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MICROBIOLOGY

Peculiarities of *Proteus mirabilis* Extracellular Metalloproteinase Biosynthesis

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Abstract—Biosynthesis of metalloproteinase by the *Proteus mirabilis* 5127-1 strain on different media and the influence of glucose and urea on biosynthesis were studied. It was found that the *P. mirabilis* 5127-1 bacteria secretes metalloproteinase in the medium in two isoforms (52 and 50 kDa). It was established that proteinase synthesis is completely suppressed during the growth of bacteria on synthetic media, as well as in the presence of glucose in the LB medium. It was demonstrated that addition of urea in the medium results in an increase of the culture productivity in the proteinase synthesis. Maximal culture productivity in the proteinase synthesis was found in the medium with natural urine. During the growth of bacteria on artificial urine, proteinase appeared in the medium only after 12 hours of growth as a single isoform.

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INTRODUCTION

Proteus mirabilis (opportunistic Gram-negative bacterium from the Enterobacteriaceae family) is a causative agent of opportunistic and hospital infections that affect the respiratory tract, skin, burn surfaces, and wounds (Endimiani et al., 2005). *P. mirabilis* is most frequent cause of urinary tract infections, especially during continuous catheter use (Jakobsen et al., 2008). Extracellular metalloproteinase is one of virulence factors for these bacteria (Senior et al., 1987). A correlation between the strain capabilities to swarm and of proteinase secretion was established. The strains that are not capable of swarming were proteolytically inactive (Senior, 1999; Walker et al., 1999). The *P. mirabilis* metalloproteinase gene (*zapA*) is included in the operon, which involves genes encoding the ATP-dependent transport system; this system allows enzyme secretion to the cultural medium. The protein encoded by the *zapA* gene belongs to the serralysin family of proteases (Wassif et al., 1995).

Transcriptome analysis of swarming *P. mirabilis* cells, showed that the *zapA* gene expression increases in swarming cells, reaching its maximum at the consolidation stage (Pearson et al., 2010). At the same time, the study of expression of the *P. mirabilis* genes in vivo in the process of urinary tract infection demonstrated that the *zapA* gene was expressed at the basic level or even lower. It is assumed that either expression of this gene can be specific to the place of localization of bacteria in the urinary tract or insignificant changes at the level of transcription may result in significant changes in the activity (Pearson et al., 2011).

The aim of this study was to characterize extracellular proteinase biosynthesis by the *P. mirabilis* 5127-1 strain on different media and effect of glucose and urea presence in the cultivation medium. The *P. mirabilis* 5127-1 strain was provided by E.S. Bozhokina (Institute of Cytology, Russian Academy of Sciences).

MATERIALS AND METHODS

Bacteria were cultivated at 37°C with an intensity of oscillation of 200 rpm (shaker, Braun, Germany). Luria-Bertani (LB) medium, M9 synthetic medium, and synthetic and natural urine were used for cultivation of bacteria. The LB medium composition (%) was the following: tripton (1), yeast extract (0.5), NaCl (0.5), pH 8.5. The LBA medium contained 2% agar (Sambrook et al., 1989). Urea in final concentrations of 4–600 mM was introduced in the nutrient medium before inoculation. 1.5% of glucose was added to the LB medium in order to study the effect of glucose. The M9 medium composition (g/L) was the following, including Na_2HPO_4 (6), KH_2PO_4 (3), NaCl (0.5), NH_4Cl (1). Glucose and glycerine in the final concentration of 0.2% were added to the M9 medium. Vitamin B1 was added to a final concentration of 1 mg/mL. 1 mL of 1 M $MgSO_4 \cdot 7H_2O$ solution and 10 mL of 0.01 M $CaCl_2$ solution were added to 1 L of the medium after autoclaving.

The composition of artificial urine (g/L) (Jones et al., 2007) was the following: pepton L37 (1), yeast extract (0.05), lactic acid (0.1), citric acid (0.4), urea (10), $CaCl_2 \cdot 2H_2O$ (0.37), NaCl (5.2), $FeSO_4$ (0.012), $MgSO_4 \cdot 7H_2O$ (0.49), $Na_2SO_4 \cdot 10H_2O$ (3.2), KH_2PO_4 (0.95), K_2HPO_4 (1.2), NH_4Cl (1.3), distilled H_2O. Child's combined urine, which was sterilized by filtration through a membrane filter with a pore diameter of 0.2 μm, was used as the natural urine. The sterility was confirmed by plating on the LB medium. A 12-h culture grown on the LB medium was used as an inoculum. A medium of the following composition (g/L): casein (5), yeast extract (5), NaCl (5), agar (20), was used for determination of caseinolytic activity.

The nucleotide sequence of the 16S rRNA gene was determined and analyzed by previously described method (Janda and Abbott, 2007). The increase in biomass was nephelometrically measured using a KFK-2 photoelectrocalorimeter at the wave length 590 nm. Biomass was expressed in light absorption 1 cm thick cuvette. The total amount of protein was determined by Bradford's assay (Bradford, 1976). Bioinformatic analysis of the *zapA* gene promoter region was conducted by means of the ASAP database (https://asap. ahabs.wisc.edu/asap/home.php) and BPROM program (www.softberry.com).

Proteolytic activity in the cultural liquid was determined according to casein decomposition by Kaverzneva's assay (Kaverzneva, 1971). The amount of enzyme that resulted in release of 1 μmole tyrosine per 1 min was taken as a unit of caseinolytic activity. Azocasein was decomposed by the previously described method (Wassif et al., 1995). The amount of enzyme that results in an increase in the optical density by 0.1 unit was taken as the unit of activity. The culture productivity relative to proteinase synthesis was determined as the ratio of proteolytic activity in the cultural liquid to biomass and was expressed in conventional units. The cultural liquid supernatant was obtained by centrifugation of the culture at 13000 rpm for 15 min.

10 mM *ortho*-phenanthroline and 2 mM PMSF were introduced in the final concentration in incubation mixtures in the experiments on the study of the effect of inhibitors.

Protein electrophoresis in polyacrylamide gel (PAAG) in the presence of sodium dodecyl sulfate was conducted according to the standard method (Laemmli, 1970). Zymography of the *P. mirabilis* cultural liquid samples in PAAG with gelatin (1 mg/mL) was conducted by a previously described method (Oliver et al., 1999). In order to conduct quantitative analysis, gels were scanned and the obtained images were processed using the Quanti Scan 2.1 program.

Statistics was done using the Microsoft Excel program. The results were considered significant if $\sigma \leq 15\%$. Student's *t*-test was used as a criterion of significance of the obtained differences ($P < 0.05$ is a reliable level of significance).

RESULTS AND DISCUSSION

The phylogenetic location of the strain 5127-1 was established by analysis of the 16S rRNA gene sequence. It was demonstrated that the strain 5127-1 belongs to the *P. mirabilis* species. The similarity of 16S rRNA genes between strains 5127-1 and the *P. mirabilis* CIFRI Ch-TSB28 was 98.9%. During the growth of bacteria of the 5127-1 strain on agar medium with casein, the strain demonstrated the ability to swarm and produced zones of hydrolysis, that indicated its proteolytic activity.

Substrates such as casein, azocasein, and gelatin were used for the study of specificity of the *P. mirabilis* 5127-1 strain proteinase. It was demonstrated that this microorganism is able to decompose azocasein, gelatin, and casein, where casein is decomposed less favorable substrate. These results are in agreement with the literature data, according to which many *P. mirabilis* strains more efficiently decompose gelatin than casein (Senior, 1999). Azocasein was used in further experiments for determination of proteolytic activity in the cultural liquid (gelatin, for zymography).

The dynamics of growth and biosynthesis of extracellular *P. mirabilis* proteinases was studied during cultivation on the LB medium at 37°C. The stationary stage of growth started after 11–12 h of cultivation. Proteinases were released into the medium after 5 h of growth of bacteria and continued secreting at all stages of the culture development (Fig. 1); they reached a maximum at the stationary stage of the growth of bacteria. Extracellular proteolytic activity was completely suppressed by 10 mM *ortho*-phenanthroline (metalloproteinase inhibitor) and was insensitive to 2 mM PMSF (serine proteinase inhibitor). Thus, extracellular *P. mirabilis* 5127-1 proteinase is a metalloproteinase.

The growth of bacteria and proteinase biosynthesis was studied on the synthetic M9 medium, which was modified by addition of different organic components. Glucose and glycerine were added to the saline basis with thiamine (1 mg/mL) as a source of carbon at a final concentration of 0.2%. It was established that bacteria grow more than two times more slowly on the modified synthetic M9 media than on the LB medium (16 h cultivation) (Fig. 2). Proteolytic activity relative to azocasein was found in all variants of media in trace amounts. However, it was no more than 2% of the enzyme activity in the cultural liquid of bacteria grown on the LB medium.

It was demonstrated that two metalloproteinase isoforms are present in the cultural liquid of different *P. mirabilis* strains (Senior, 1999; Pearson et al., 2008). Zymography of the cultural liquid proteins allowed the detection of isoforms of extracellular *P. mirabilis* proteinase with molecular weight 52 and 50 kDa (Oliver et al., 1999). The reasons for generation of two isoforms are not known. It is assumed that the isoform with $M = 50$ kDa is generated due to the enzyme degradation (Oliver et al., 1999). The dynamics of of pro-

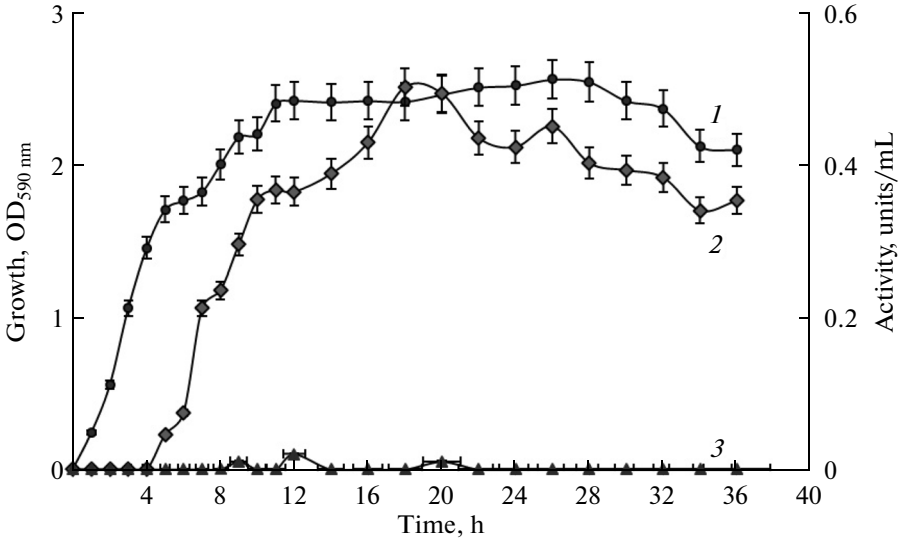

Fig. 1. Dynamics of growth (*1*) and *P. mirabilis* extracellular proteolytic activity using azocasein as a substrate (*2*), as well as proteolytic activity in the presence of 10 mM *ortho*-phenanthroline (*3*). $OD_{590\ nm}$, optical density of the culture at 590 nm.

teinase isoforms appearance in the cultural liquid of the *P. mirabilis* isolate 5127-1 (LB medium) over time was studied by the method of zymography (Fig. 3). Extracellular proteolytic activity was low after 6 h of the culture growth, and only one enzyme isoform with $M = 52$ kDa was observed in the cultural liquid; proteolytic activity increased after 12 h, and the isoform with $M = 50$ kDa appeared. The activity of the isoform with $M = 50$ kDa was insignificant. Two enzyme isoforms, the activity of which remained after 20–24 h, were found after 18 h of growth. The gel scanning demonstrated that the isoform with $M = 52$ kDa is more expressed as compared with the isoform with $M = 50$ kDa. The fact that the protein with $M = 52$ kDa is mainly observed in the medium in the early hours of cultivation and that 50 kDa form appears only after 12 h of culture growth and then its amount increases allows us to hypothesize that the 50 kDa form of proteinase appears as a result of particular proteolysis of the main form.

Synthesis of many enzymes is regulated by catabolite repression (that is, suppressed in the presence of easily metabolizable sources of carbon (for example, glucose) (Vázquez et al., 2009). The *P. mirabilis* bacteria were cultivated on the LB medium containing 1.5% of glucose. The presence of glucose resulted in a decrease in the growth of bacteria as compared with the control LB medium by 20% and in a decrease in proteolytic activity relative to azocasein by 90–95% after 16 and 24 h of cultivation, respectively (Fig. 4). Bioinformatic analysis of the *zapA* gene promoter region allowed us to identify the presumable binding site for catabolite repression activator protein (CRP) (Fig. 5). The consensus of the CRP factor binding site in *Escherichia coli* cells is the sequence 5'-AAATGTGATCTAGATCACATTT-3' (Leuze et al., 2012). The sequence of presumable *P. mirabilis* site 5'-TCAAACTT-3' is located between conservative –10 and –35 promoter sites and is similar by 75% with the *E. coli* sequence TCACATTT. The results obtained

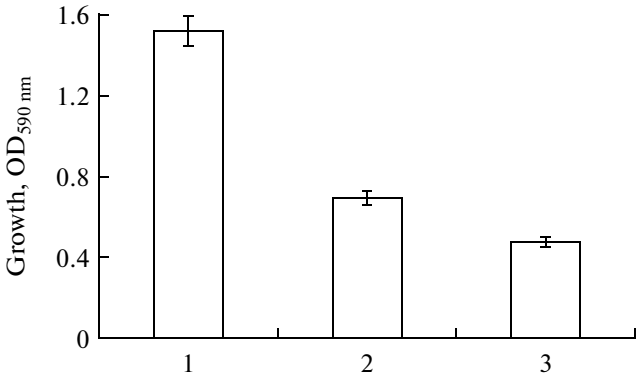

Fig. 2. Growth of *P. mirabilis* on different media. 1, LB medium; 2, synthetic M9 medium + thiamin + glycerine; 3, M9 medium + thiamin + glucose.

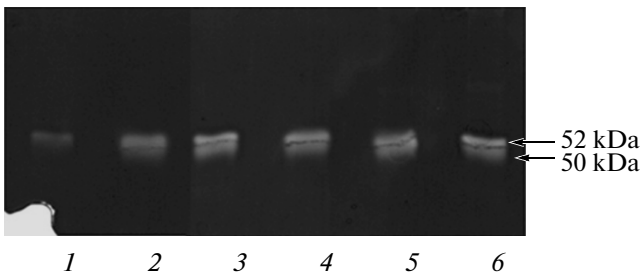

Fig. 3. Zymograms of *P. mirabilis* spent media during growth on the LB medium at 37°C. *1*, 6; *2*, 12; *3*, 18; *4*, 20; *5*, 22; *6*, 24 h. Substrate, gelatin.

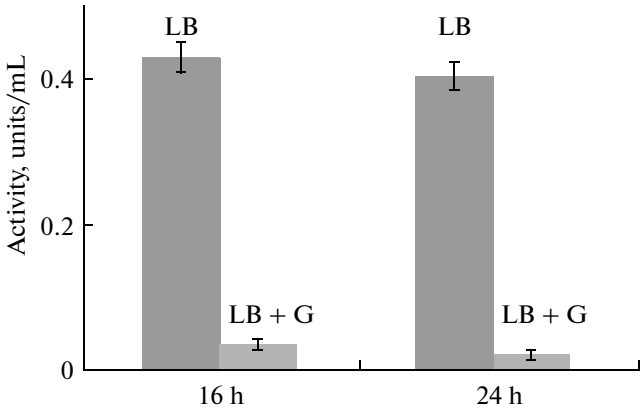

Fig. 4. Proteolytic activity in *P. mirabilis* spent LB media and LB with 1.5% glucose (LB + G) media.

AATATTATTATTACTATAGAAATAAAACAAACATCAAACATCAAACATCAAACATCAAACATCAAACATCAAACA

−35 *crp* −10

ACTTAACATAATAAATGATACATTAATTTTAAACAAACCCATTAA**TTAAAT**TAAAAA*TCAAACTT***AAGTAAAA**TT

TTCCTTTCAATTATAAAACAATAACTAACAATAGAAATCATCTAAAAATAAAATATAACTATACTTATATTCACCT

CATTAAATTATTCTGACTTAAAACACACTTTACTTCACAATTATTTAAAAACTTATTTTACCCCCCTAGATTAATAA

TAATTAATGTCTATCTTTATCCTCAATGGCATTCGCCAGATAAATATATTTTCAGTATGAAAATAAACTTAACCTAA

SD

ATTTATATCTGGAGGATATT**ATG**GGATCT

Fig. 5. Putative CRP transcription factor binding site 5'-TCAAACTT-3' in the promoter region of the *P. mirabilis zapA* gene. SD, Shine–Dalgarno sequence. ATG, start codon.

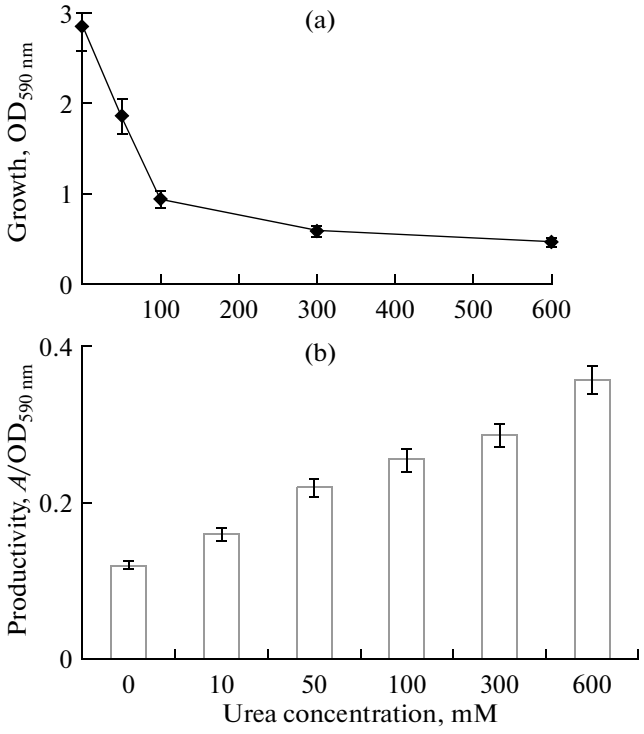

Fig. 6. Dynamics of *P. mirabilis* bacteria growth (a) and productivity of extracellular proteinase biosynthesis (b) in the presence of urea in the medium. Bacteria were cultivated 16 h at 37°C on the LB medium containing 0–600 mM urea. *A*, azocasein is used as a substrate, units/mL; for Figs. 6 and 7.

indicate that biosynthesis of the *P. mirabilis* extracellular proteinases can be regulated by catabolite repression.

It is known that the *P. mirabilis* bacteria cause urinary tract infection. Synthesis of extracellular enzymes can be regulated by different components of the medium. Accumulation of extracellular proteinase in the medium was studied in the presence of urea. The urea content in adult urine normally reaches 400–700 mM (Liu et al., 2012). We studied the effect of urea (10–600 mM) on the strain growth and metalloproteinase activity during the *P. mirabilis* cultivation for 16 h. Urea in the concentration of 50–100 mM resulted in a sharp inhibition of the culture growth (by 50–70% of the control) (Fig. 6a). The optical density of the culture did not exceed 15–20% of the control in the presence of 300 and 600 mM urea. In these conditions, the culture productivity (by extracellular proteinase synthesis) increased 2.5–3 times (Fig. 6b).

Accumulation of the *P. mirabilis* proteinase was studied during culture growth on artificial and natural urine as compared with those on LB medium with 50 mM urea and without urea (Fig. 7). Maximal growth of bacteria was observed on the LB and LB with 50 mM urea media. The growth on artificial and natural urine was 20–30% of the growth on the LB medium (Fig. 7a). Low culture productivity by extracellular proteinase was observed on the control medium (LB medium). The culture productivity

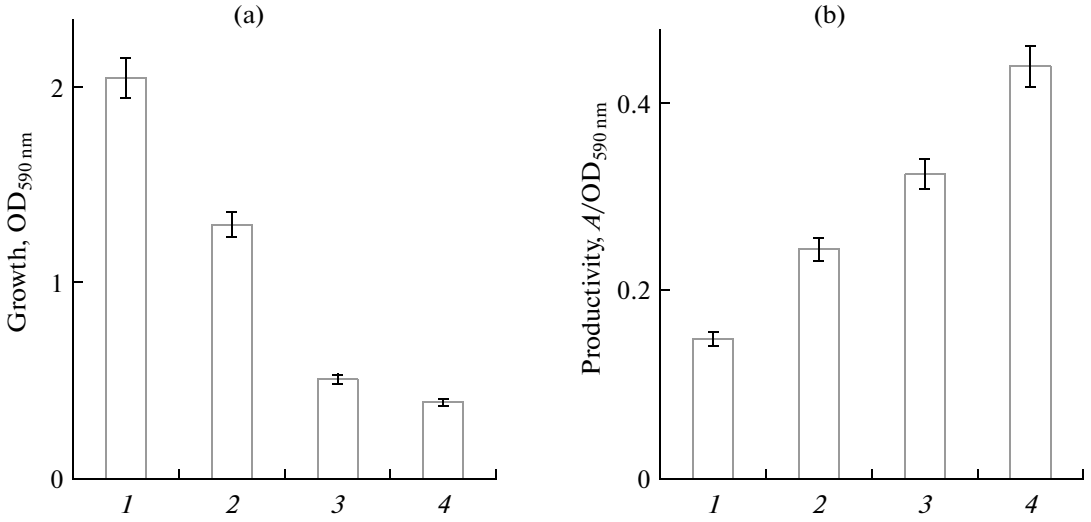

Fig. 7. *P. mirabilis* growth (a) and culture productivity on proteinase biosynthesis (b) on different media. *1*, LB medium; *2*, LB medium + 50 mM urea; *3*, artificial urine; *4*, natural urine.

increased by 60–65% in the presence of 50 mM urea in the medium. The culture productivity by proteinase on artificial and natural urine was higher by 2–2.5 and 3 times, respectively, as compared with the control (Fig. 7b). The data obtained allow us to conclude that urea is one of the factors influencing the induction of the *P. mirabilis* extracellular metalloproteinase biosynthesis.

The dynamics of proteolytic activity accumulation during the growth of bacteria on artificial urine was also studied (Fig. 8). According to zymography data, the activity was detected in the medium after 12 h of growth and reached a maximum after 48 h. As compared with this, proteolytic activity was found after 6 h of growth of bacteria and reached a maximum after 12 h of growth on the LB medium (Fig. 3). It was previously demonstrated that the active enzyme can be found in urine of patients with a urinary tract infection caused by bacteria from the *Proteus* genus (Senior et al., 1991). An important difference consists in the fact that two metalloproteinase isoforms (52 and 50 kDa) were generated during the growth of bacteria on the LB medium. Only one isoform (52 kDa) was observed during the growth of bacteria on artificial urine, indicating differences in accumulation of proteinase isoforms on different media.

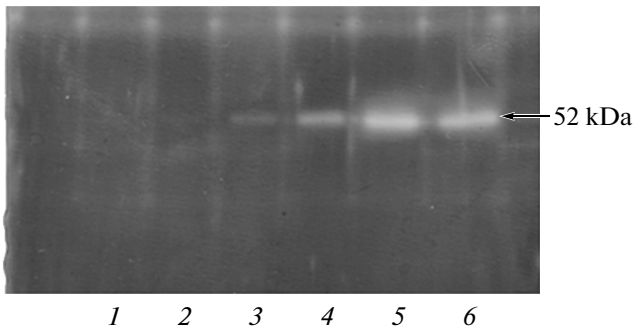

Fig. 8. Zymograms of *P. mirabilis* spent media during the growth of bacteria on artificial urine. *1*, 4; *2*, 8; *3,* 12; *4*, 24; *5*, 48; *6*, 72. Substrate, gelatin.

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