A Fluorescent Probe Response to the Interaction of Pyocin R1 with Sensitive Cells

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Addition of pyocin R1, a bacteriocin of \textit{Pseudomonas aeruginosa}, to sensitive cells caused a fluorescence increase of 8-anilino-1-naphthalenesulfonate (ANS) in the cell suspension. The reaction was rapid, starting with a short time lag after adsorption of pyocin onto the cells and finishing within several minutes. The fluorescence response was attributed to the interaction of the cell body and ANS, not to that of the medium outside the cells and ANS. The maximal amplitude of fluorescence after pyocin addition was dependent on temperature, and the relation appeared to be biphasic. Similarly, Arrhenius plots of the initial rate of fluorescence change were biphasic. The transition of slopes in both cases occurred in the temperature range between 18 and 19°. These results suggest that ANS interacts with lipids in the cell envelope and that pyocin causes a structural change of the cell envelope leading to increased fluorescence of ANS.

Pyocin R1, one of the bacteriocins produced by \textit{Pseudomonas aeruginosa}, is a protein particle which has a complex structure resembling a bacteriophage tail with a contractile sheath, and its molecular weight is approximately $1.2 \times 10^7$ daltons (1-3). The pyocin particle is adsorbed on the surface of the envelope of a sensitive cell and contraction of its sheath takes place. Subsequently pyocin R1 causes a rapid and complete inhibition of macromolecular syntheses—DNA, RNA, and protein syntheses—as well as of the uptake of sugars and amino acids into the sensitive cell, and a gradual leakage of a 260 nm-adsorbing substance occurs (4, 5; Iijima, personal communication). Ultimately, pyocin R1 causes the death of the sensitive cell. These actions of pyocin R1 are very similar to those of colicin E1 or K, a bacteriocin of \textit{Escherichia coli}, which has been purified and identified as a protein of molecular weight less than 100,000 (6-10). To investigate the action of colicin E1, Cramer and Phillips (11, 12) have used a fluorescent dye, 8-anilino-1-naphthalenesulfonate (ANS), or a more lipophilic dye, N-phenyl-1-naphthylamine (NPN), as a probe for colicin-induced structural changes in the envelope of sensitive cells, and observed the probe fluorescence response induced by colicin E1. Colicin E1 causes an increase in fluorescence intensity and a blue shift in the emission maxima of ANS and NPN bound to sensitive cells. The initial rate of fluorescence increase is temperature-dependent and Arrhenius plots of the rate of fluorescence change are biphasic. These data suggest a structural change of the cell envelope induced by colicin E1 and an involvement of envelope lipid in the transmission mechanism of colicin E1 (13). It has also been reported that addition of bacteriophages T2–T7 to host cells

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; NPN, N-phenyl-1-naphthylamine.
causes a response of the fluorescent probe ANS (14).

In the present paper, we investigated the response of a fluorescent probe, ANS, to the interaction of pyocin R1 with sensitive cells. Pyocin R1 induced an increase in fluorescence intensity after addition of a cell-ANS suspension. It is assumed that the fluorescence increase is largely attributable to an increase in the amount of ANS molecules bound to sensitive cells. The initial rate of fluorescence change at various temperatures showed biphasic Arrhenius plots. The probe fluorescence response casts light on one of the early reactions of sensitive cells to pyocin R1.

MATERIALS AND METHODS

Bacterial Strains and Media—Pseudomonas aerugionosa P14 was used as a pyocin R1-sensitive bacterium for most of these experiments. The cells were cultivated in G-medium at 37° and harvested at the early to middle logarithmic phase (15). The cells were washed once with a medium containing 0.1 M NaCl and 0.01 M Tris-buffer (pH 7.5) and suspended in the same medium. This medium will be referred to as saline buffer.

Pyocinogenic strain P15 was used for the preparation of pyocin R1.

Preparation of Pyocin R1—Pyocin R1 was prepared according to the procedure described by Kageyama (J).

Killing Activity—Surviving cells after treatment with pyocin R1 were determined by the usual method of plating on nutrient agar plates. The multiplicity of pyocin R1, i.e., the average number of pyocin particles adsorbed on a sensitive cell, was calculated from the survival using the zero term of the Poisson distribution equation, $e^{-m}$ (S).

Fluorimetry—Fluorescence measurements were carried out using a Shimadzu RF502 corrected recording spectrophotometer with a wavelength compensator attachment. A 1 x 1 cm or 1 x 0.5 cm quartz cell was used in a constant-temperature cell holder connected to a temperature-controlled water bath. Unless otherwise indicated, fluorescence of ANS and NPN was excited at 350 and 330 nm and measured at 510 and 415 nm, respectively. The final concentrations of fluorescent probes ANS and NPN in the sample solution were 50 and 5 μM, respectively.

RESULTS

Emission Spectrum of ANS after Addition of Pyocin R1—Figure 1(a) shows emission spectra of ANS under various conditions. When excited at 350 nm, cells alone (curve 1) did not show any emission in the range between 400 and 550 nm, whereas ANS alone (curve 2) showed an emission spectrum with low fluorescence intensity and a broad peak around 535 nm. The emission spectrum of ANS with pyocin R1 was quite similar to that of ANS alone (not shown). Addition of cells to ANS solution caused a small increase in fluorescence intensity and a slight blue shift in the peak of the emission spectrum from 535 to 533 nm. The excitation spectra of ANS alone, of ANS and cells, and of ANS and cells with pyocin R1, are shown in Fig. 1 (b). The peak shifted from 357 to 366 nm on adding pyocin R1. ANS molecules, when located in a micro-environment with smaller polarity and/ or higher viscosity, give rise to an increase in the quantum yield of fluorescence and a blue shift in the emission maximum (16, 17). Therefore, the pyocin-induced fluorescence response—the fluorescence increase, a blue shift in the emission maximum, and a red shift in the peak of the excitation spectrum—strongly suggests that ANS molecules bind to region(s) with smaller polarity and/or higher viscosity and cause the increase in fluo-
FLUORESCENCE CHANGE INDUCED BY PYOCIN R1

Fig. 1. a: Fluorescence emission spectra of ANS from cell suspensions with and without pyocin R1. *P. aeruginosa* P14 was grown in G-medium, concentrated, and resuspended in saline buffer. Curve 1, cells alone; curve 2, 50 μM ANS alone; curve 3, cells and 50 μM ANS; curve 4, 17 min after pyocin R1 addition to cells plus ANS. The emission maxima of spectra 2, 3, and 4 are at approximately 535, 533, and 510 nm, respectively. Temperature, 37°C; cell concentration before addition pyocin R1, 2×10^6 cells/ml; cell survival after pyocin R1 addition, 2%. Excitation at 350 nm.

b: Excitation spectra of ANS from cell suspensions with and without pyocin R1. Curve 1, 50 μM ANS alone; curve 2, cells and 50 μM ANS; curve 3, cells and ANS with pyocin R1. Emission was fixed at 510 nm.

When pyocinogenic strain P15 was used in place of sensitive strain P14, pyocin R1 did not cause any fluorescence change of ANS. It appears that the pyocinogenic cells do not adsorb pyocin R1 on the cell surface.

Next, a cell suspension containing ANS was excited at a wavelength of 290 nm (Fig. 2). Excitation at 290 nm provides information on the conformation of tryptophanyl residues in proteins and on the possibility of energy transfer from these residues to ANS molecules (18). The emission spectrum of cells alone showed a single peak at 345 nm (curve 1) and that of cells with ANS exhibited two maxima in the fluorescence spectrum at approximately 345 and 520 nm (curve 2). Therefore, it is considered that the emission maxima of 345 and 520 nm are due to tryptophanyl residues in cell proteins and to the fluorescent probe ANS, respectively. After addition of pyocin R1, the fluorescence intensity and the position of the peak at 345 nm were not altered, whereas in a longer wavelength region, an increase in fluorescence intensity and a blue shift of the peak from 520 to 485 nm were observed (curve 3). From these data, it seems clear that pyocin R1 causes no fluorescence response of tryptophanyl residues and no energy transfer from these residues to ANS molecules. The fluorescence decrease at 345 nm caused by addition of ANS to cells may be due to a shielding effect.

Fig. 2. Fluorescence emission spectra excited at 290 nm from cell-ANS suspensions before and after pyocin R1 addition. Curve 1, cells alone; curve 2, cells and 50 μM ANS; curve 3, cells and 50 μM ANS with pyocin R1. Temperature, 37°C; cell survival after pyocin treatment, 2%. Glass filters cutting scattered light below 300 nm, Corning 0–54, Hoya Glass UV-32, and UV-34, were placed in the emission path.
effect of ANS, absorbing the emission of tryptophanyl residues (Fig. 1b).

**Time Course of Pyocin R1-induced Fluorescence Change**—The time course at 37° of the fluorescence change after pyocin R1 addition to ANS-cell suspension is shown in Fig. 3. The increase in fluorescence intensity reached a saturated level within 5 min after addition of pyocin R1. The fluorescence intensity at the saturated level remained constant for more than 30 min. In a reverse procedure in which ANS was added to a cell suspension previously treated with pyocin R1, the fluorescence intensity increased rapidly to the saturated level in less than 30 sec after the addition of ANS. The results suggest that the rate of binding of free ANS molecules to the area causing the increase in fluorescence yield is much higher than that of the appearance of this area induced by pyocin R1.

The pyocin R1-induced fluorescence enhancement followed the process of adsorption of pyocin on the cell surface with a short delay (Fig. 3). Since the two processes, pyocin adsorption on cells and fluorescence enhancement, were almost indistinguishable at 37°, the experimental temperature was lowered. At 12.7°, the fluorescence increase occurred more slowly than pyocin adsorption and was completed 8 min after the addition of pyocin R1. Figure 4 shows time courses of cell survival plotted on a logarithmic scale and the fluorescence change recalculated as \(1 - DF/DF_{\text{max}}\), where \(DF_{\text{max}}\) is the final (maximal) fluorescence increase after pyocin R1 addition. Plots of the two time courses showed a time lag of about 30 sec between the initial phases of the two. The above result indicates that the fluorescence increase occurs with a very short delay after pyocin binding to the cell surface.

**Turbidity of the Cell Suspension and Fluorescence Change**—A sensitive cell suspension is intrinsically turbid, and the turbidity increases with cell growth. It has been reported that the addition of pyocin R1 to growing cells causes an immediate inhibition of cell growth, halts the increase in turbidity, and even brings about a decrease in cell turbidity when a high concentration of pyocin R1 is employed (4). In the present study, the turbidity of cells suspended in saline buffer, not in nutrient broth, remained constant, but after addition of pyocin R1, the turbidity decreased remarkably (Fig. 5). The decrease continued for more than 30 min, while the fluorescence enhancement caused by pyocin R1 ceased within 3 min. It has been found that the addition of MgCl₂ to the cell suspension prevents the decrease in turbidity observed with pyocin R1 (Iijima, personal communication). Therefore, the effect of MgCl₂ on the pyocin-induced fluorescence change was studied. In the
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Fig. 5. a: Pyocin R1-induced fluorescence increase in the presence of 50 mM MgCl₂. Solid line, 0 mM MgCl₂; dashed line, 50 mM MgCl₂; temperature, 37°C; cell survival: 0 mM, 7%; 50 mM, 10%. b: Pyocin R1-induced turbidity change at 620 nm in the absence and presence of 50 mM MgCl₂. Cell suspensions contained 50 μM ANS. Solid line, 0 mM; dashed line, 50 mM; temperature, 37°C; cell survival: 0 mM, 10%; 50 mM, 10%.

In the presence of 50 mM MgCl₂, the absorbance at 620 nm and fluorescence intensity at 510 nm after pyocin R1 addition were compared. The absorbance remained constant, while the fluorescence intensity increased more slowly than in the case without MgCl₂ and finally reached a higher level. Therefore, the fluorescence increase is not related to the turbidity change of cell suspension. The two events are the results of different phases of pyocin action.

Relation of Fluorescence Change and Killing Activity—Various concentrations of pyocin R1 were added to cell suspensions at a fixed cell concentration. In each case, the fluorescence was recorded and viable cells were counted (Fig. 6a). The total amplitude of the pyocin-induced fluorescence change, measured 10 min after pyocin addition, was a linear function of the number of killed cells, calculated from the count of viable cells (Fig. 6b). The proportional relationship between the two shows that the fluorescence change induced by pyocin R1 corresponds to the fraction of killed cells, but not to the number of pyocin particles adsorbed on a single cell. In addition, the total amplitude of fluorescence change as a function of multiplicity reached a maximal level at a multiplicity of over 4, when more than 98% of the cells were killed (data not shown).

The region where ANS molecules change fluorescence yield was investigated by separating the...
pyocin-treated cell suspension into two parts, the supernatant and cell pellet, by centrifugation. After centrifugation, the supernatant showed a fluorescence intensity almost equal to that of ANS alone (Table I). The pellet suspension, which was resuspended in saline buffer without ANS to the same volume as the original, did not show any fluorescence. On the other hand, the cell suspension after recentrifugation and suspension in saline buffer containing ANS showed a higher fluorescence intensity than pyocin-untreated cells. These data show that fluorescence enhancement takes place not in the medium outside the cell body but on the cell body itself and that the association constant of ANS molecules for the cell body appears to be small.

**Fluorescence Changes at Various Temperatures**

Kageyama et al. (5) found that a reversible adsorption of pyocin R1 occurs at 0°: at that temperature pyocin R1 is adsorbed on sensitive cells but cannot kill bacteria. That is, at 0° the reaction does not pass the reversible stage, and pyocin dissociates from the bacteria on dilution for plating. Therefore, the fluorescence response was examined under these conditions. The experiment was carried out at 1.8°. After addition of pyocin R1, no fluorescence increase was observed for more than 10 min (data not shown), and the survival obtained after dilution was 67 to 100%, while at 37° where the fluorescence change occurred readily, the survival was 3%. Therefore, it is considered that the fluorescence response does not occur at the reversible adsorption stage, but after the reaction proceeds to the subsequent irreversible stage.

The fluorescence intensity of ANS-cell suspension was dependent on temperature. Measurements were carried out in the absence and presence of pyocin R1 at various temperatures between 10 and 30°. The values were plotted against 1/T (Fig. 7). The intensity of fluorescence was extrapolated to 100% death (calculated from the maximal intensity and survival obtained). The fluorescence with or without pyocin increased greatly or slightly on lowering the temperature, respectively. However, both plots appear to be biphasic, indicating that a turning point or discontinuity exists between 18 and 19°. The existence of the inflection in both plots seems to reflect a characteristic of the membrane structure rather than temperature-dependent association-dissociation equilibrium between ANS molecules and binding sites on the cell membrane (19).

The time courses of fluorescence change after addition of pyocin R1 were recorded at various temperatures from 10 to 30°. The apparent initial rates of fluorescence increase were obtained from

<table>
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<th>TABLE I. Fluorescence intensity at 510 nm of cell-ANS suspensions before and after centrifugation.</th>
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<td>Centrifugation</td>
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<tr>
<td>Beforeb</td>
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<td>(pellet+ANS)d</td>
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a Suspensions were centrifugated at 6,000 rpm for 10 min at 25°. b Viable cell counts of suspensions in the absence and presence of pyocin R1 were 6.0×10⁸ and 4.6×10⁸ cells/ml, respectively. Survival was 0.75%. c Each pellet was suspended in 2 ml of saline buffer containing 50 μM ANS. d Excitation was carried out at 350 nm. The temperature was 37°.
the measurements at each temperature. However, the initial rate depended on two factors, the multiplicity of pyocin and the temperature-dependent fluorescence yield. At 26.8 and 11.8°, a linear relationship between the initial rate and multiplicity was found in the region of multiplicity of 0 to 4 (data not shown). Therefore, the magnitude of the apparent initial rate obtained at each temperature was corrected for the two factors to obtain the value at a multiplicity of one and the initial rates were calibrated relative to the final level of fluorescence. Figure 8 shows Arrhenius plots of the initial rate thus calculated. The plots approximated to two straight lines, with one turning point between 18 and 19°. The existence of the transition temperature at about 18 to 19° in the Arrhenius plots suggests that the fluorescence response induced by pyocin R1 involves the cell envelope lipids, phase transition of which occurs in the same temperature region (13, 20).

N-Phenyl-1-naphthylamine as a Fluorescent Probe in Place of ANS—N-Phenyl-1-naphthylamine (NPN) is a derivative of ANS lacking the sulfonic acid residue and is considered to be a fluorescent probe with strong hydrophobicity, having no intrinsic electric charge (12, 17). The emission spectrum of NPN in the presence of sensitive cells and the time course of fluorescence of NPN after addition of pyocin R1 were observed. The addition of pyocin R1 caused a large increase in fluorescence and a blue shift of the peak from 450 to 415 nm (Fig. 9) and, at the same time, a red shift of the peak in the excitation spectra (Table II). As in the case of ANS, the fluorescence response after pyocin addition reached a stationary level within a few minutes. NPN as a fluorescent probe exhibits the fluorescence response induced by pyocin R1 more markedly than ANS.

![Fig. 8. Arrhenius plots of the initial rate of the pyocin R1-induced fluorescence increase. See the text for details of the calculation of the initial rate. On the ordinate, k is the initial rate of fluorescence increase (arbitrary units); actual cell survival levels were between 5-23%.](https://academic.oup.com/jb/article-abstract/81/2/333/875592)

![Fig. 9. Fluorescence emission spectra of NPN in cell suspensions with and without pyocin R1. Curve 1, cells alone; curve 2, 5 μM NPN alone; curve 3, cells and 5 μM NPN; curve 4, 17 min after pyocin R1 addition. 10^-4 M NPN dissolved in ethanol was diluted with saline buffer and added to a cell suspension to provide a final concentration of 5 μM. Excitation was at 330 nm. Initial cell concentration, 4.2×10^8 cells/ml; temperature, 37°; survival after pyocin treatment, 0.12%.](https://academic.oup.com/jb/article-abstract/81/2/333/875592)

**Table II.** The position of the maximum in emission and excitation spectra of N-phenyl-1-naphthylamine (NPN) added to cell suspensions.*

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<th>Emission^b</th>
<th>Excitation^c</th>
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<tr>
<td>NPN alone</td>
<td>485 nm (broad)</td>
<td>346 nm</td>
</tr>
<tr>
<td>with cells</td>
<td>450 nm (broad)</td>
<td>347 nm</td>
</tr>
<tr>
<td>with cells and pyocin R1^d</td>
<td>415 nm</td>
<td>354 nm</td>
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* Concentration of NPN, 5 μM. Fluorescence was measured at 37°. ^b Excitation at 330 nm. ^c Emission at 415 nm. ^d The initial cell concentration was 4.2×10^8 cells/ml. Survival after pyocin treatment was 0.12%.
DISCUSSION

In the present study, we found that pyocin R1 induced a fluorescent probe response after the addition of pyocin R1 to the cell-probe suspension. The fluorescence change of ANS as a probe is assumed to reflect an effect of pyocin R1 on the cell-ANS interaction. It was observed that pyocin R1 induced a decrease in the turbidity of the cell suspension. Therefore, the effect of the intrinsic turbidity of cell suspensions on fluorescence and the relationship between turbidity and fluorescence changes induced by pyocin R1 were investigated under conditions which separated the two events. In the presence of a high concentration of magnesium chloride, pyocin R1 was able to cause fluorescence response without turbidity decrease. Thus, it is assumed that the turbidity of the cell suspension has little, if any, influence on the fluorescence measurements and that these changes induced by pyocin R1 represent two different modes of action of pyocin R1. At present, it is not clear whether the decrease in turbidity is due to the lysis of cells caused by pyocin R1 or to other effects, and the role of magnesium chloride in the repression of turbidity decrease also remains unknown.

The next problem is the significance of the fluorescence change resulting from pyocin R1 action. First, the increase in fluorescence yield and the blue shift in the maximum of the emission spectrum of ANS are due to interaction of ANS molecules with cells and not with extracellular medium after the addition of pyocin R1. Therefore, it is suggested that pyocin R1 causes an alteration of properties of the cell so as to increase the amount of ANS binding to the cells and to increase the quantum yield of the ANS molecule.

Second, the results obtained provide some indications of the properties of the ANS-binding site on the cell. ANS has been employed as a probe binding to hydrophobic and/or positively charged regions and for detecting microenvironmental changes near the molecules (21). In whole bacterial cells, proteins and phospholipids in the cell envelope or cell membranes are thought to be the regions where ANS molecules are able to associate. As regards the problem of which proteins or phospholipids are the ANS binding sites, several results have been obtained as follows: (i) there is an inflection at 18-19° in the temperature-dependent fluorescence intensity of ANS in the presence of pyocin R1, (ii) there is a discontinuity at the same temperature of 18-19° in Arrhenius plots of the initial rate of pyocin R1-induced fluorescence increase, (iii) there is no enhancement of fluorescence by tryptophanyl residues in cell proteins and no energy transfer from these residues to ANS molecules, and (iv) there is a fluorescence increase of NPN, a derivative of ANS lacking a charged group, induced by pyocin R1. The temperature of 18-19° obtained in (i) and (ii) corresponds to that of the phase transition characteristic of phospholipids in the bacterial envelope (11-13, 19, 23). Therefore, the above findings indicate that though ANS molecules may bind to the cell proteins, the main contribution to the fluorescence change of ANS caused by pyocin R1 seems to be due to the association of ANS molecules with phospholipids rather than proteins. In particular, result (iv), that pyocin R1 causes an increase in the fluorescence yield of NPN, suggests that fluorescent probes such as ANS or NPN associate with the envelope by hydrophobic interaction rather than by electrical interaction.

Third, it has been shown that the magnitude of fluorescence intensity is proportional to the number of cells killed by pyocin R1. This suggests that the fluorescence response is an "all-or-none" event in which even one pyocin particle can induce a structural change of the cell envelope or membranes over a whole cell.

Based on the above findings it is considered that pyocin R1 adsorbed on the cell surface causes a structural change of the cell envelope or cell membranes, which mainly consist of phospholipids and proteins, so as to increase the amount of ANS associating with the lipids in the cell envelope.

The envelope of Pseudomonas aeruginosa, however, consists of two layers, cell wall and cytoplasmic membrane (24). Both layers contain lipids, although the cytoplasmic membrane contains considerably more lipids than the cell wall (25). Therefore, several possibilities exist regarding the mechanism of pyocin R1 action which induces the structural change and fluorescence response: (i) a structural change of the cell wall and/or cytoplasmic membrane may be caused by pyocin R1, resulting in the binding of ANS to the altered membrane regions, (ii) pyocin R1 may only induce a structural change...
of the cell wall, a barrier which prevents ANS molecules from approaching and associating with the cytoplasmic membrane, permitting ANS molecules to penetrate the cell wall and associate with the cytoplasmic membrane. However, in the present study, the identity of the ANS binding region remains unsettled.

Regarding the action process of pyocin R1, several stages have been found after the addition of pyocin R1 to the cell before pyocin R1 shows inhibitory effects on cellular metabolism, leading to cell death. The first stage should be a reversible adsorption at the receptor site on the surface of the cell, followed by irreversible adsorption with sheath contraction and core penetration through the cell wall, and finally the occurrence of inhibitory reactions after an unknown interaction of pyocin R1 with the cytoplasmic membrane (4, 5).

Since fluorescence response does not occur at the reversible stage but starts within 30 sec after the addition of pyocin to the cell, the structural change of the cell envelope may occur at the subsequent irreversible adsorption stage at the earliest.

As well as pyocin R1, colicins E1 and K, or bacteriophages T2 to T7 and T4 phage ghost devoid of head DNA cause probe fluorescence response (11-14, 23, 26). Furthermore, some effects of pyocin R1 other than fluorescence response—rapid inhibition of macromolecular syntheses and uptake of sugars and amino acids—are similar to those of colicin E1, or T4 ghost, although pyocin R1 is analogous in morphology to T4 ghost rather than to colicins E1 or K (6-9, 27-29). However, there is a difference, though it is small, between the effects of pyocin R1 and colicin E1. The time lag from the addition of pyocin R1 to the rise of fluorescence is shorter than that with colicin E1, a difference which is more marked at low temperature, i.e., 10° (13).

However, it is too early to compare the action of pyocin R1 with that of T4 ghost on the fluorescence response, since detailed results in case of T4 ghost are not yet available.

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