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Induction of β-Lactamase Production in *Pseudomonas aeruginosa* Biofilm

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Imipenem induced high levels of β-lactamase production in *Pseudomonas aeruginosa* biofilms. Piperacillin also induced β-lactamase production in these biofilms but to a lesser degree. The combination of β-lactamase production with other protective properties of the biofilm mode of growth could be a major reason for the persistence of this sessile bacterium in chronic infections.

Foreign-device-associated infections and chronic *Pseudomonas aeruginosa* bronchopulmonary infection in patients with cystic fibrosis are not eradicated despite normal immune defenses and aggressive antibiotic treatment, even when an apparently susceptible strain of *P. aeruginosa* is cultured from a patient prior to and during treatment. One important resistance factor is the inherent resistance of bacterial microcolonies and biofilms to antibiotics, antibodies, and phagocytic leukocytes (2, 5). Another important resistance factor is the production of chromosomal class I β-lactamase by the infecting organism (3, 6, 11). The interplay of β-lactamase production and the biofilm mode of growth could be a major reason for the persistence of this bacterium. The purpose of this study was to investigate the abilities of imipenem (Merck Sharp & Dohme, Copenhagen, Denmark) and piperacillin (Lederle, Copenhagen, Denmark) to induce β-lactamase production in cells of *P. aeruginosa* growing in biofilms.

P. aeruginosa (nonmucoid [5]) isolated from the sputum of a patient with cystic fibrosis was used for biofilm production. The isolate was fully susceptible to imipenem and piperacillin (MIC < 3.1 μg/ml).

A log-phase culture of *P. aeruginosa* was pumped at a rate of 40 ml/h through a modified Robbins device at 22°C to establish biofilms on silicone disks (5, 9). After 72 h, inducer was added to the modified Robbins device systems at concentrations as follows for 16-h β-lactamase induction: imipenem, 350, 100, 5, and 0.25 μg/ml; and piperacillin, 100, 50, 5, and 0.5 μg/ml.

The disks bearing induced or noninduced biofilms were removed, rinsed with saline to remove free bacteria, and used for β-lactamase quantitation, protein determination, and bacterial enumeration. The biofilm was scraped off the disks, mixed into 2 ml of saline, and sonicated for 10 min at 60 W (Bransonic; Branson Europa B.V., Soest, Holland), and the bacteria were counted by plating and microscopy. Control experiments showed that this kind of sonication procedure did not reduce the viability of the bacteria (5).

The susceptibility of suspensions of biofilm bacteria to piperacillin and imipenem, prior to and after induction, was investigated by the agar diffusion method (Neosensitabs; Rosco, Copenhagen, Denmark).

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β-Lactamase was released by freeze-thawing of the disks bearing induced or noninduced biofilms, and the activity of the suspended homogenized biofilms was quantitated by a direct spectrophotometric method using a dual-beam spectrophotometer (UV 160; Shimadzsu, Kyoutu, Japan) with nitrocefin (Becton Dickinson, Cockeysville, Md.) as the substrate (4, 10). Sonication was found to yield lower values of activity and was therefore not used as a method of releasing the β-lactamase (data not shown). For protein investigation, the freeze-thawed biofilm suspension was sonicated on ice for six periods of 30 s in the assay buffer (phosphate buffer, pH 6.9). Protein content was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Federal Republic of Germany). Basal β-lactamase activity in planktonic *P. aeruginosa* cells removed from the disks prior to and after induction (22°C) was also determined. Cells were induced for 2.5 h (benzylpenicillin, 500 mg/ml), washed once in the assay buffer, and sonicated in iced water. The supernatants of the sonicated extracts were used for protein and β-lactamase determinations (3).

β-Lactamases were focused in Ampholine PAG plates (LKB Instruments, Bromma, Sweden). The positions of enzyme bands were demonstrated with nitrocefin. For inhibition studies, filter paper soaked with inhibitor, clavulanic acid (0.1 mM), and cloxacillin (0.1 mM) was placed on the surface of the focused gel. After 20 s, the paper was removed and the nitrocefin overlay was applied (12).

For statistical analysis, the Wilcoxon test (two tailed) for paired data was used.

Figure 1 shows the results of β-lactamase activity in biofilm prior to and after induction. β-Lactamase activity in biofilm increased eightfold to high values of 1,860 nU (median) when induced with 5 to 350 μg of imipenem per ml. When 0.25 μg of imipenem per ml was used as the inducer, a significantly lower ($P < 0.03$) increase in median β-lactamase activity was found (from 230 to 720 nU). The highest median value of β-lactamase activity in biofilms (1,860 nU) was significantly lower ($P < 0.02$) than the highest median value achieved in fully induced cells of *P. aeruginosa* growing planktonically (2,400 nU) (Fig. 1).

Piperacillin also induced a significant increase in β-lactamase production (two- to fourfold) in the biofilm bacteria ($P < 0.02$), but its inducing activity at high concentrations was significantly lower than that of imipenem ($P < 0.02$).

No detectable β-lactamase activity was found in the

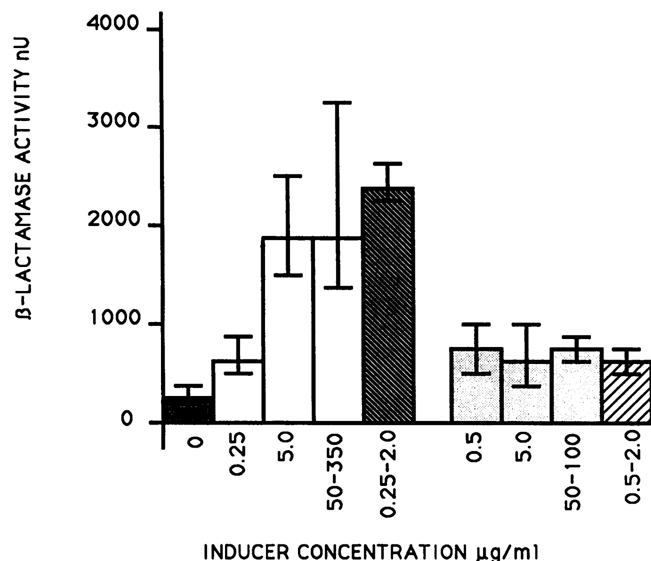

FIG. 1. β-Lactamase induction in *P. aeruginosa* biofilms and planktonically grown cells of *P. aeruginosa*. β-Lactamase activity is reported as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein at 37°C (nU). Each column represents the median value ($n \geq 5$). Error bars show ranges. ▤, Basal β-lactamase activity in biofilms; ▭, imipenem-induced β-lactamase activity in biofilms; ▦, piperacillin-induced β-lactamase activity in biofilms; ▧, imipenem-induced β-lactamase activity in planktonically grown cells of *P. aeruginosa*; ▨, piperacillin-induced β-lactamase activity in planktonically grown cells of *P. aeruginosa*.

supernatant from the outflow of the modified Robbins device (<5 nU) in any of the induction experiments.

The β-lactamase produced by the induced *P. aeruginosa* biofilm showed a single band visualized at pH 7.6. The β-lactamase activity was inhibited by cloxacillin but not by clavulanic acid, indicating a chromosomal class I enzyme according to Richmond and Sykes (10a). The mean number of CFU at the time of induction was 4.9×10^6 ($\pm 2.5 \times 10^6$) per disk. A significant 1,000-fold decrease ($P < 0.04$) in CFU was seen after induction with 100 and 350 µg of imipenem per ml, whereas no CFU change was seen in the other induction experiments (data not shown). A few bacteria in the biofilms thus survived a concentration of 350 µg of imipenem per ml, and all bacteria survived 100 µg of piperacillin per ml. Phase microscopy showed normal bacterial shape and motility at all the antibiotic concentrations used. Cells suspended from the induced biofilm populations remained fully susceptible to both antibiotics (MIC < 3.1 µg/ml).

Our results demonstrate that imipenem and piperacillin are able to induce β-lactamase production in *P. aeruginosa* biofilms and, therefore, that they must be able to penetrate the biofilms in accordance with the mathematical models which were previously constructed by Nichols et al. to estimate time courses of penetration of tobramycin and cefsulodin into biofilms and microcolonies of *P. aeruginosa* (8). Imipenem was a strong inducer of β-lactamase production in biofilms, even though the highest median β-lactamase activity achieved was lower in biofilms than in induced planktonic bacteria. Even though we did not detect any β-lactamase activity in the supernatants from the outflow of the modified Robbins devices, this does not exclude a transport or diffusion of β-lactamase from the biofilms into the media. Technical differences in preparing the β-lactamases from biofilms and planktonically grown bacteria are also possible causes of differences in the maximum β-lactamase activity. Further studies are needed to clarify this.

Among the newer β-lactams, imipenem is a strong inducer of class I β-lactamases and is normally sufficient to induce maximal β-lactamase production in cells of *P. aeruginosa* growing planktonically in conventional batch cultures (7). This did not occur in the cells in the biofilms.

There are several possible explanations for our results, including slow penetration and/or hydrolysis of the inducer as it passes through the biofilm. It could be hypothesized that the concentration of inducer decreases toward the bottom of the biofilm and that β-lactamase activity varies throughout this matrix-enclosed adherent population.

According to the mathematical models of Nichols et al. (8), calculations predicted that the cefsulodin concentration at the base of a biofilm would rise to 90% of the external concentration in 29 s and 41% at the base of a biofilm of bacteria synthesizing enhanced levels of β-lactamase. A decreased response to the inducer when the bacteria grow within a biofilm, including different growth rates in different layers of the biofilm (1, 13), also could explain such results. Clearly, the answer must await further studies.

Piperacillin also induced β-lactamase production in biofilms to rather high levels, but this agent proved to be a less efficient inducer, as expected (7). *P. aeruginosa* removed from the disks after induction did not exhibit elevated basal β-lactamase production, thus excluding the selection of stable derepressed mutants.

We conclude that the exposure of biofilm cells of *P. aeruginosa* to β-lactamase inducers triggers the production of the β-lactamase enzyme that remains associated with the biofilm. The presence of such levels of β-lactam-degrading enzymes in a biofilm would be expected to afford these sessile cells a large measure of protection from β-lactam antibiotics. The mechanism of this proposed protection was previously described by Nichols et al. (8). The interplay of β-lactamase production with the other protective properties of the biofilm mode of growth (2) could be a major reason for the persistence of these bacteria in chronic *P. aeruginosa* infections in cystic fibrosis patients and foreign-device-associated infections.

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