LOCALIZATION OF CEPHALOSPORINASE IN *Enterobacter cloacae* BY IMMUNOCYTOCHEMICAL EXAMINATION

YOSHIKAZU ISHII[†], MASATAKA ICHIKAWA

Pharmacy Department, Nagasaki University Hospital,
7-1 Sakamoto-machi, Nagasaki 852, Japan

KEIZO YAMAGUCHI[††]

Clinical Laboratory, Nagasaki University Hospital,
7-1 Sakamoto-machi, Nagasaki 852, Japan

KUNIO TAKANO

Department of Oral Histology, Nagasaki University School of Dentistry,
7-1 Sakamoto-machi, Nagasaki 852, Japan

MATSUHISA INOUE

Laboratory of Drug Resistance in Bacteria, Gunma University, School of Medicine,
3-39-22 Shouwa-machi, Maebashi 317, Japan

(Received for publication February 25, 1991)

Enterobacter cloacae NUH10 was isolated at Nagasaki University Hospital in 1987. *E. cloacae* NUH10 is a mutant strain which produces high levels of cephalosporinase. *E. cloacae* ATCC 23355 is known to be sensitive to so-called third generation cephems and produces an inducible cephalosporinase.

The polyclonal antibody to cephalosporinase extracted from *E. cloacae* NUH10 was utilized in post-embedding immunogold labeling in order to localize this protein in *E. cloacae* ATCC 23355 and *E. cloacae* NUH10. Immunocytochemical localization of the cephalosporinase in both strains was observed with and without incubation with an inducer. Cephalosporinase was detected in both the cytoplasm and periplasmic space of *E. cloacae* ATCC 23355 and *E. cloacae* NUH10 incubated in medium including cefoxitin as an inducer. In the case of incubation without the inducer, a small quantity of cephalosporinase was located in the periplasmic space in either strain of bacteria. Western blot analysis showed that cephalosporinase was predominantly localized in the periplasmic space rather than in the cytoplasmic space.

In a previous investigation on the localization of β-lactamase in bacteria, 85% to 90% of the enzyme activity synthesized by *Bacillus cereus* was detected extracellularly[1], while much β-lactamase blocks activity has been found to be restricted to the periplasmic space in Gram-negative bacteria[2]. Enzyme localization is very important for bacterial cells, because β-lactamase blocks β-lactam antibiotics from reaching the penicillin-binding proteins on the inner surface of the bacterial cell wall, and in this way bacteria acquire resistance to such antibiotics[2].

It has been known for about 45 years that β-lactam antibiotics are hydrolyzed by β-lactamases.

Present address: [†] Department of Oral Histology, Nagasaki University School of Dentistry, 7-1 Sakamoto-machi, Nagasaki 852, Japan.
[††] Department of Bacteriology, Toho University School of Medicine, 5-21-16 Ohmorinishi, Ohta-ku, Tokyo 143, Japan.

Accordingly, a number of antibiotics stable against β-lactamases have been developed during the last two decades. Resistance to these new β-lactam antibiotics has now developed in many species of Gram-negative bacteria because of their frequent use in various clinical fields[3~5].

Induction of cephalosporinase, which is produced by Gram-negative bacteria including Enterobacteriaceae, is controlled by bacterial chromosomal gene(s)[6]. Cephalosporinase produced in such bacteria is grouped among the class I β-lactamases[2]. Furthermore, it is known that inducible cephalosporinase can be transformed to production by derepression[3~5], and the mechanism of this has been clearly determined by gene analysis[6~9]. The derepression of cephalosporinase production originates from the mutation of *amp*D or *amp*R, which are chromosomal regulatory genes. Cephalosporinase production by derepressed bacteria is increased 100- to 1,000-fold more than the production of inducible cephalosporinase[10]. This is why many derepressed bacteria show a high resistance to β-lactam antibiotics which are stable against β-lactamase activity.

Trapping of β-lactamase is believed to be another mechanism of resistance to β-lactam antibiotics[5]. This hypothesis was suggested by the detection of β-lactamase in the periplasmic space[11].

Accordingly, precise information about the localization of β-lactamase in bacteria is needed to further study the mechanisms of resistance to β-lactam antibiotics. For this reason, we examined the localization of β-lactamase in Enterobacteriaceae using immunocytochemistry.

Materials and Methods

Bacterial Strains

E. cloacae ATCC 23355 was used as the sensitive strain and *E. cloacae* NUH10 was used as the resistant strain. It was obtained from the clinical laboratory of Nagasaki University Hospital. The organisms were cultured in Mueller-Hinton broth (Difco Lab., Detroit, U.S.A.) or on Mueller-Hinton agar (Difco Lab., Detroit, U.S.A.).

Antibiotics

Antibiotics were obtained from the following sources: Ampicillin was from Meiji Seika Kaisha, Ltd. (Tokyo, Japan); carbenicillin, cefazolin, and ceftizoxime were from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan); cephaloridine, cephalothin, and latamoxef were from Shionigi & Co., Ltd. (Osaka, Japan); cefmetazole was from Sankyo Co., Ltd. (Tokyo, Japan); cefoxitin was from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan); cefotiam, and cefmenoxime were from Takeda Chemical Industries, Ltd. (Osaka, Japan); cefotaxime was from Hoechst Japan Limited (Tokyo, Japan); ceftazidime was from Tanabe Seiyaku Co., Ltd. (Osaka, Japan); aztreonam was from Squibb Japan Inc. (Tokyo, Japan).

Susceptibility Tests

The MIC of the drugs was determined by the agar dilution method. Bacterial strains cultured overnight in Mueller-Hinton broth were diluted to a final concentration of about 5×10^6 cfu per ml with buffered saline containing gelatin. About $3 \sim 4 \times 10^4$ cfu were inoculated onto Mueller-Hinton agar plates containing serial 2-fold dilutions of the antibiotics with a microplanter (Sakura Co., Ltd., Tokyo, Japan). The MIC was defined as the lowest concentration preventing visible growth after incubation for 18 hours at 37°C.

Inducibility of Cephalosporinase Production and Cephalosporinase Assay

For the preparation of bacteria without an inducer, organisms were grown overnight at 37°C in Mueller-Hinton broth, then diluted 10-fold with the same fresh broth. The cells were cultured at 37°C under aerobic conditions for 5 hours on a rotary shaker (type TB-16T, Takasaki Kagaku Ltd., Saitama, Japan). When cefoxitin was used as the inducer, it was added to the medium at a final concentration of

$\frac{1}{2}$ MIC after 2 hours of incubation, and incubation was performed for further 3 hours with shaking. The cells were collected by centrifugation for 10 minutes at 7,000 × *g* at 4°C and washed twice with 0.05 M sodium phosphate buffer (pH 7.0) to remove residual culture medium. After disruption of the cells, crude enzyme solution was obtained from the supernatant produced by centrifugation for 30 minutes at 100,000 × *g* at 4°C. The enzyme activity was determined by spectrophotometric assay[12,13], which involved measuring the decrease in absorbance at 262 nm with cephalothin as the substrate in a temperature-controlled spectrophotometer (30°C) (model U3200, Hitachi Ltd., Tokyo, Japan). One unit of activity was defined as the amount of enzyme that hydrolyzed 1 μmol of cephalothin as a substrate in 1 minute at 30°C. Protein concentration was determined by the method of LOWRY *et al.*[14], using bovine serum albumin Fraction V (Sigma Chemical Co., St. Louis, U.S.A.) as the standard.

Preparation of Periplasmic and Cellular β-Lactamase

The osmotic shock method used was described by NEU and HEPPEL[15]. After centrifugation, cells were washed twice in half the volume of the culture medium with 0.01 M Tris-HCl buffer (pH 8.0). Then 200 mg of cells was resuspended in 8 ml of 40% sucrose - 0.33 M Tris-HCl buffer (pH 7.3). The suspension was shaken for 10 minutes at room temperature in the presence of 0.002 M EDTA. After centrifugation at 12,000 rpm for 10 minutes, the cells were resuspended in the same volume of ice-cold water. This suspension was then shaken for 10 minutes at 4°C, followed by centrifugation for 10 minutes at 12,000 rpm. The supernatant was cautiously removed and the cells were resuspended in the same volume of 0.01 M Tris-HCl buffer (pH 7.3). An aliquot of this suspension was sonicated, the resulting suspension was centrifuged for 10 minutes at 12,000 rpm, and the supernatant was removed. The "periplasmic fraction activity" was defined as the activity found in the supernatant resulting from cold water treatment, while the "cellular fraction activity" was defined as that found in the supernatant resulting from the disruption.

Purification of Cephalosporinase

The crude enzyme solution was applied to a CM-Sephadex ion exchange column (Pharmacia Fine Chemical., Uppsala, Sweden) with 0.005 M sodium phosphate buffer (pH 7.0) and eluted with the 0.05 M sodium phosphate buffer (pH 7.0), by the method described previously[16]. The cephalosporinase activity of the purified enzyme extract was determined by a spectrophotometric technique[12,13]. The enzyme was purified over 120-fold from an extract of *E. cloacae* NUH10. The active fraction showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with more than 95% purity. The eluate of the active fraction was concentrated by ion exchange chromatography and then used as an antigen in further studies.

Cephalosporinase Antiserum

Two mg of purified cephalosporinase in 0.5 ml of saline was emulsified with the same volume of FREUND's complete adjuvant (Nakarai Chemical Co., Ltd., Kyoto, Japan). The emulsion was injected subcutaneously into male Japanese white rabbits, and 3 weeks following the first injection a booster injection containing 0.5 mg of protein was administered intramuscularly. Antiserum was obtained 1 week after the second injection. The crude globulin fraction was prepared by precipitation with $\frac{1}{2}$ saturated ammonium sulfate (Ishizu Pharmaceutical Co., Ltd., Osaka, Japan). Purified rabbit immunoglobulin was collected by application of the crude globulin fraction to an Affi-Gel Protein-A MAPS II Kit (Bio Rad Lab., California, U.S.A.). The specificity of the antibody was investigated by Western blot analysis[17] using whole-cell extract and purified cephalosporinase, and the antibody titer was shown to be 256-fold by the double immunodiffusion method[18]. This experiment was performed at the Laboratory of the Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Western Blot Analysis

Whole-cell extract, the periplasmic fraction, and the cytoplasmic fraction were separated by the method of LAEMMLI[19] in SDS-PAGE using 10% polyacrylamide gels. Following SDS-PAGE, the protein was transferred to clear blot membrane-p (ATTO Ltd., Tokyo, Japan) by the method of TOWBIN *et al.*[17]. The membrane was blocked with 10% bovine serum albumin solution overnight. The membrane was then washed twice for 15 minutes at a time with 0.01 M sodium phosphate buffer (pH 7.4) - 0.02% polyoxyethene

(20) sorbitan monolaurate (Bio Rad Lab.), followed by two more washes for 15-minute in the same buffer with 0.02% Nonidet P-40 (Sigma Chemical Co.). Antibody diluted 1:10 in 1% bovine serum albumin was added, and the blot was incubated at 37°C for 1 hour. The antibody was removed, and the blot was washed again as before. This was followed by the addition of an appropriate concentration of ^{125}I-labeled Protein-A (Amersham Japan, Tokyo) and incubation at 37°C for 2 hours. The blot was washed as described above and autoradiographed at −70°C with X-ray film (Konica Trading Co., Tokyo, Japan).

Preparation of Cells for Electron Microscopy

The cells cultured in Mueller-Hinton broth were centrifuged at 7,000 × *g* for 15 minutes and washed twice in 0.05 M cacodylate buffer (pH 7.4). Cells were then fixed for 4 hours in the fixative which was 0.05 M cacodylate buffer (pH 7.4) plus 2% glutaraldehyde (TAAB Ltd., Aldermaston, U.S.A.) and 3% paraformaldehyde (Nakarai Chemical). After fixation, cells were dehydrated in a graded series of alcohols and embedded in LR white (London Resin Co., London, England).

Immunocytochemistry

Thin sections were collected on uncoated nickel grids (Nishin EM Co., Ltd., Tokyo, Japan). Sections were washed in 0.05 M phosphate buffer (pH 7.2) and then incubated in bovine serum albumin for 30 minutes at room temperature. The sections were then incubated with antibody for 30 minutes at room temperature. After rinsing with the same buffer, these sections were incubated for 30 minutes in Protein A-gold (particle size = 5, 10, or 15 nm) solution (Janssen Life Science Products, Beerse, Belgium). The grids were next rinsed and counter stained with uranyl acetate and lead citrate. Specimens were observed using a Hitachi H-800 electron microscope (Hitachi Ltd.).

Quantitation of Labeling

Gold particles (particle size = 5 nm) were counted manually, and density of labeling was expressed as average number of gold particles per square micrometer of cell surface. The average number of background particles was deducted from the total number of gold particles coating each cell.

Results

Antibacterial Activity

The MICs of cefmenoxime, ceftizoxime and latamoxef for *E. cloacae* ATCC 23355 were each 0.1 μg/ml, while the respective MICs for *E. cloacae* NUH10 were 400, 400 and 25 μg/ml or more (Table 1).

Cephalosporinase Production with or without an Inducer

Without an inducer, the β-lactamase activity of *E. cloacae* ATCC 23355 and *E. cloacae* NUH10 was 0.001 and 0.617 unit/mg protein, respectively. Enzyme activity was increased in both bacterial strains after incubation with cefoxitin. The induction ratio of cephalosporinase in both bacterial strains is shown in Table 2. The specific enzyme activity of *E. cloacae* ATCC 23355 was increased about 12-fold by induction, but only a 1.2-fold increase in activity was seen for *E. cloacae* NUH10.

Western Blots of β-Lactamase Activity

β-Lactamase was found in the whole-cell, and periplasmic fractions obtained from *E. cloacae* NUH10. Both with and without the inducer, the Western blot showed the same pattern (Fig. 1). β-Lactamase activity in the cytoplasmic fraction was very low in comparison with that in the periplasmic fraction (Table 3).

Immunocytochemistry

Fig. 2(A) shows an ultrathin section of *E. cloacae* ATCC 23355 cells incubated without an inducer. One to three Protein A-gold particles per cell showing the localization of cephalosporinase were found in the cytoplasm, and the periplasmic space was not labeled with the antibody. However, after the induction

Table 1. Antibiotic susceptibility of *Enterobacter cloacae* strains against β-lactams.

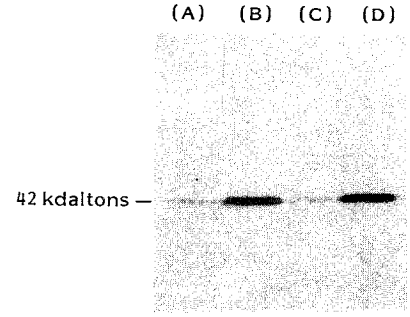
Antibiotics	MICs (μg/ml)	
	E. cloacae ATCC 23355	*E. cloacae* NUH10
Ampicillin	100	>400
Carbenicillin	1.56	200
Cephaloridine	50	>400
Cefazolin	100	>400
Cefmetazole	25	>400
Cefotiam	1.56	>400
Cefotaxime	<0.1	>100
Cefmenoxime	<0.1	>400
Ceftizoxime	<0.1	>400
Latamoxef	<0.1	25
Ceftazidime	<0.1	25
Aztreonam	<0.1	50

Table 2. Inducibility of cephalosporinase production in *Enterobacter cloacae* strains.

Strains	Specific enzyme activity (unit/mg protein)		Induction ratio[a]
	Without inducer	With inducer	
E. cloacae ATCC 23355	0.001	0.012	12.0
E. cloacae NUH10	0.617	0.708	1.2

[a] Induction ratio was calculated as the enzyme activity after induction divided by the activity in the absence of induction.

Fig. 1. Western blots of the whole-cell extract, cytoplasmic fraction, and periplasmic fraction of *Enterobacter cloacae* NUH10.



Twenty μg/ml of protein was applied (10 μl per sample). Film exposure was for 24 hours at −70°C. Lanes: A, cytoplasmic fraction without inducer; B, periplasmic fraction without inducer; C, cytoplasmic fraction with inducer; D, periplasmic fraction with inducer.

Table 3. β-Lactamase activity of cytoplasmic fraction and periplasmic fraction from *Enterobacter cloacae* NUH10.

Strain	Inducer	Enzyme activity (unit/mg of protein)	
		Periplasm (SEM)	Cytoplasm (SEM)
E. cloacae NUH10	+	0.152 (±0.017)	0.005 (±0.001)
	−	0.129 (±0.015)	0.004 (±0.001)

Fig. 2. Electron micrographs of *Enterobacter cloacae* ATCC 23355.

(A) Cephalosporinase was found in the cytoplasmic space (arrowhead) without inducer. Bar = 0.2 μm. (B) Cephalosporinase was found in both the cytoplasmic space (arrowhead) and the periplasmic space (arrow) in the presence of cefoxitin (5 μg/ml) as an inducer of cephalosporinase. Bar = 0.2 μm.

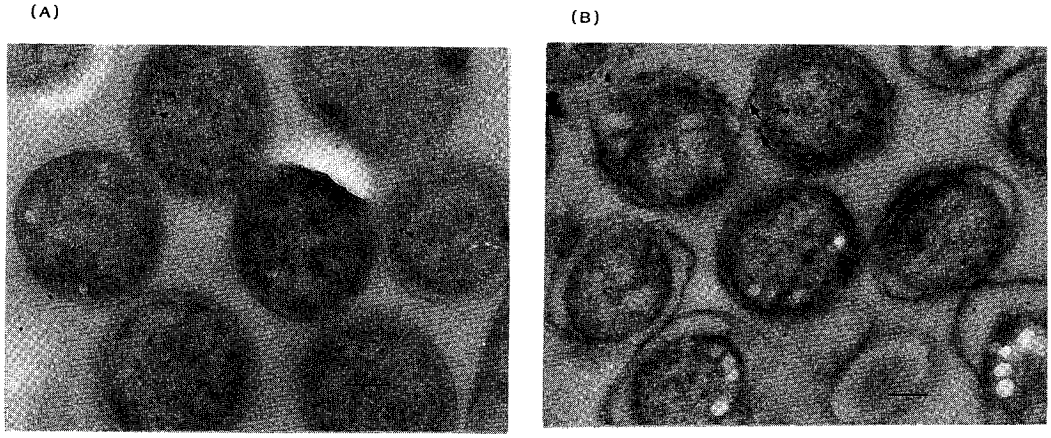


Fig. 3. Electron micrographs of *Enterobacter cloacae* NUH10.

(A) Cephalosporinase was found in both the cytoplasmic space (arrowhead) and the periplasmic space (arrow) without inducer. Bar=0.2 μm. (B) Cephalosporinase was found in both the cytoplasmic space (arrowhead) and the periplasmic space (arrow) in the presence of cefoxitin (50 μg/ml) as an inducer of cephalosporinase. Bar=0.2 μm.

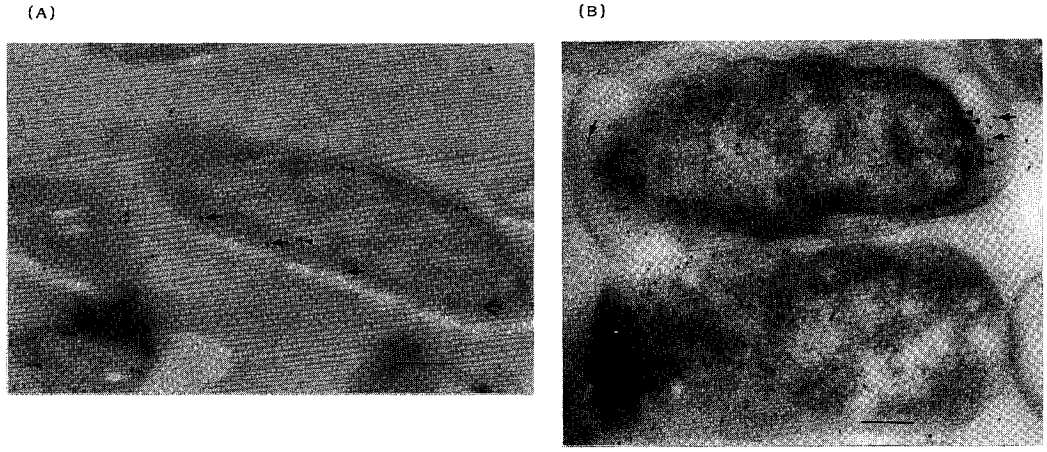


Table 4. Label distribution after treatment of *Enterobacter cloacae* section with cephalosporinase-antibody.

Strains[a]	Inducer	No. of gold particles/μm² of cell profiles (Mean±SEM)[b]		
		Cytoplasm	Periplasm	Total
E. cloacae ATCC 23355	−	2.7±0.3 (104)	0.3±0.1 (104)	3.1±0.3 (104)
	+	3.2±0.6 (90)	6.5±0.3 (90)	10.7±0.5 (90)
E. cloacae NUH10	−	6.5±0.8 (98)	1.5±0.1 (98)	8.0±0.7 (98)
	+	18.4±0.8 (81)	10.1±0.4 (81)	28.5±1.0 (81)

[a] Cells probed for cephalosporinase were embedded in LR white resin.
[b] The parenthesis indicated the number of cells counted.

of cephalosporinase by cefoxitin, gold particles labeled both the cytoplasm and the periplasmic space (Fig. 2B).

In *E. cloacae* NUH10 cells incubated without inducer, numerous gold particles were scattered in the cytoplasm near the inner cell wall, but few were found in the periplasmic space (Fig. 3A). In contrast, numerous gold particles labeled both the cytoplasm and periplasmic space when the bacteria were incubated in the presence of cefoxitin. In addition, more gold particles were observed in the peripalsmic space than in the cytoplasm (Fig. 3B).

Quantitation of Labeling

With *E. cloacae* ATCC 23355 in the periplasmic space, incubation with the inducer increased labeling approximately 22-fold compared with incubation without the inducer. With *E. cloacae* NUH10 in the periplasmic space, incubation with the inducer increased labeling by about 7-fold (Table 4).

Discussion

Immunocytochemical examination is an experimental method that can visualize the cellular proteins[20].

The immunocytochemical distribution of cephalosporinase, as shown by the immunogold technique, is presented in this study. However, the fact that separation and fractionation of the cell contents abolishes the structural coherence of the organism hampers the study of the spatial correlations between the cell envelope and the cytoplasmic contents. To correlate the production of β-lactamase with the resistance of bacteria to antibiotics, a suitable method for the localization of β-lactamase needs to be employed.

If the size of the bacterium *E. cloacae* NUH10 is taken as $0.5 \times 1.0\,\mu m$ and the thickness of the thin section is 0.5 nm, then our rough calculation gives about 8×10^4 molecules of cephalosporinase per cell in *E. cloacae* NUH10 from the electron microscopy data (Table 4). SANDERS and SANDERS, Jr.[10] have reported that derepressed *E. cloacae* strains have 10^5 molecules of cephalosporinase per cell, so our calculated result and that of SANDERS show good agreement.

NEU and HEPPEL[15] have reported the localization of alkaline phosphatase, cyclic phosphodiesterase, β-galactosidase, 5′-nucleotidase, and ribonuclease by the osmotic shock procedure. From experiments using the osmotic shock procedure, cephalosporinase activity was observed in the periplasmic fraction obtained from Gram-negative bacteria[11,21], and was designated as a periplasmic enzyme. However, their evidence[15] suggested that the enzymes which are released occur at or near the cell surface.

In contrast, we detected cephalosporinase in the cytoplasm of both *E. cloacae* ATCC 23355 (inducible) and *E. cloacae* NUH10 (derepressed) after incubation without or with an inducer. These results are in contrast to those of previous reports[11,21], where data were obtained by biochemical methods[15]. Our study also showed that most of the β-lactamase activity was periplasmic and that little of it was cellular. By Western blot analysis, β-lactamase was detected in both the periplasmic and cellular fractions, but the majority of it was localized in the periplasmic fraction.

ROGGENKRAMP *et al.*[22] have reported that the precursor was synthesized on free polysomes *in vivo*, but could be processed by rough endoplasmic reticulum in a cell-free translation system. The relationship between our immunocytochemical results and the results of biochemical procedures may be explained by ROGGENKRAMP's study[22]. We think that biochemical studies detected only mature cephalosporinase activity, while immunocytochemical studies can also detect precursor inside the cellular fraction.

EVERETT *et al.*[23] have reported an induction mechanism of the *Citrobacter freundii* group I β-lactamase. They stated that leaderless β-lactamase was active in the cytoplasmic space. LAMINET *et al.*[24] investigated the effect of GroEL/ES on the folding of β-lactamase and its precursor. Those reports support our deduction that precursor or nascent β-lactamase exists in the cytoplasmic space.

E. cloacae NUH10 produced high levels of cephalosporinase. Such strains were called derepressed or constitutive types. But this strain was not the constitutive type, because cephalosporinase activity was increased about two times in the presence of an inducer such as imipenem (R. OKAMOTO; personal communication). For these reasons, after the induction of cephalosporinase by cefoxitin, numerous gold particles were observed in *E. cloacae* NUH10.

Cephalosporinase was found to be distributed near the cytoplasmic membrane in the periplasmic space by immunocytochemical studies, suggesting that in *E. cloacae* cephalosporinase is synthesized by membrane-associated ribosomes. Furthermore, this study suggested that cephalosporinase activity is exhibited in the periplasmic space as a result of secretion.

Acknowledgments

We are most grateful to MASAKI HIRAOKA, TADASHI FUJII, SOMAY, Y. MURAYAMA and RYOICHI OKAMOTO for their helpful advice with regard to this research.

References

1) ABRAHAM, E. P. & S. G. WALEY: β-Lactamase from *Bacillus cereus*. *In* The Beta-Lactamase. *Ed.*, J. M. T. HAMILTON-MILLER *et al.*, pp. 311～338, Academic Press Inc., 1979
2) RICHMOND, H. M. & R. B. SYKES: The β-lactamase of gram negative bacteria and their possible physiological role. Adv. Microb. Physiol. 9: 31～88, 1973
3) GOOTZ, T. D.; D. B. JACKSON & J. C. SHERRIS: Development of resistance to cephalosporins in clinical strains of *Citrobacter* spp. Antimicrob. Agents Chemother. 25: 591～595, 1984
4) QUINN, J. P.; C. A. DIVINCENZO & J. FOSTER: Emergence of resistance to ceftazidime during therapy for *Enterobacter*

cloacae infections. J. Infect. Dis. 155: 942~947, 1987

5) THEN, R. L. & P. ANGEHRN: Trapping of nonhydrolyzable cephalosporins by cephalosporinase in *Enterobacter cloacae* and *Pseudomonas aeruginosa* as a possible resistance mechanism. Antimicrob. Agents Chemother. 21: 711~717, 1982
6) MURAYAMA, S. Y.; T. YAMAMOTO, I. SUZUKI & T. SAWAI: Mutation of *Escherichia coli* capable of expressing gene(s) for β-lactamase production of *Citrobacter freundii*. Antimicrob. Agents Chemother. 29: 707~709, 1986
7) GALLENI, M.; F. LINDBERG, S. NORMARK, S. COLE, N. HONORE, B. JORIS & J. M. FRERE: Sequence and comparative analysis of three *Enterobacter cloacae ampC* β-lactamase genes and their products. Biochem. J. 250: 753~760, 1988
8) LINDBERG, F. & S. NORMARK: Common mechanism of *ampC* β-lactamase induction in Enterobacteria: Regulation of the cloned *Enterobacter cloacae* P99 β-lactamase gene. J. Bacteriol. 169: 758~763, 1987
9) LINDQUIST, S.; F. LINDBERG & S. NORMARK: Binding of the *Citrobacter freundii* ampR regulator to a single DNA site provides both autoregulation and activation of the inducible *ampC* β-lactamase gene. J. Bacteriol. 171: 3746~3753, 1989
10) SANDERS, C. C. & W. E. SANDERS, Jr.: Microbial resistance to newer generation β-lactam antibiotics: clinical and laboratory implications. J. Infect. Dis. 151: 399~406, 1985
11) BUSH, K. & R. B. SYKES: Interaction of β-lactam antibiotics with β-lactamase as a cause for resistance. *In* Antimicrobial Drug Resistance. *Ed.*, L. E. BRYAN, pp. 1~31, Academic Press Inc., 1984
12) HIRAI, K.; S. IYOBE, M. INOUE & S. MITSUHASHI: Purification and properties of a new β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 17: 355~358, 1980
13) WALEY, S. G: A spectrophotometric assay of β-lactamase action on penicillins. Biochem. J. 139: 780~789, 1974
14) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265~275, 1951
15) NEU, H. C. & L. A. HEPPEL: The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. 240: 3685~3692, 1965
16) MINAMI, S.; M. INOUE & S. MITSUHASHI: Purification and properties of cephalosporinase from *Enterobacter cloacae*. Antimicrob. Agents Chemother. 18: 853~857, 1980
17) TOWBIN, H.; T. STAEHELIN & J. GORGON: Electrophoresis transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76: 4350~4354, 1979
18) OUCHTERLONY, O.: Immunodiffusion and immunoelectrophoresis. *In* Handbook of Experimental Immunology. *Ed.*, D. M. WEIRB, pp. 655~706, Blackwell Scientific Publications, 1967
19) LAEMMLI, U. K: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680~685, 1970
20) BAYER, M. H.; W. KECK & M. E. BAYER: Localization of penicillin-binding protein 1b in *Escherichia coli*: immunoelectron microscopy and immunotransfer studies. J. Bacteriol. 172: 125~135, 1990
21) ITO, K.; P. J. BASSFORD, Jr. & J. BECKWITH: Protein localization in *E. coli*: is there a common step in the secretion of periplasmic and outer-membrane proteins? Cell. 24: 707~717, 1981
22) ROGGENKRAMP, R.; D. HARAL & C. P. HOLLENBERG: Precursor of β-lactamase is enzymatically inactive: Accumulation of the preprotein in *Saccharomyces cerevisiae*. J. Biol. Chem. 260: 1508~1512, 1985
23) EVERETT, M. J.; I. CHOPRA & P. M. BENNET: Induction of the *Citrobacter freundii* group I β-lactamase in *Escherichia coli* is not dependent on entry of β-lactam into the cytoplasm. Antimicrob. Agents Chemother. 34: 2429~2430, 1990
24) LAMINET, A. A.; T. ZIEGELHOFFER, C. GEORGOPOULOS & A. PLUCKTHUN: The *Escherichia coli* heat shock proteins GroEL and GroES modulate the folding of the β-lactamase precursor. EMBO J. 9: 2315~2319, 1990