# LOCALIZATION OF AMINOGLYCOSIDE 3′-PHOSPHOTRANSFERASE II ON A CELLULAR SURFACE OF R FACTOR RESISTANT *ESCHERICHIA COLI*

Sir:

In previous papers, we have reported the presence of three aminoglycoside 3′-phosphotransferases (APH(3′)-I, II and III) in resistant bacteria[1~4], they differ in their substrate specificities. Moreover, APH(3′)-II can be differentiated immunologically from the other phosphotransferases, and APH(3′)-II enzymes of different origins such as *E. coli* and *Pseudomonas* were identical in immunological behavior.[5] In this paper, we report on the localization of the APH (3′)-II on (or near) the cellular surface.

*E. coli* JR66/W677 which produced APH(3′)-II was grown in 1% peptone, 0.5% meat extract and 0.3% sodium chloride, pH 7.4, at 37°C and at the early logarithmic phase, cells were harvested from the culture (1,000 ml) by centrifugation. The cells were washed twice in 250 ml of 0.9% sodium chloride, and the washed cells (3.7 g, wet weight) were suspended in 300 ml of 30 mM tris-HCl buffer (pH 8.0) containing 0.5 M sucrose at room temperature and 1 mM disodium ethylenediaminetetraacetate. After stirring for 10 minutes, the cells were collected by centrifugation, suspended in water (300 ml) at 4°C and the suspension was stirred for 10 minutes. Thus, the cells underwent cold osmotic shock[6,7] and the suspension was separated into the cold shock cells and the supernatant (Sup I) by centrifugation.

The cold shock cells and the washed cells (2.5 g, wet weight) which did not undergo the cold osmotic shock were suspended in cold water and disrupted by passing a French pressure cell (1,200 kg/cm$^2$). The disrupted suspensions were centrifuged and supernatants were prepared. The supernatant prepared from cells which underwent osmotic shock was designated Sup II and that from cells without osmotic shock was designated Sup III. As shown in Table 1, the APH(3′)-II activities[8] were found in Sup I and Sup III but not in Sup II. Thus, the cold osmotic shock was shown to release all APH(3′)-II. 5′-Nucleotidase activity[9] was also found in Sup I but not Sup II. However the activity of inorganic pyrophosphatase[10] was found in Sup II but not in Sup I. The results suggested that APH(3′)-II in *E. coli* JR66/W677 is located near the cellular surface with 5′-nucleotidase. It is known that the 5′-nucleotidase in *E. coli* is located near the cellular surface[11] and the inorganic pyrophosphatase in *E. coli* is located in the cytoplasm[12].

The presence of APH(3′)-II on the cellular surface was shown by an indirect immunofluorescent staining method. Cells of *E. coli* JR66/W677 were grown in nutrient agar for 18 hours, at 30°C, and suspended in 0.9% sodium chloride. One drop of this cell suspension (0.3 mg of wet cells/ml) was placed on a glass slide. After drying in air for 15 minutes, 100 μl of rabbit antiserum to APH(3′)-II, or normal rabbit serum was added and the slide incubated for 20 minutes in a humidity chamber at 37°C. The glass slide was washed in 1% Triton X-100 in phosphate buffer saline (PBS; 0.02 M phosphate buffer, pH 7.4, containing 0.9% sodium chloride) twice for 5 minutes. The cells on the glass slide thus treated were incubated for 20 minutes with fluorescein isothiocyanate-conjugated antiserum (purchased from Boehringer Mannheim) to the rabbit IgG and washed with 1% Triton X-100 in PBS and with PBS successively. The slide was dipped into water to remove salts and mounted with buffered glycerol (0.5 M potassium phosphate buffer, pH 7.4-glycerol, 1:1) and a cover glass. Photographs

Table 1. The activities of the APH(3′)-II, the 5′-nucleotidase and the inorganic pyrophosphatase

| Fraction | APH(3′)-II u/g of cell weight | 5′-Nucleotidase u/g of cell weight | Inorganic pyrophosphatase u/g of cell weight |
|---|---|---|---|
| Sup I | 2,108 (101.3%) | 346.4 (92.8%) | 1.1 (4.6%) |
| Sup II | 122 (5.9%) | 4.5 (1.2%) | 22.3 (92.9%) |
| Sup III | 2,080 (100%) | 373.4 (100%) | 24.0 (100%) |

APH(3′)-II activity was assayed by the method described in a previous paper[8]. One unit was defined as the amount of enzyme which phosphorylated 1 μ mole of butirosin A per an hour.

5′-Nucleotidase activity was assayed according to the method of NEU[9]. One unit was defined as the amount of enzyme which hydrolyzed 1 μ mole of 5′-AMP per an hour.

Inorganic pyrophosphatase was assayed according to the method of NEU and CHOU[10]. One unit was defined as the amount of enzyme which hydrolyzed 10 μ mole of sodium pyrophosphate per an hour.

were taken on Kodak Tri-X film using a Leitz Orthoplan fluorescence microscope, equipped with a Ploem vertical illuminator producing incident blue UV light (an activation filter KP490 which produces an activating wave length at the peak of fluorescein isothiocyanate, a red suppression filter BG38, and a barrier filter K530) and with a mercury vapour lamp as the light source. As shown in Fig. 1, specific immunofluorescence was observed on the surface of cells treated with the antiserum. In the case of cells treated with normal rabbit serum, no specific fluorescence was observed. In the case of *E. coli* W677 (which does not carry an R factor and does not form APH (3′)-II), the fluorescence was not observed. Based on these data, it can be concluded that at least part of the molecule of APH(3′)-II which is involved in binding with its antibody appears on the cell surface. This immunofluorescent method will be a useful tool for the identification of APH (3′)-II in resistant bacteria.

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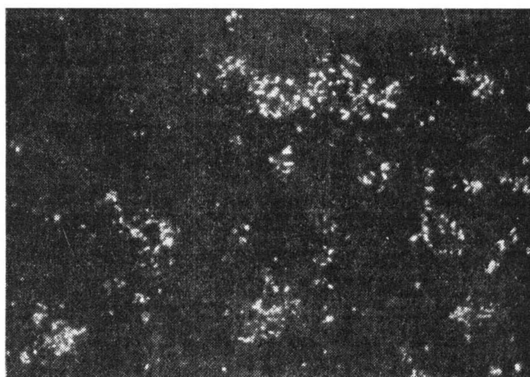
(Received May 17, 1976)

Fig. 1. Immunofluorescent micrograph specific for the APH(3′)-II on *E. coli* JR66/W677.



Specific immunofluorescence is observed around the cells. ×680

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