) obtained similar results and also demonstrated quantitative differences in induction of \(\beta\)-lactamase with other \(\beta\)-lactams at empirically selected concentrations of 10 \(\mu\)M and 100 \(\mu\)M. As well as demonstrating a difference in induction of \(\beta\)-lactamase at equivalent concentrations of the \(\beta\)-lactams used in the study Farmer & Reading (1987) found that with some antibiotics induction of \(\beta\)-lactamase at a concentration of 100 \(\mu\)M was less than that at a concentration of 10 \(\mu\)M. Similar results were demonstrated previously by Minami *et al.* (1980) with the group of \(\beta\)-lactam antibiotics that they tested, and they noted also that with some \(\beta\)-lactams at a concentration of 1000 mg/l induction of \(\beta\)-lactamase was less than that at a concentration of 100 mg/l of the same antibiotic. With a variety of \(\beta\)-lactams at either a quarter of their minimum inhibitory concentration (MIC), as determined by agar dilution, or at a concentration of 100 mg/l, Sanders &
Sanders (1986) also found quantitative differences in induction of β-lactamase. Together, these observations suggest that for each β-lactam there is a concentration at which induction of β-lactamase is optimum and although it is possible that this concentration may relate to the inherent antibacterial activity of the β-lactam it may not necessarily relate to the MIC as determined by agar dilution. If the concentration of a β-lactam is critical, one possible explanation for the previous failures to demonstrate that aztreonam induced β-lactamase may be that the empirically selected concentrations of the antibiotic did not approach the level necessary to achieve optimum induction.

Another explanation for the failure to demonstrate induction of β-lactamase with aztreonam may relate to its capacity to bind β-lactamase. The results of Farmer & Reading (1987) and Sanders & Sanders (1986) showed that with cultures of E. cloacae exposed to aztreonam, the β-lactamase activity was less than that of cultures grown in the absence of aztreonam. These results support the suggestion of Bush & Sykes (1986) that the binding of β-lactamase by the inducer may mask β-lactamase activity. In order to overcome this effect Bush & Sykes (1986) proposed that sonicated samples from induced cultures be either dialysed to remove excess inducer or incubated overnight at room temperature to allow hydrolysis of the inducer. However these methods may fail to demonstrate fully the presence of induced β-lactamase. Farmer & Reading (1987) dialysed their sonicated samples, but were unable to show that aztreonam induced β-lactamase.

In the present study we investigated the effect of aztreonam on induction of Class I β-lactamase in E. cloacae ATCC 13047 and compared this with that of imipenem, which was previously shown to be an efficient inducer of Class I β-lactamase in the same bacterial species (Yang, Livermore & Williams, 1988). A wide range of concentrations of each β-lactam was used to examine the effects on growth and the kinetics of induction of Class I β-lactamase and to determine whether there was a level for optimum induction of β-lactamase. Cell samples were also treated before the assay of β-lactamase to overcome any inhibition of β-lactamase activity by the inducer.

Materials and methods

Bacterial strain

E. cloacae ATCC 13047 was used throughout. This organism was previously shown, by isoelectric focusing, to produce Class I β-lactamase (Gatus, Bell & Jimenez, 1988).

Induction of β-lactamase with aztreonam and imipenem

Twenty-ml volumes of SensitTest Agar (Oxoid, CM 409) in 90 mm diameter Petri dishes (Bunzl Medical and Laboratory Products) were flooded with 0.15 M saline suspensions containing 10⁷ cfu/ml of E. cloacae ATCC 13047 (Bell, 1975). The excess suspensions were drained and, after drying, the plates were incubated aerobically for 20 h at 35°C. Cell suspensions were prepared in 0.15 M saline and the absorbance at 640 nm was adjusted to 1.0 using a Bausch and Lomb Spectronic 20 spectrophotometer.

Ten millilitre volumes of the bacterial suspension were added to 190 ml volumes of Tryptone Soya Broth (Oxoid, CM 129) prewarmed to 35°C in 500 ml capacity Erlenmeyer flasks (Pyrex). The broths were incubated at 35°C in a shaking water bath (Heto Laboratory Equipment) with a throw of 4 cm at 100 rpm. Aztreonam (Squibb) and imipenem (Merck, Sharp and Dohme) at final concentrations ranging from 0.004
800 mg/l were added separately to the broth cultures after 1 h and incubation was then continued for a further 3 h.

**Extraction of intracellular β-lactamase**

*From unwashed cells.* Ten-ml volumes of culture were removed at 30 min intervals following the addition of aztreonam and imipenem and were centrifuged at 2000 g for 5 min. The cell pellets were resuspended in 5-ml volumes of phosphate buffered saline (pH 7·0) and were sonicated on ice for 2 min using a Branson Sonifier Cell Disruptor B 30 at output control 5, 50% pulsed duty cycle.

*From washed cells.* In parallel experiments 10 ml volumes of broth were removed from the cultures at 30 min intervals following the addition of aztreonam and imipenem and the samples were centrifuged at 2000 g for 5 min. The supernatants were discarded and the cell pellets were washed three times in 10-ml volumes of phosphate buffered saline (pH 7·0) by centrifugation at 2000 g for 5 min. The resulting cell pellets were resuspended in 5-ml volumes of phosphate buffered saline (pH 7·0) and were sonicated on ice for 2 min.

**Effects of incubating the sonicates at 4°C**

Immediately after sonication gentamicin (Schering) at a final concentration of 20 mg/l was added to each sonicated sample to inhibit the growth of any cells which might have survived sonication and the samples were then incubated at 4°C. The absorbance at 640 nm, the protein content (assayed by the method of Bradford, 1976), the viable cell counts (method of Miles & Misra, 1938) and the β-lactamase activity of each sample were measured at two-day intervals for up to 14 days.

**Assay of β-lactamase**

The β-lactamase activity of each sonicate was determined using nitrocefin (Glaxo 87/312) as substrate according to the method of O'Callaghan et al. (1972) which was previously modified (Gatus, Bell & Jimenez, 1986). β-Lactamase activity was expressed as μmoles of nitrocefin hydrolysed/min/mg of protein.

**Measurement of the effect of aztreonam and imipenem on growth**

*Minimum inhibitory concentrations on agar.* A standard agar dilution method (Ericsson & Sherris, 1971) with an inoculum of 10⁴ cfu was used to determine the MICs of aztreonam and imipenem.

*Inhibitory concentrations in broth cultures.* The growth-inhibitory effects of aztreonam and imipenem under the conditions of broth culture that were used for induction experiments were determined by removing 3-ml volumes of broth from the cultures at 30 min intervals and measuring their absorbance at 640 nm with a Bausch and Lomb Spectronic 20 spectrophotometer.

**Results**

**Effect of incubating the sonicates at 4°C**

*Stability of the sonicates.* When cell sonicates were incubated at 4°C for 14 days there was no change in either the absorbance at 640 nm or the protein content and there was no growth of organisms, as was indicated by unchanged viable cell counts.
Figure 1. $\beta$-Lactamase activity related to the period of incubation at 4°C of (□) the sonicates of washed cells and (■) the sonicates of unwashed cells obtained at 120 min from cultures induced at 200 mg/l of aztreonam. Data are mean ± S.E.M. of triplicate experiments.

$\beta$-Lactamase activity of aztreonam-induced washed and unwashed cells. The $\beta$-lactamase activity of the sonicates of washed cells from cultures induced at each concentration of aztreonam progressively increased during the period of incubation at 4°C. An example of the increase in the $\beta$-lactamase activity of the sonicates that were obtained 120 min after induction at 200 mg/l of aztreonam is shown in Figure 1. Activity increased from 2.2 μmoles of nitrocefin hydrolysed/min/mg of protein immediately after sonication to a maximum of 18.5 μmoles of nitrocefin hydrolysed/min/mg of protein by the eighth day of incubation at 4°C. No $\beta$-lactamase activity was detected in the sonicates of unwashed cells induced at any concentration of aztreonam when assayed immediately after sonication or at any period during the 14 days of incubation at 4°C (Figure 1).

$\beta$-Lactamase activity of imipenem-induced washed and unwashed cells. Incubation at 4°C had minimal effect on the $\beta$-lactamase activity of the sonicates that were obtained from imipenem-induced washed and unwashed cells. The $\beta$-lactamase activity of all the sonicates of washed cells that were obtained from cultures induced at all concentrations of imipenem was maximal immediately after sonication and did not increase further during incubation at 4°C. The initial $\beta$-lactamase activity of the sonicates of unwashed cells from cultures induced at each concentration of imipenem was lower than that of the sonicates of washed cells but increased to an equivalent level within 2 days of incubation at 4°C. An example of the increase in the $\beta$-lactamase activity of sonicates
P-Lactamase induction in E. cloacae

Figure 2. β-Lactamase activity related to the period of incubation at 4°C of (□) the sonicates of washed cells and (■) the sonicates of unwashed cells obtained at 120 min from cultures induced at 0.25 mg/l of imipenem. Data are mean ± S.E.M. of triplicate experiments.

of unwashed cells which were obtained 120 min after the addition of 0.25 mg/l of imipenem to the cultures is shown in Figure 2. Activity increased from 15.0 μmoles of nitrocefin hydrolysed/min/mg of protein immediately after sonication to a maximum of 18.8 μmoles of nitrocefin hydrolysed/min/mg of protein by day 2 of incubation at 4°C.

Comparison of β-lactamase activity and the kinetics of induction at different concentrations of aztreonam and imipenem

The β-lactamase activity at various times and at the five most effective inducing concentrations of aztreonam and imipenem is shown in Tables I and II. There was insignificant enzyme activity in cultures exposed to levels equal to or less than 6.25 mg/l of aztreonam and 0.008 mg/l of imipenem. Induction of β-lactamase increased progressively with an increase in the concentration of both antibiotics and peaked at 200 mg/l of aztreonam and at 0.25 mg/l with imipenem. There was a fall in the enzyme activity as the concentration of each antibiotic was increased from the optimum level for induction up to the almost completely inhibitory level of 800 mg/l for aztreonam and 1 mg/l for imipenem. Although the maximal levels of induction of β-lactamase achieved with aztreonam and imipenem were similar (Figure 3) the concentrations of the two antibiotics with which this was achieved were considerably different.
Table I. β-Lactamase activity of washed cell sonicates of *E. cloacae* ATCC 13047 related to the period of induction with different concentrations of aztreonam. The absorbance at 640 nm of cultures 3 h after the addition of aztreonam is also shown (results are the mean of triplicate experiments)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Aztreonam concentration (mg/l)</th>
<th>β-lactamase activity*</th>
<th>Abs640 at 3 h*</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>50</td>
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<tr>
<td>180</td>
<td>0-04</td>
<td>3-8</td>
<td>4-7</td>
</tr>
<tr>
<td>Abs 640 at 3 h*</td>
<td>1-1</td>
<td>0-97</td>
<td>0-97</td>
</tr>
</tbody>
</table>

*μmoles of nitrocefin hydrolysed/min/mg of protein.

With both antibiotics at their optimum concentration for induction the β-lactamase activity rose rapidly during the first 60 min to reach a peak at 120 min. Thereafter the β-lactamase activity rapidly declined and by 180 min was well below the peak level observed.

**Effects of aztreonam and imipenem on growth**

**MICs on agar.** The MIC of aztreonam was 2 mg/l and that of imipenem was 0-25 mg/l when determined by an agar dilution method.

Table II. β-Lactamase activity of washed cell sonicates of *E. cloacae* ATCC 13047 related to the period of induction with different concentrations of imipenem. The absorbance at 640 nm of cultures 3 h after the addition of imipenem is also shown (results are the mean of triplicate experiments)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Imipenem concentration (mg/l)</th>
<th>β-lactamase activity*</th>
<th>Abs640 at 3 h*</th>
</tr>
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</tr>
<tr>
<td>Abs 640 at 3 h*</td>
<td>1-1</td>
<td>1-0</td>
<td>1-0</td>
</tr>
</tbody>
</table>

*μmoles of nitrocefin hydrolysed/min/mg of protein.

*Initial absorbance at 640 nm of cultures = 0-15.
\[ \beta-Lactamase \] induction in \textit{E. cloacae} 

Figure 3. \( \beta \)-Lactamase activity of the sonicates of washed cells related to the period of induction with aztreonam (■) at a concentration of 200 mg/l and (□) imipenem at a concentration of 0.25 mg/l. Data are mean ± S.E.M. of triplicate experiments.

Inhibitory concentrations in broth cultures. With each increase in the concentrations of aztreonam above 100 mg/l and imipenem above 0.125 mg/l there was a progressive decrease in the absorbance at 640 nm of the broth cultures when measured 3 h after the addition of the antibiotics (Tables I and II). The addition of 800 mg/l of aztreonam to broths inhibited growth of the culture to the extent that the absorbance at 640 nm at 3 h was similar to that which was observed before the addition of the antibiotic. An almost similar inhibition of growth was observed after the addition of 1 mg/l of imipenem.

Discussion

In all previous studies aztreonam was found to induce Class I \( \beta \)-lactamase poorly or not at all in \textit{E. cloacae} (Sykes & Bonner, 1985; Sanders & Sanders, 1986; Farmer & Reading, 1987; Then, 1987). In the present study by extending the concentrations of antibiotics used and removing the residual inhibitory effect of the inducer on \( \beta \)-lactamase activity it was shown that aztreonam and imipenem can induce Class I \( \beta \)-lactamase in \textit{E. cloacae} ATCC 13047 to a similar extent. However at identical concentrations aztreonam was far less efficient as an inducer than imipenem. Optimum induction by aztreonam required a concentration of 200 mg/l. This was 800 fold greater than the concentration of 0.25 mg/l of imipenem which resulted in optimum induction.
The differences in the concentrations of aztreonam and imipenem which were required to give optimum induction of β-lactamase were closely related to the differences in the inherent antibacterial activities of the antibiotics only when these were determined under the conditions of broth culture and not by the conventional method of agar dilution MIC. However the differences between the inherent antibacterial activities of the antibiotics under the conditions of broth culture did not resemble those observed when the relative activities were assessed by agar dilution MIC determination. In the system used to study induction of β-lactamase the antibacterial activity of aztreonam was 800 times less than that of imipenem whereas on agar with an inoculum size of $10^4$ cfu the antibacterial activity of aztreonam was eight-fold less than that of imipenem.

Farmer & Reading (1987) dialysed cell sonicates in an attempt to overcome inhibition of β-lactamase activity by aztreonam but were unable to demonstrate that aztreonam induced β-lactamase. Sykes & Bonner (1985) and Sanders & Sanders (1986) used washed cell samples from aztreonam-induced cultures but they too were unable to demonstrate induction of β-lactamase. In the present study the inhibition of induced β-lactamase activity by aztreonam was overcome by using sonicates of washed cells and incubating these at 4°C for a prolonged period. During this time the increase in β-lactamase activity was most probably due to the slow release of the enzyme from aztreonam to which it was bound. Low concentrations of aztreonam were shown to inhibit Class I β-lactamase activity (Barry et al., 1985) and the rate of hydrolysis of aztreonam by β-lactamase was found to be extremely slow (Sykes & Bonner, 1985). The mechanism of inhibition of β-lactamase by a relatively stable attachment to aztreonam was also demonstrated in the present study by the failure to remove the inhibitory effect of the antibiotic by washing alone. Although with the technique used aztreonam was diluted at least $10^{-6}$ by the process of washing, without prolonged incubation at 4°C there was still an appreciable inhibition of β-lactamase activity.

When the findings of the present study are applied broadly they suggest that there is a concentration of an antibiotic that will optimally induce β-lactamase in E. cloacae and it may be necessary to determine this concentration by extensive titration. If the concentrations of antibiotics are simply selected empirically or on the basis of an agar-dilution MIC there may be difficulty in demonstrating optimum induction of β-lactamase in broth culture. This difficulty may be compounded if the residual but variable inhibitory effect of different inducers is not overcome by washing and incubation at 4°C. These findings will also be of particular relevance to those studies which attempt to compare the efficacy of β-lactams as inducers of Class I β-lactamase.

References


\textbf{\textit{\beta-Lactamase induction in \textit{E. cloacaec}}}


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