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# Microbial Sensor for New-Generation Cephalosporins Based in a Protein-Engineered β-Lactamase

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## ABSTRACT

A protein-engineered β-lactamase, constructed by site-directed mutagenesis in *Escherichia coli* (E104M/G238S), and having broadened specificity, was able to degrade cephalosporins of first, second, and third generations. Manipulations of culture conditions allowed an increase in β-lactamase specific activity by up to twofold. The resultant bacteria were used to construct an immersable whole-cell biosensor for the detection of new-generation cephalosporins. Cells were immobilized on agar membranes, which in turn were attached to the surface of a flat pH electrode, thus constituting a biosensor based on the detection of pH changes. The sensor was able to detect second- and third-generation cephalosporins: cefamandole (0.4–4 m*M*), cefotaxime (0.4–3.5 m*M*), and cefoperazone (0.3–1.85 m*M*). Response times were between 3.5 and 11 min, depending on the kind of cephalosporin tested. The biosensor was stable for at least 7 d, time during which up to 100 tests were performed.

**Index Entries:** β-lactamase mutant; cephalosporin sensor.

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## INTRODUCTION

The determination of β-lactamic antibiotics (penicillin and cephalosporins) is very important in fields such as human health and quality control operations in fermentation and food industries. Conventional techniques for penicillin and cephalosporin determination are usually tedious; therefore, simple, fast, reliable, and highly specific determination methods for these antibiotics are needed. An alternative has been the use of biosensors. The biosensors constructed for β-lactamic antibiotics have been based in the use of either immobilized enzymes or cells, and then combined with an electrochemical sensor. The principle of operation is the use of β-lactamases, which are specific for penicillins and cephalosporins. The enzymatic reaction produces penicilloic and cephalosporinic acid, respectively. An electrochemical sensor detects the pH change produced by the reaction, which is proportional to the antibiotic concentration. Several enzyme electrodes for penicillin, using immobilized β-lactamase in combination with pH sensors, have been reported in the literature *(1–14)*. Optical fibers have been used as well in combination with immobilized β-lactamase *(15–19)*. Thermistors and field-effect transistors have also been reported for β-lactamic antibiotic detection *(11,12,20,21)*.

In all previous reports, wild-type β-lactamases (TEM-1) have been used, therefore restricting the detection to the relatively limited substrate affinity exhibited by these enzymes. Recently, advances in protein engineering techniques have made possible the design of new β-lactamases *(22–24)*, which can be used for the construction of biosensors having new, exceptional properties. Nicolini *(25)* has reviewed the possibilities offered by protein design in biocatalysis and for the construction of molecular biodevices, namely, biotransistors and biosensors. To the authors' knowledge, this is the first report on the construction of a biosensor for second- and third-generation cephalosporins based on a protein-engineered enzyme, which exhibits a wider substrate spectrum.

The biosensor was based on the immobilization of *Escherichia coli* cells harboring a recombinant plasmid with a double-mutant β-lactamase gene (E104/G238S), attached to the vicinity of a combination flat pH electrode. This strain was obtained using random combinatorial mutagenesis of a TEM β-lactamase *(23,24)*. The constructed biosensor is able to measure, by direct immersion in an antibiotic solution, cephalosporins of first, second, and third generation in wide linear ranges in less than 10 min. The results of the double-mutant β-lactamase biosensor for new-generation cephalosporins were also compared with a biosensor constructed using a TEM β-lactamase of a wild-type *E. coli*.

## MATERIALS AND METHODS

### Strains and Culture Conditions

The double mutant β-lactamase, present in a plasmid (E104M/G238S), was constructed by site-directed mutations introduced via polymerase chain reaction procedures, as described previously *(23,26)*. The double-mutant E104M/G238S exhibited a higher hydrolytic rate against cefotaxime, compared with the corresponding single mutation. A nearly 1000-fold higher $k_{cat}/K_m$ value was observed for the double mutant when compared with that of the wild-type *(24)*. For comparison purposes, a wild-type *E. coli* (which produces a TEM-1-type β-lactamase) was used as well.

Fresh colonies of the mutant and the wild-type *E. coli* were grown in 500-mL Erlenmeyer flask containing 150 mL of Luria-Bertani broth (10 g/L triptone, 5 g/L yeast extract and 10 g/L NaCl, and containing 25 μg/mL kanamicin) in a shaker for 12–14 h, at 250 rpm and 30°C. A second culture condition was used to improve the specific activity (SA) of the biocatalyst using a modified Luria-Bertani broth containing 5 g/L triptone; 2.5 g/L yeast extract and 5 g/L NaCl.

### Biocatalyst Construction and Electrodes Assembly

The cell content in 25 mL of broth, harvested after 3 h of culture, and centrifuged (10 min, 27,200*g*), was resuspended in 1 mL of agar solution (2%) at 65 ± 5°C. The cells and agar were mixed immediately to avoid solidification, and 80 μL of this solution (equivalent to 2.06 ± 1.0 mg of cells) were placed on the surface of a combination flat pH electrode (Orion, Cambridge, MA, 8135 BN). This mixture was maintained in contact with the electrode for 2 h at room temperature, until the membrane was dry. To avoid damage to this membrane, it was covered with a cellophane membrane (26 μm in thickness, International Dental, Mexico City, México). This membrane system was fixed to the pH electrode with an O-ring, and secured with parafilm. Although extreme care was taken in constructing the membranes, it was practically impossible to make two identical membranes. Differences inherent to the membranes (probably its actual wet thickness) caused different starting pHs in the experiments. However, this was not a problem because the relevant parameter was the pH drop, not the absolute value of the pH.

The biosensors, using the double-mutant strain and the wild-type strain, were connected to a potentiometer (Orion, 920-A), and allowed to stabilize in phosphate buffer (0.1 *M*, pH 7.0) for 2 h. This buffer molarity was chosen because, in previous experiences *(13)*, the most reliable

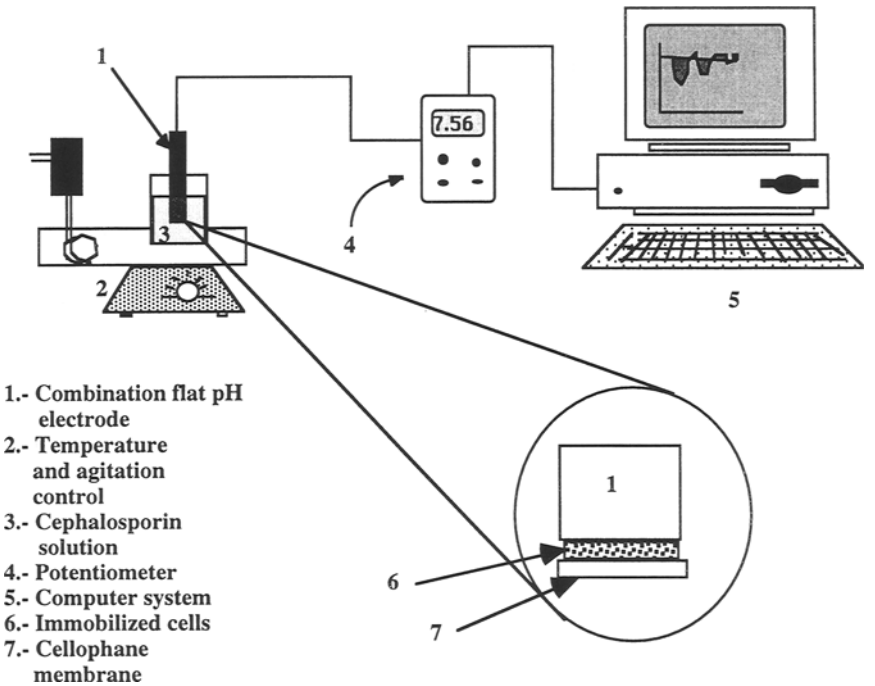


Fig. 1. Experimental setup of the cephalosporins microbial sensor.

response, as well as the widest linear range, were obtained for a microbial penicillin biosensor. The potentiometer was used in combination with a personal computer (AcerMate, Acer, Mexico City, Mexico, 286/16) and a Quickbasic software, to log and display the readings of pH every 3 s. Figure 1 shows, schematically, the experimental arrangement.

## Assay Procedures

β-lactamase activity of the culture was determined following the procedure described by Ross and O'Callaghan (*27*). Biomass concentration was estimated by optical density at 620 nm and correlated to dry wt by a standard curve. One unit of cephalosporinase was defined as 1 nmol of cephaloridine degraded per min.

## Biosensor Characterization

The biosensor was allowed to reach a stable pH value in the buffer solution free of cephalosporin. This procedure was repeated after every determination. When the buffer solution reached a stable pH value, different cephalosporin stock solutions (in a final concentration range of 0.1–4

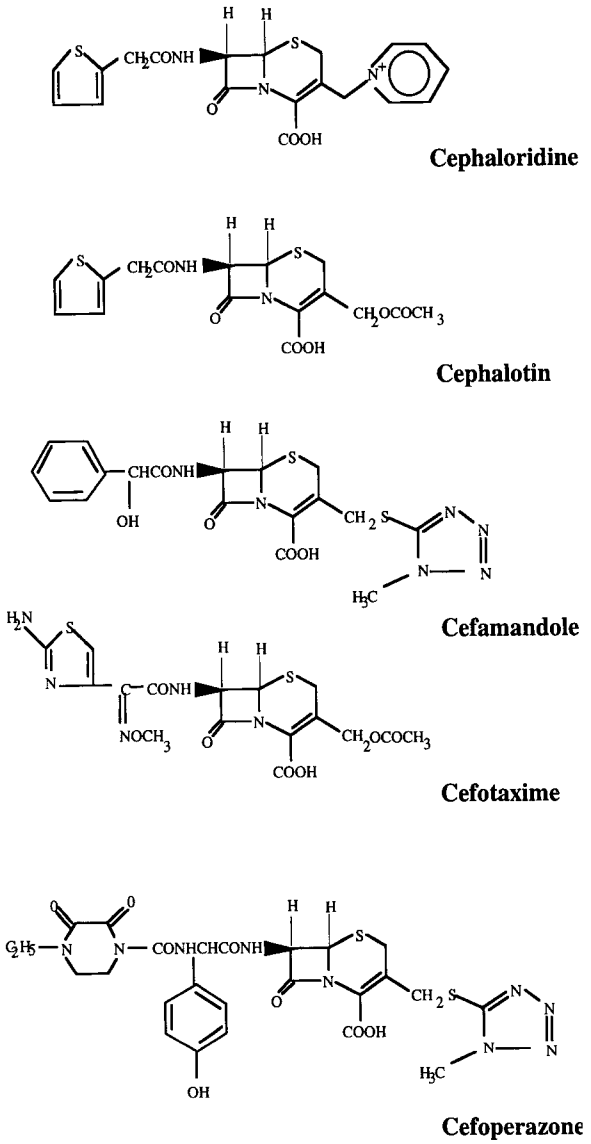


Fig. 2. Chemical structures of the cephalosporins used.

m*M*) were added to the buffer (phosphate, 0.1 *M*, pH = 7.0), in which the biosensor was immersed. Cephaloridine, cephalotin, cefamandole, cefotaxime, and cefoperazone were obtained from Sigma (St. Louis, MO), and their chemical structures are shown in Fig. 2.

All measurements were conducted in a thermostatic bath (Wesstechnick, GMBH, ET5), at 37 ± 0.5°C in 50-mL beakers containing a stirred magnet at 100 rev $min^{-1}$. All chemicals used were of reagent grade, and were prepared with distilled water.

The response curves of the system were stored and displayed in a personal computer. Response curves typically showed two stages: one characterized by the decrease and leveling of the pH signal, and a second one showing the recovery of the original signal. The response time is defined as the time required by the biosensor to reach a plateau after the cephalosporin injection. The recovery time is defined as the time required by the biosensor to reach the original stable pH value in the buffer solution free of cephalosporin. Calibration curves were prepared from the response curves, taking into consideration the difference between the stable pH value and the steady-state pH after every cephalosporin solution was injected.

To evaluate the level of noise or false-positive response, membranes were also constructed using heat-treated cells (121°C, 20 min), having no β-lactamase activity.

Working lifetime of the biosensor was determined by making a number of tests with a solution containing cephaloridine (200 μg/mL), and recording the response. Between measurements, the biosensor was left at room temperature and immersed in the phosphate buffer (0.1 *M*, pH 7.0) free of cephalosporin, unstirred.

## RESULTS AND DISCUSSION

### Enzyme Production

In order to find out the β-lactamase activity achievable in shake-flask cultivations, as well as to determine the best harvest time, experiments were conducted in which the evolution of the SA was measured. Such results are shown in Fig. 3A. Cephalosporinase activity increased during the first 6 h of the culture, reaching a maximum value of about 7 U/mg of cells. The synthesis of the enzyme was maximal during the early exponential phase, a similar phenomenon occurring in other *E. coli* (GM-32) β-lactamases *(28)*. The SA of the enzyme dropped after reaching a peak at the sixth hour of culture. This was coincident with the late logarithmic phase, a phenomenon also observed by other plasmid-encoded β-lactamases *(29–31)*. The decrease of the SA can be associated either with lower production rate and/or proteolysis occurring at the end of the batch culture.

Cells harvested at the third hour of the above described culture were used to construct a biosensor, as described in presently experimental materials and methods. A very unstable response was obtained (Fig. 4A), characterized by the inability to reach either a plateau value in the pH (after injection of the cephalosporin) and the basal signal (when the electrode was immersed in a buffer solution with no antibiotic). Evidently, the amount of enzyme present in the gel membrane was not enough to yield

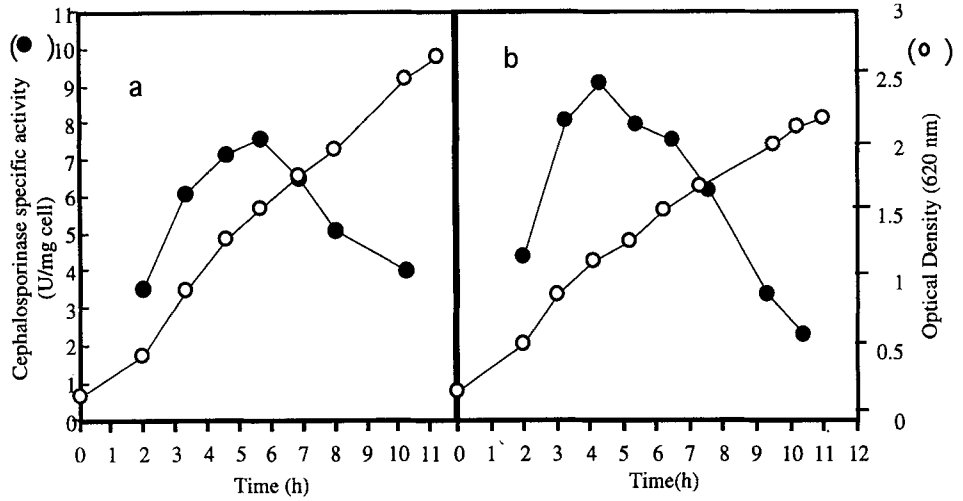


Fig. 3. Evolution of β-lactamase SA and growth curve of *E. coli* E104M/G238S cultured in undiluted **(A)** and diluted **(B)** Luria-Bertani broth in shake flasks.

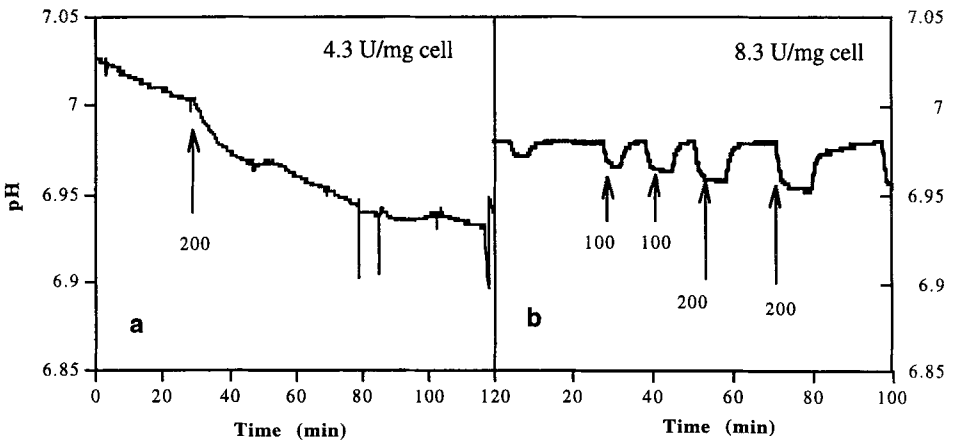


Fig. 4. Response curves obtained with the biosensors constructed using *E. coli* cells grown in undiluted **(A)** and diluted **(B)** Luria-Bertani broth. Numbers indicate the final concentration (in mg/mL) of cephaloridine injected.

a reliable and reproducible response. Therefore, for the cells to be used as the biocatalyst (without further purification of the enzyme), an improvement in the SA was necessary.

The manipulation of culture conditions can improve dramatically the expression of proteins in recombinant strains *(32)*. Making the culture broth poorer in terms of complex nitrogen sources, such as peptone and yeast extract, has been a successful approach for improving the yield of recombinant systems *(33)*. Therefore, a twofold dilution of the Luria-

Bertani broth was assessed. The results are shown in Fig. 3B. The SA profile was very similar to that observed in the undiluted Luria-Bertani broth, i.e., both culture media showed an increase during the early stages of the fermentation, followed by a period in which the specific enzymatic activity decreased. However, higher enzyme production rates were observed when the diluted medium was used. The maximum SA obtained with the diluted medium was 9 U/mg of cells, representing an improvement of 1.3-fold (considering the maximum peaks), and about twofold (if taken after 3 h of culture), if compared with the undiluted Luria-Bertani broth. The use of these latter cells, with higher cephalosporinase activity, in the construction of the biocatalytic gel membrane, allowed the system to produce clearly defined response and recovery curves (Fig. 4B).

## Biosensor Characterization

Figure 5 shows an example of the responses of the biosensor using the TEM-1 β-lactamase and the biosensor using the double-mutant enzyme. As expected, the protein-engineered enzyme hydrolyzes either first- and third-generation β-lactams. At the other hand, the TEM-1 β-lactamase is not able to degrade third-generation cephalosporins as efficiently.

The response curves of the cephalosporins tested are shown in Figs. 6 and 7, and a summary of the biosensor response characteristics is shown in Table 1. As shown in Figs. 6 and 7, the starting pH value was different for the different tests. This is likely to be caused by the difficulty in making two identical constructions (gel/cellulose membranes). The decrease in pH readings reached a plateau as a function of the cephalosporin concentration. These pH drops were reproducible, regardless of the starting pH values.

Figure 6A shows the response curve for cephaloridine (a first-generation cephalosporin), in which the plateau is reached after 3.5 min. This response time is shorter than that (10 min) reported by Matsumoto et al. *(4)*, using a biosensor for this cephalosporin using *Citrobacter freundii* cells having cephalosporinase activity. These authors *(4)* reported a linear range for cephaloridine from 0.12 to 0.72 m*M*. Using the microbial sensor described in this work, a wider linear range (0.4–1.9 m*M*) was achieved (Fig. 8; Table 1).

As shown in Fig. 6B, the plateau pH value for cephalotin was reached after 4.5 min. Matsumoto et al. *(4)* also reported the detection of this first-generation cephalosporin as having a response time of 10 min. The linear range obtained using both the wild-type and the engineered β-lactamase was higher as well (from 0.2 to 2.0 *M*; Table 1; Fig. 6B), if compared with that reported (0.12–0.72 m*M*) by Matsumoto et al. *(4)*. Decristoforo and Danielsson *(20)* reported an enzyme thermistor for the determination of

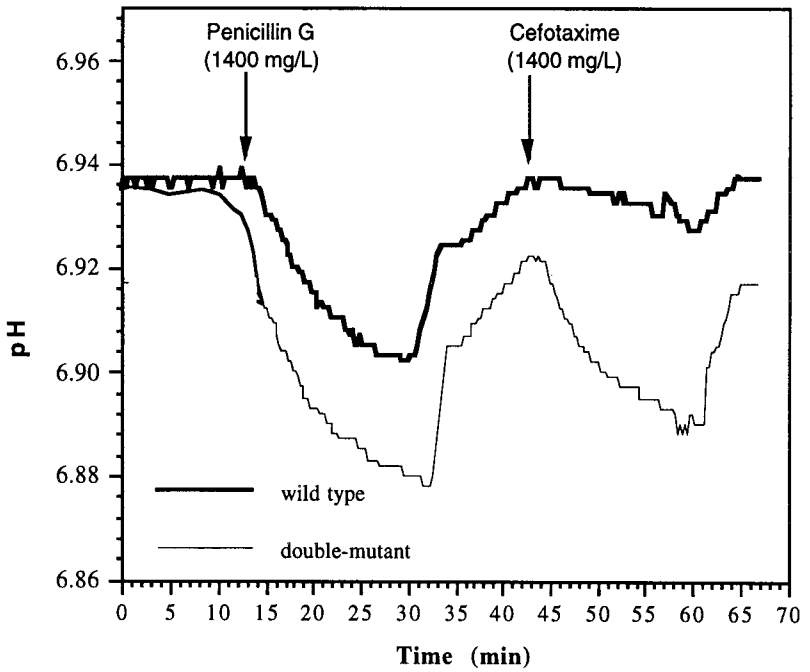


Fig. 5. Response curves obtained with the biosensors constructed with the double-mutant *E. coli* and with the wild TEM-1 *E. coli.*

first-generation cephalosporins. In that work, the linearity was very wide (0.24–24 m*M*), and showed a fast response time (80 s).

It should be pointed out that, in the microbial sensors described here, antibiotic concentrations can be determined directly, without the need for pumping the sample through a relatively large reactor (where the microorganisms or enzymes are immobilized), as was the case of the sensors reported by Matsumoto et al. *(4)* and Decristoforo and Danielsson *(20)*.

When the microbial recombinant sensor was tested with the second-generation cephalosporin cefamandole, the response time was 5 min, and the recovery time was 7 min. (Fig. 6C). The linear response range was 0.4–4 m*M* (Fig. 8). This is the first report on the determination of this type of cephalosporin by a biosensor, and therefore no comparisons can be made.

When the microbial recombinant sensor was tested with third-generation cephalosporins (cefotaxime; Fig. 7A), the response time was 7 min and the recovering time 5 min. Figure 7B shows the results of the response of the biosensor when inactivated cells were used in the construction. Although the signal shows some noise, no significant pH drop was evident, even for high cefotaxime concentrations. Using cefoperazone (data not shown), the response curve was similar to that with cefotaxime. The

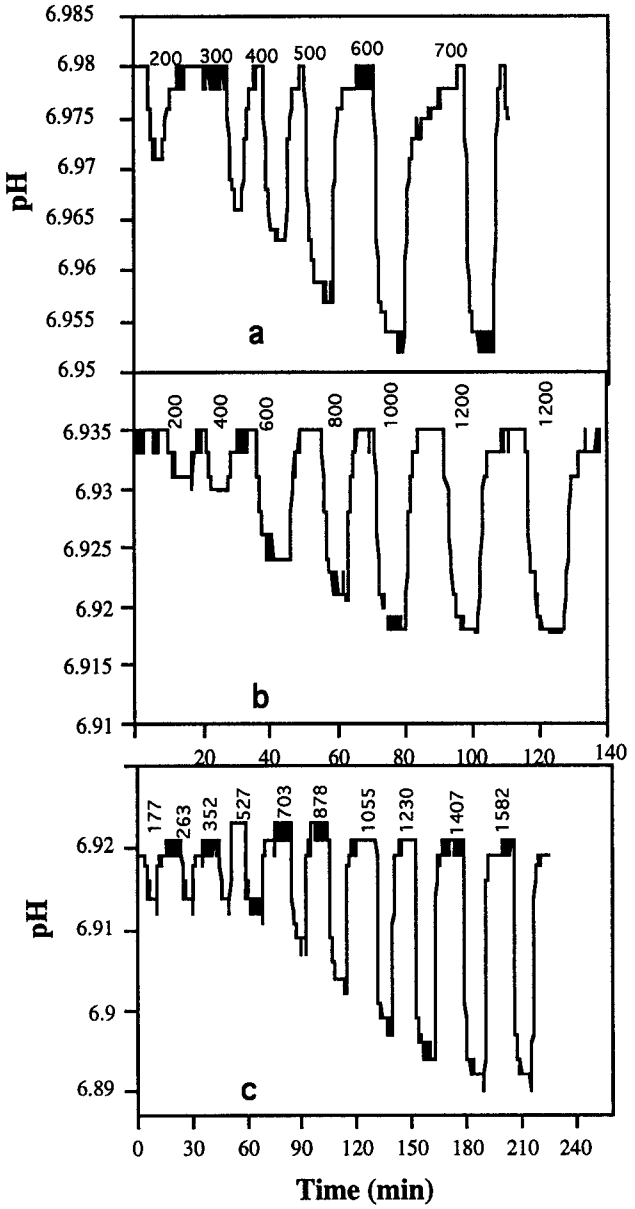


Fig. 6. Typical response curves obtained using different cephalosporins. **(A)** cephaloridine, **(B)** cephalotin, and **(C)** cefamandole. Numbers indicate cephalosporin concentrations in mg/mL.

linear-response range for cefotaxime was 0.4–2.85 m*M*, and, for cefoperazone, 0.3–1.85 m*M* (Fig. 8).

Figure 9 shows that the response of the electrode (measured as the pH difference for a given concentration of cephaloridine) was quite stable for a period of about 7 d, during which more than 100 tests were carried out.

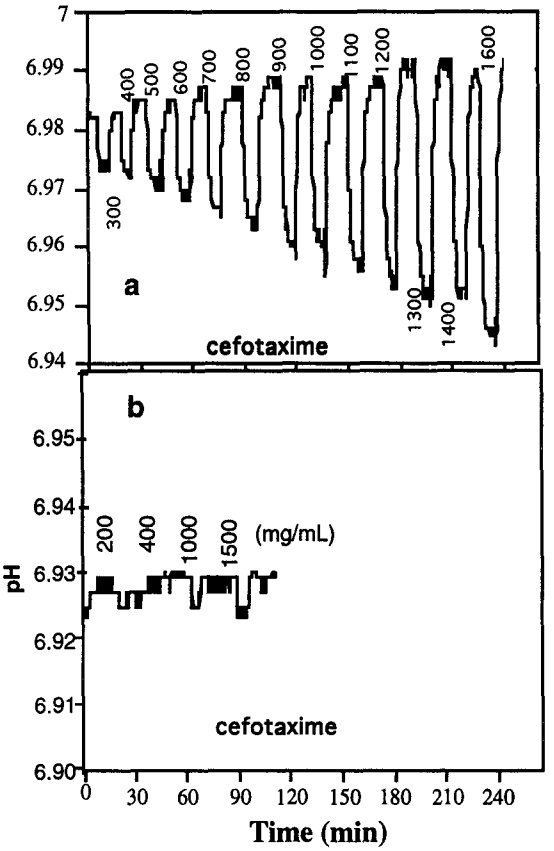


Fig. 7. Typical response curves for cefotaxime using the active biosensor **(A)**, and using a biosensor constructed with heat-treated cells **(B)**. Numbers indicate cephalosporin concentrations in mg/mL.

Table 1
Response Characteristics of the Biosensors

| Cephalosporin | Type of antibiotic (generation) | Response (min) | | Recovery time (min) | | Linear response range (m*M*) | |
|---|---|---|---|---|---|---|---|
| | | $B_{dm}$ | $B_{wt}$ | $B_{dm}$ | $B_{wt}$ | $B_{dm}$ | $B_{wt}$ |
| Cephaloridine | First | 3.5 | 5.0 | 4.5 | 5.0 | 0.4–1.9 | 0.2–2.0 |
| Cephalotin | First | 4.5 | — | 4.5 | — | 0.5–3.0 | — |
| Cefamandole | Second | 7.0 | ND | 7.0 | ND | 0.4–4.0 | ND |
| Cefotaxime | Third | 5.0 | ND | 7.0 | ND | 0.4–3.5 | ND |
| Cefoperazone | Third | 3.5 | ND | 5.0 | ND | 0.3–1.85 | ND |

ND, non detectable; $B_{dm}$, biosensor constructed with the double mutant strain; $B_{wt}$, biosensor constructed with the wild-type strain.

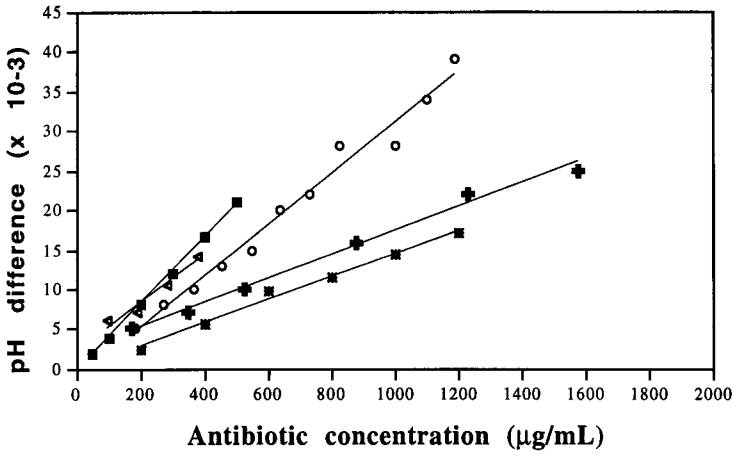


Fig. 8. Calibration curves of the cephalosporins determined with the biosensor, (■) cephaloridine, (Δ) cephalotin, (+) cefamandole, (○) cetotaxime, and (*) cefoperazone.

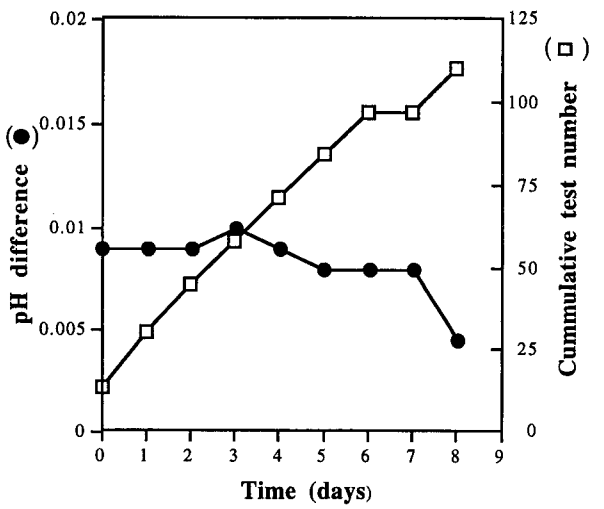


Fig. 9. Operational stability of the microbial sensor constructed with the double-mutant *E. coli*. Tests were conducted using a range of cephaloridine solutions containing from 200 to 700 mg/mL.

Demonstrated here is the possibility of measuring second- and third-generation cephalosporins using a β-lactamase engineered in samples diluted in a buffer solution. Further evaluation of this biosensor in complex media is necessary to determine its usefulness, and to establish possible false-positive response, as reported for other pH-based biosensors *(34–35)*.

## CONCLUSIONS

The microbial sensor described in this work (using a protein engineered β-lactamase having broadened specificity) is able to measure second- and third-generation cephalosporins, including broad-spectrum cephalosporins (like cefotaxime), which is not possible using wild-type β-lactamases. The immobilization procedure of cells was simple, and avoided the use of the purified enzyme.

## ACKNOWLEDGMENTS

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