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**EXPERIMENTAL ARTICLES**

# Biosynthesis of *Proteus mirabilis* Nuclease

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**Abstract**—The culture liquid and periplasm of *Proteus mirabilis* contained nuclease, an enzyme with DNase and RNase activities. The nuclease was most actively synthesized in the early exponential and stationary growth phases. Nuclease synthesis was regulated by nucleic acids (induction by substrate) and inorganic phosphate (end-product inhibition). The synthesis and secretion of nuclease by *P. mirabilis* was induced by mitomycin C, an inducer of the SOS functions of cells. This suggests the involvement of SOS-response proteins in the regulation of nuclease synthesis.

*Key words*: *Proteus mirabilis*, nuclease, induction, repression

Bacteria of the genus *Proteus* are very adaptable microorganisms found in many environments, such as animal and human feces, manure, and soils rich in putrefying organic substances.

A high level of nuclease activity was observed earlier in the culture liquid and cell-free extract of *Proteus mirabilis* [1]. Although the biochemical and physicochemical properties of secretory bacterial nucleases are sufficiently studied, little is known about the synthesis regulation of these enzymes.

The aim of the present work was to study the dynamics of the synthesis of the secretory nuclease of *P. mirabilis* and the regulatory mechanisms involved.

## MATERIALS AND METHODS

The strain *Proteus mirabilis* 4 used in this work was obtained from the collection of microorganisms of the Department of Microbiology, Kazan State University.

The strain was grown in a nutrient broth supplemented with 0.1% casein hydrolysate (this medium contained 23.3 mg/100 ml inorganic phosphate ($P_i$)) or in a low-phosphate semisynthetic medium containing 0.8 mg $P_i$/100 ml [2]. Cultivation was carried out in flasks one-fifth full of the medium in a temperature-controlled shaker (200 rpm; 37°C). Cultivation media were inoculated with cells from overnight cultures grown on tryptose agar and resuspended in 0.14 M NaCl. Inoculum was added to give them a cell density of 0.05–0.10 OD unit/ml. In experiments with washed cells, they were harvested by centrifugation from a 20-h culture, washed with and resuspended in 0.14 M NaCl to equal the cell density of the culture.

After cultivation, the accumulated biomass was estimated by measuring the optical density of the culture at $\lambda = 650$ nm in a 1-cm path-length cuvette. To determine enzyme activity, cells were precipitated by centrifugation at 10000 *g* for 15 min and resuspended in sterile 0.01 M Tris–HCl buffer (pH 7.4) to a cell density of 10 g wet biomass/ml. An aliquot of this suspension was hydrolyzed by boiling it in 0.4 M NaOH for 30 min and centrifuging it at 10000 *g* for 20 min. Protein concentration in the supernatant was measured using the method of Lowry. The rest of the suspension was subjected to several freeze–thaw cycles to extract periplasmic proteins [1] and to determine enzyme activity.

The dry weight of the biomass was determined after drying it to a constant weight at 105°C. Viable cells were enumerated by the method of plating on 4% nutrient agar. The data obtained were expressed as the logarithm of the number of viable cells (log NVC). Specific growth rate was measured as described by Pirt [3].

Nuclease activity was assessed by the formation of DNA or RNA products soluble by hydrolysis in 2% $HClO_4$. The concentration of such products was estimated by measuring the optical density ($\lambda = 260$ nm) of the supernatant after precipitation of the products of nucleic acid hydrolysis that are insoluble in 2% $HClO_4$. The reaction mixture (1 ml) contained 1 mg/ml DNA (RNA), 0.1 ml of the tested material, and 6 mM $MgCl_2$ in Tris–HCl buffer (pH 8.5). One unit of activity was defined as the amount of enzyme required to increase the optical density of the experimental samples by one OD unit (as compared to the control) after 1 hour of incubation at 37°C [4]. The activity of the enzyme was expressed per mg protein (to characterize its specific activity) or per mg dry biomass (to characterize the producing ability of culture).

High-polymeric DNA, chloramphenicol, and mitomycin C were purchased from Serva (Germany), and all other reagents were domestically produced. The peptone used contained 0.8 mg $P_i$ per 100 g [2].

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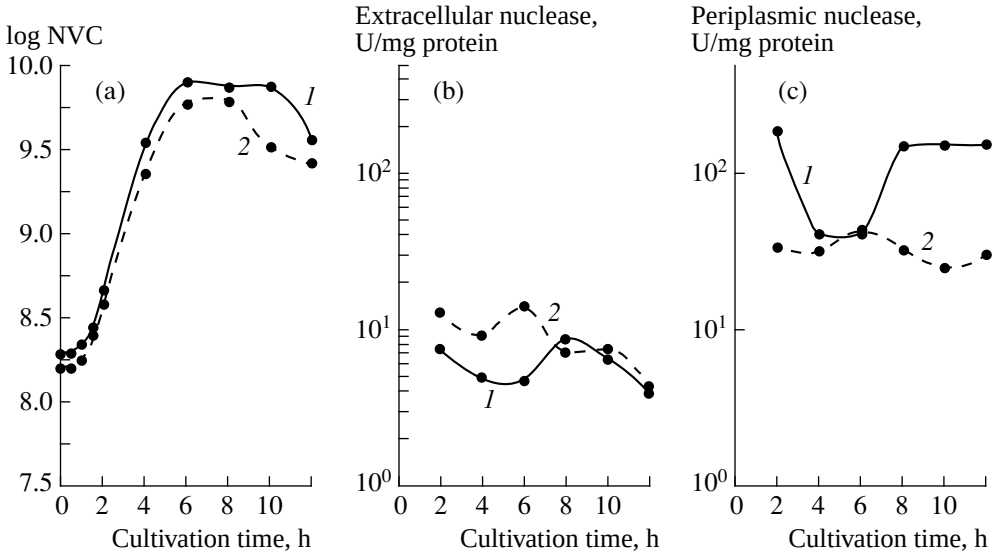


**Fig. 1.** (a) Growth and nuclease activity in (b) the culture liquid and (c) periplasm of *P. mirabilis* 4 cells grown in (*1*) nutrient broth and (*2*) low-phosphate semisynthetic medium.

## RESULTS AND DISCUSSION

The culture liquid (CL) and cell-free extract of *P. mirabilis* contained an enzyme possessing both DNase and RNase activities, i.e. nuclease. The cell-bound enzyme was located in the periplasm. To discern the possible regulatory mechanisms involved in the regulation of nuclease synthesis, we studied the effect of various concentrations of DNA, RNA, and $P_i$ (as one of the products of the total degradation of nucleic acids), as well as of mitomycin C (an inducer of the SOS functions of cells), on enzyme activity. In growth experiments, the dynamics of nuclease synthesis and secretion in different media were investigated.

The comparative study of the growth parameters of *P. mirabilis* cultivated in a low-phosphate semisynthetic medium and a nutrient broth (Fig. 1) showed that the bacterium grew better in the latter medium. The generation times were 53 and 42 min and the specific growth rates were 0.76 and 1.01 $h^{-1}$, respectively. Correspondingly, the final culture density was also higher in the latter medium. In both media, maximum nuclease activity in the periplasm and CL occurred in the early exponential and in the stationary growth phases, and minimum activity occurred in the mid-exponential phase (Fig. 1). It can also be seen in Fig. 1 that growth in the low-phosphate medium provided for two times higher enzyme activity in the culture liquid and considerably lower enzyme activity in the periplasm than growth in the nutrient broth.

Effect of different concentrations of inorganic phosphate ($P_i$) in the semisynthetic medium on the biomass and the activity of periplasmic nuclease in 20-h *P. mirabilis* 4 cells

| $P_i$ concentration, mg/100 ml | Dry biomass, mg/ml | Nuclease activity, % of the control |
|---|---|---|
| 0.8 (control)* | 100 | 100 |
| 5 | 120 | 47 |
| 10 | 100 | 50 |
| 100 | 100 | 49 |
| 200 | 100 | 60 |

* Control corresponds to the semisynthetic medium without supplementary inorganic phosphate.

Experiments in vitro showed that $P_i$ did not suppress the activity of nuclease even at a concentration of 200 mg/100 ml. Upon batch cultivation in a semisynthetic medium, the addition of $P_i$ at the moment of inoculation to a final concentration of 5 mg/100 ml led to a twofold decrease in the total (extracellular plus periplasmic) nuclease activity of the culture (see table).

The next set of experiments was carried out with washed *P. mirabilis* cells. For these experiments, the cells were grown in the semisynthetic medium with 5 mg $P_i$/100 ml, washed and then placed, in equal amounts, in three flasks. Flask 1 contained the same medium, while the two other flasks contained the media and 0.8 mg $P_i$/100 ml. In flask 3, the medium was additionally supplemented with 10 μg/ml chloramphenicol (an inhibitor of protein synthesis). The incubation of these flasks for 4 h led to a 60% decrease in the nuclease activity of the cells in flask 1 and to a 30% increase in the nuclease activity of the cells in flask 2. The nuclease activity of cells incubated in the presence of chloramphenicol (flask 3) did not change. These results suggest that the increase in the nuclease activity of cells incubated in the low-phosphate medium (flask 2) was related to the de novo synthesis of nuclease. In

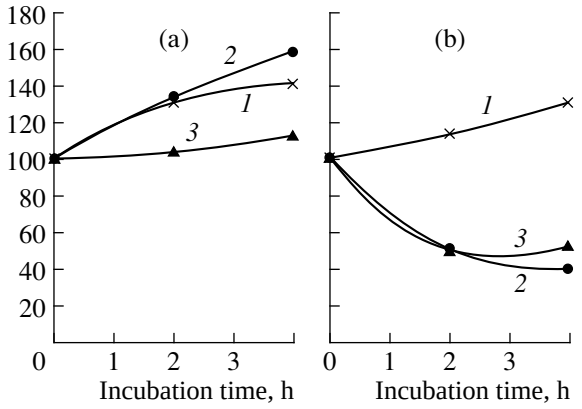


**Fig. 2.** Changes in (a) the protein content and (b) nuclease activity of washed cells of *P. mirabilis* 4 incubated in media with (*1*) 0.8 mg $P_i$/100 ml, (*2*) 5 mg $P_i$/100 ml, and (*3*) 0.8 mg $P_i$/100 ml and 10 μg/ml chloramphenicol. Protein content and nuclease activity at zero time points were taken to be 100%.

other words, nuclease synthesis in *P. mirabilis* 4 is induced by phosphate deficiency in the medium.

The effect of DNA and RNA on nuclease synthesis was studied in similar experiments with washed *P. mirabilis* cells. As shown in Fig. 3, the addition of DNA or RNA at a concentration of 1 mg/ml did not influence growth of the culture, slightly augmented the protein content of cells, and did not induce enzyme synthesis. At the same time, when *P. mirabilis* was incubated in media with 1 mg/ml DNA or RNA as the sole source of carbon and phosphorus, growth was noticeably suppressed, protein content of the cells slightly increased, and nuclease activity rose by two–three times (Fig. 3). These data suggest that nuclease synthesis is induced in the medium containing poorly metabolizable sources of nitrogen and phosphorus.

Previous experiments with *Serratia marcescens* showed that the synthesis of extracellular endonuclease by this bacterium is induced by mitomycin C [5, 6]. In these experiments with *P. mirabilis*, mitomycin C added to the nutrient broth at the moment of inoculation at a concentration of 0.5 μg/ml augmented the nuclease activity of CL and did not affect nuclease activity in the periplasm (Fig. 4b). In this case, the protein content of cells increased by 50%. Chloramphenicol (10 μg/ml) added to the medium in addition to mitomycin C prevented the increase in nuclease activity induced by mitomycin C. These data provide further evidence that the increase in the nuclease activity of CL is related to the de novo synthesis of the enzyme.

In the next set of experiments, mitomycin C (0.5 μg/ml) was added, either by itself or in combination with 10 μg/ml chloramphenicol, to the growth medium in the late exponential phase. The culture biomass, the protein content of cells, and the nuclease activity were measured 1 and 3 h after the addition of the antibiotics (Figs. 5a, 5b). It is evident that the addition of mitomycin C in the late exponential phase led to a considerable increase in the nuclease activity of CL and virtually did not influence the enzyme level in the periplasm. The inducing effect of mitomycin C was prevented by chloramphenicol.

Thus, mitomycin C induced the synthesis and secretion of nuclease by *P. mirabilis* cells. In this case, the nuclease activity of the periplasm changed insignificantly; therefore, excess nuclease was secreted into the medium. Chloramphenicol prevented the inducing effect of mitomycin C, suggesting that nuclease was synthesized de novo. Taking into account that mitomy-

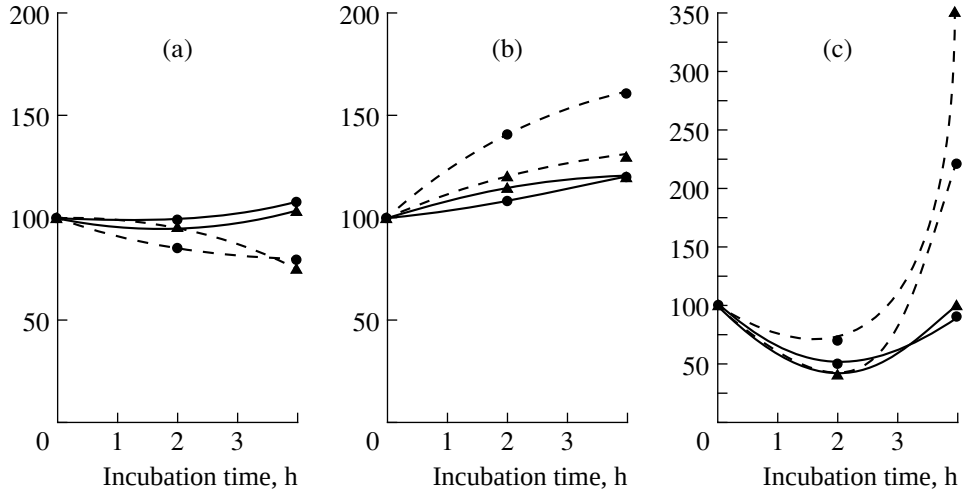


**Fig. 3.** Changes in (a) the biomass ($OD_{650}$), (b) the protein content, and (c) the nuclease activity of washed cells of *P. mirabilis* 4 incubated in media supplemented with DNA (circles) or RNA (triangles) as the supplementary sources (solid lines) or the sole sources of nitrogen and phosphorus (dashed lines). Biomass, protein content, and nuclease activity at zero time points were taken to be 100%.

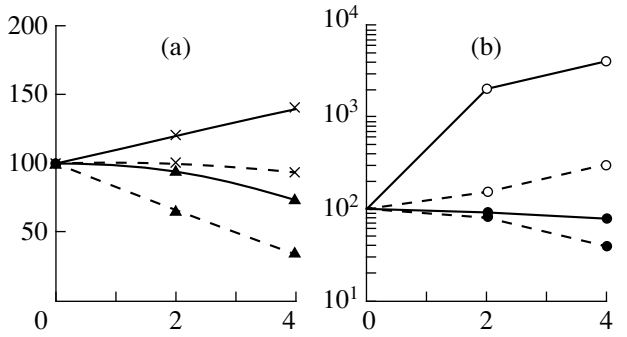


**Fig. 4.** Changes in (a) the biomass (triangles), the protein (crosses) activity, and (b) the nuclease activity in the incubation medium (open circles) and the periplasm (dark circles) of washed cells of *P. mirabilis* 4 incubated in the presence of mitomycin C (solid lines) or mitomycin C and chloramphenicol (dashed lines). Biomass, protein content, and nuclease activity at zero time points were taken to be 100%.

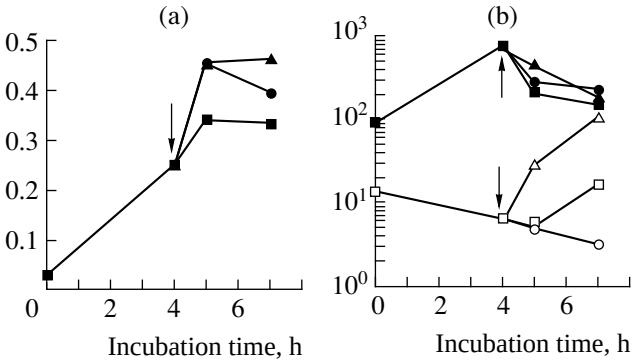


**Fig. 5.** Changes in (a) protein concentration (mg/ml) and (b) nuclease activity (U/mg protein) in the incubation medium (open symbols) and the periplasm (dark symbols) of washed cells of *P. mirabilis* 4 incubated in the absence of antibiotics (circles) and in the presence of mitomycin C (triangles) or mitomycin C and chloramphenicol (squares). Arrows indicate the time of addition of antibiotics.

cin C is an inducer of the SOS functions of cells [7], the results obtained in this study provide evidence that the synthesis of *P. mirabilis* nuclease, like that of *S. marcescens* endonuclease, is induced in response to the blocking of the DNA repair system and is regulated similarly to the SOS response of cells.

There are grounds to believe that nuclease synthesis in *P. mirabilis* cells is regulated by nucleic acids (induction by substrate) and inorganic phosphate (end-product inhibition). The synthesis and secretion of nuclease by *P. mirabilis* cells were induced by mitomycin C, suggesting the involvement of the SOS-response proteins in the regulation of this secretory nuclease.

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