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EXPERIMENTAL ARTICLES

Disruption of Bacterial Biofilms Using Recombinant Dispersin B

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Abstract—A synthetic gene encoding dispersin B of *Aggregatibacter actinomycetemcomitans* was cloned and expressed in *Escherichia coli* cells. Procedure for purification of recombinant dispersin B was developed, and its in vitro activity was determined. The enzyme was used in experiments on disruption of the biofilms formed by various microorganisms. It exhibited high activity against *Staphylococcus epidermidis* biofilms. The biofilms formed by *Burkholderia cenocepacia* and *Achromobacter xylosoxidans* were more resistant to the recombinant enzyme.

Keywords: Aggregatibacter actinomycetemcomitans, Escherichia coli, Staphylococcus epidermidis, Burkholderia cenocepacia, Achromobacter xylosoxidans, recombinant dispersin B, heterologous expression, biofilm disruption

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Biofilms formed by several groups of microorganisms aid their survival in changing environment. The matrix of microbial biofilms is a complex heterogeneous structure which involves proteins, nucleic acids, lipids, phospholipids, and other substances. The major components of the matrix are exogenous polysaccharides synthesized by microorganisms [1]. Enzymatic degradation of the matrix is regarded as a promising method for biofilm disruption [2] to fight microbial contamination and pollution.

For some bacteria, the polysaccharide component of the matrix was found to contain poly-β-1,6-*N*-acetyl-D-glucosamine [3, 4]. The only enzyme known to disrupt poly-β-1,6-*N*-acetyl-D-glucosamine is dispersin B, synthesized by *Aggregatibacter actinomycetemcomitans*, a pathogenic bacterium inhabiting human oral cavity [5, 6]. Dispersin B is a 40 kDa protein, a glycosyl hydrolase of the 20th family of β-hexosaminidase (EC 3.2.1.52) [6, 7].

Disruption of bacterial biofilms with dispersin B could be achieved using either the enzyme isolated from its natural source (*A. actinomycetemcomitans*) [7] or recombinant dispersin B [8, 9]. From the biotechnological point of view the latter is advisable.

The goal of the present work was to obtain purified recombinant dispersin B and to study its influence on the biofilms formed by various species of bacteria. For this purpose, a synthetic gene coding for dispersin B from *A. actinomycetemcomitans* was cloned in *Escherichia coli* cells. The protein accumulated by the producing strain was isolated and purified. Its effect on the biofilms of *Staphylococcus epidermidis, Burkholderia cenocepacia*, and *Achromobacter xylosoxidans* was studied.

MATERIALS AND METHODS

Subjects of study and cultivation conditions. The following microorganisms were used in the study: *Achromobacter xylosoxidans SCCH3:Ach 33-1365 glt allele 2; Burkholderia cenocepacia SCCH2:Bcn33-1220 ST709* (strains from collection of Institute of Pulmonology, Ministry of Health of the Russian Federation, Moscow); *Staphylococcus epidermidis* 210 (strain from collection of Laboratory of Molecular Epidemiology of Nosocomial Infections, N.F. Gamaleya Federal Scientific Research Center of Epidemiology and Microbiology, Ministry of Health of the Russian Federation).

S. epidermidis was grown in Mueller-Hinton broth; other bacteria were grown in L-broth at 35°C with intense aeration on thermostatic shakers for 16 h in 200-mL flasks with 20 mL of the medium. Grown cultures were used as inocula.

Cloning of the *dspB* gene coding for dispersin B from *A. actinomycetemcomitans*. The synthetic gene sequence used for cloning in *E. coli* was planned in the view of codon composition of *E. coli* and the absence of pronounced mRNA secondary structure. For subsequent cloning, the sequence was flanked with restriction sites *Nco*I and *Kpn*2I. The amino acid sequence, encoded by the synthetic gene, corresponded to the sequence of dispersin B glycosyl hydrolase from *A. actinomycetemcomitans* (DspB) (NCBI

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Reference Sequence: ZP 11583848.1). Synthesis of oligonucleotides and gene assembly were carried out at Evrogen (Moscow, Russia). For cloning, the vector pQE 13 and strain *E. coli* M15 [pREP4], (F^-, Nal^s Str^s Rif^s lac^- ara^- gal^- mtl^-) (Quagen, United States) were used.

Nucleotide sequence identity of the cloned gene to that of the dispersin B gene was confirmed by sequencing. Molecular mass of recombinant dispersin B calculated by amino acid sequence was 43.3 kDa.

Isolation and purification of recombinant dispersin 6His-DspB. To obtain the biomass, 2 mL of 16-h culture of producer strain *E. coli* M15 (6His-DspB) was used to inoculate 200 mL of liquid LB medium with kanamycin (25 μg/mL) and ampicillin (150 μg/mL) in 500-mL Erlenmeyer flasks. The cultures were grown on a thermostatic rotary shaker (700 rpm, 37°C) to OD_{600} 1.0–1.5. Optical density was measured on an Ultrospec 1100 pro spectrophotometer (Amersham, United States) in 1-cm cuvettes. Then isopropyl-β-D-thiogalactoside (IPTG) was added to the final concentration 0.2 mM and cultivation was continued for another 12 h at 25°C. The biomass was collected by centrifugation in a 4K15 Sigma centrifuge (United States) at 6000 *g* for 15 min and washed from growth media with 0.9% NaCl solution. Biomass yield was approximately 4 g/L. Obtained biomass was used further for isolation of the target protein.

For this purpose, 600 mg of wet cell biomass was suspended in 2.5 mL of the buffer solution (50 mM Tris-HCl, pH 7.5 and 50 mM NaCl), and the cells were lysed with lysozyme (4 mg/200 μg) at room temperature for 30 min. The lysate was treated with ultrasound on ice for 2 min at impulse mode with 5 s intervals and impulse power 30% of maximum on Bandelin Sonopuls sonicator (Bandelin Electronics, Germany). The volume of the suspension was adjusted to 4 mL with buffer, the debris was removed by centrifugation on a 4K15 Eppendorf 5424 (Germany) centrifuge (15 min, 4°C). The supernatant volume was adjusted to 4 mL with the buffer, and 121 μL of the buffer containing 1 M imidazole-HCl (pH 8.0) was added. Then 1.33 mL 50% suspension of affine sorbent Workbeads Ni Bio-Works 40 was added, preliminarily equilibrated with 30 mM imidazole-HCl buffer, pH 8.0. After 30 min incubation at 4°C, the mixture was transferred onto a chromatographic column (diameter 10 mm, height 10 cm). Chromatography was carried out at room temperature on BioLogicLP (Bio-Rad, United States). The column was washed with 12 mL of 1 M NaCl solution in 20 mM Tris-HCl, pH 7.5 with 30 mM imidazole. Then the protein was eluted with 1 M imidazole-HCl buffer solution, pH 8.0. The fraction containing the target protein was collected, its volume and protein concentration were 2.5 mL and 9 mg/mL, respectively, as well as the following fractions: total volume of 3 mL and protein concentration 1.8 mg/mL. Obtained fractions were dialyzed during 16 h against 20 mM Tris-HCl buffer, pH 8.0 (2 changes of 500 mL). Protein content was measured by the Lowry method.

Lyophilization of purified dispersin B was carried out from 200 μL volume (solution in Tris-HCl 20 mM, pH 8.0 buffer) in concentrations of 2.5, 6, and 9 mg/mL. After drying, the flasks were sealed under vacuum.

Activity determination of isolated dispersin B preparation. The enzymatic reaction was carried out according to the previously developed method [6], in 1.5 mL of the reaction mixture. The mixture contained 50 mM K-phosphate buffer, pH 5.9, 100 mM NaCl and 10–100 μg of purified target protein. The reaction was started by addition of 0.5 mL 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide (4 mg/mL) solution (Sigma) in phosphate buffer (of the composition reported above) to the mixture heated to 37°C. The reaction was conducted at 37°C. The process was stopped by addition of 10 μL 10 N NaOH. Optical density of the solution (E) was measured at 420 nm. The specific enzyme activity was calculated according to the formula $A = E_{420}/(0.0045 \times C \times T)$, (where C is the protein amount, T is reaction time (min), and 0.0045 is the molar extinction coefficient of 4-nitrophenol at 420 nm).

Biofilms formation. Bacteria were grown on the medium containing 1.8% agar (L-agar) for 24 h. Obtained bacterial cultures were collected with a loop and used to inoculate 5 mL broth. Liquid cultures were grown under intense aeration at 35°C during 16 h. Experiments on biofilm formation were carried out as reported previously [10, 11] in sterile 96-well plates (Costar, United States). Sample volume was 100 to 200 μL. Bacterial concentrations in every well of the plate were 10^5 CFU/mL for *S. epidermidis* (in Mueller-Hinton broth) and 10^6 CFU/mL for *A. xylosoxidans* and *B. cenocepacia* (in M9 medium with addition of 0.4% casamino acids). The plates were incubated for 24–72 h at 35°C and elevated humidity without mixing. The biofilm formed on the well walls was stained for 30 min with 0.1% crystal violet solution (for *S. epidermidis*, with 0.1% gentian violet solution) at room temperature and washed twice with 0.9% NaCl solution. Then 250 μL 30% acetic acid was added (for *S. epidermidis*, 100 μL 96% ethanol). Optical density of solutions obtained in the wells was measured with Multiscan FC microplate photometer (Thermo Scientific, United States). It is important to note that the rate of formation of stable biofilms in the wells differed for the cultures used in study: it was 48 h for *A. xylosoxidans* and *B. cenocepacia* and 72 h for *S. epidermidis*.

Effects of dispersin B on biofilms. Activity of the isolated recombinant enzyme dispersin B in prevention of biofilm formation, as well as its effect on already formed biofilm, was determined. In the first case, dispersin B, dissolved in growth medium in vari-

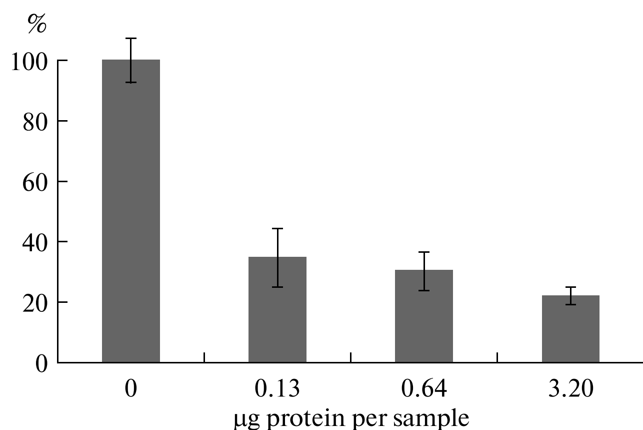

Fig. 1. Disruption of mature biofilms formed by *S. epidermidis* by dispersin. 100% corresponds to $E_{540} = 0.75$ for gentian violet.

ous concentrations, was added to the wells (10% vol/vol), together with culture suspension. Then incubation was continued at 35°C. The same amount of growth media without the enzyme was added to the control wells.

In the second case, 2 h before formation of mature biofilms, suspension of planktonic culture was carefully removed from each of the wells, and dispersin solution in various concentrations in 60 mM Na-phosphate buffer, pH 7.4, was added to the wells (no more than 10% of the sample volume). The volume was adjusted to the former value (200 μL) with growth medium, and the plates were incubated for 2 h at 35°C. After incubation the biofilms were stained with crystal violet or gentian violet as described previously.

RESULTS AND DISCUSSION

Cloning and expression of the dispersin gene. Isolation and purification of recombinant protein. In contrast to earlier studies dedicated to production of recombinant dispersin by *E. coli* [9], in the present work we used an approach based on cloning of a synthetic gene sequence with codon composition optimized for expression in *E. coli*. This resulted in obtaining an efficient dispersin producer, with the level of enzyme synthesis at IPTG induction reaching 10 to 20% of the total cell protein. Six histidine residues located on the N-end of the recombinant dispersin B molecule made it possible to isolate an electrophoretically homogeneous protein with molecular mass of 43.3 kDa by means of metal-ion affine chromatography. Molecular mass of the protein was close to those previously reported for recombinant dispersins [8] and is consistent with the calculated data based on its amino acid sequence. The fractions collected from the column were combined, and the protein concentration in the resulting sample was 5.8 mg/mL.

Enzymatic activity of recombinant dispersin in vitro. The β-glycosyl hydrolase activity of enzyme was studied using the substrate nitrophenyl-*N*-acetyl-β-D-glucosaminide (NAGA). Specific activity of the enzyme was 84.3 ± 2.1 nmol 4-nitrophenol/mg protein per minute at 37°C.

Lyophilization of the enzyme with subsequent dissolving in phosphate buffer, single freezing of the protein solution, or its storage as a solution in phosphate buffer for 2 weeks at 4°C did not result in a loss of its activity in vitro (data not shown).

Effect of recombinant dispersin on bacterial biofilms. Dispersin B was previously shown to disrupt bacterial biofilms [5, 6, 11]. Recombinant protein isolated in the present work was able to disrupt the biofilms formed by various species of model bacteria.

In our in vitro experiments with the *S. epidermidis* clinical strain, the recombinant dispersin actively disrupted mature biofilms even at relatively low concentrations of the protein (less than 0.3 μg protein in the sample (Fig. 1). This agrees well with the information of recombinant dispersin activity against *S. epidermidis* biofilms reported by other authors [9]. The revealed activity against biofilms correlated with substrate specificity of the protein because the main matrix exopolysaccharide of *S. epidermidis* biofilms is poly-*N*-acetylglucosamine, the substrate of dispersin B [11].

Investigation of the lytic activity of recombinant dispersin B against biofilms formed by clinical strains of *A. xylosoxidans* and *B. cenocepacia* demonstrated that in those cases activity was lower than in the case of *S. epidermidis* (Fig. 2a). It was previously found that bacteria of the genus *Burkholderia* also form biofilms with poly-*N*-acetylglucosamine-containing matrix. However, unlike *Staphylococcus*, this polysaccharide is not the major component of the matrix [4]. Bacteria of the genus *Burkholderia* also produce other polysaccharides, which are incorporated into the biofilm matrix, including cepacian, which is not a substrate for this glycosyl hydrolase [4]. Activity of dispersin B in *Burkholderia* might be low due to the fact that the enzyme disrupts only a part of the polysaccharide matrix. The same explanation might apply to the matrix of *A. xylosoxidans* biofilms as a target to enzymatic attack.

Activity of recombinant dispersin B in prevention of biofilm formation by *A. xylosoxidans* and *B. cenocepacia* was also low (Fig. 2b).

Thus, an efficient producer of recombinant dispersin B based on *E. coli* was obtained. The purified protein possessed enzymatic activity identical to the activity of native dispersin B from *A. actinomycetem-*

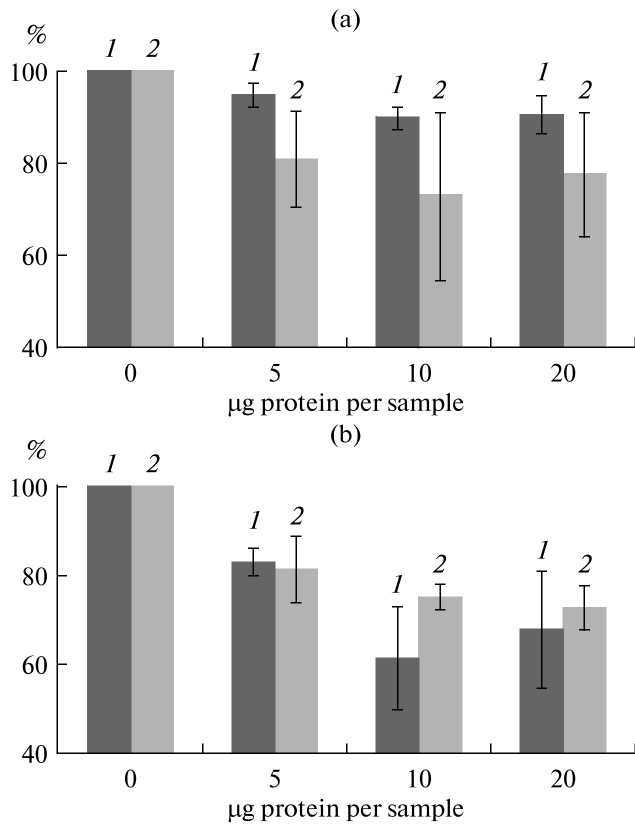

Fig. 2. Effect of dispersin B on biofilms formed by *B. cenocepacia* (*1*) and *A. xylosoxidans* (*2*): disruption of mature biofilms by dispersin, with 100% for *B. cenocepacia* and *A. xylosoxidans* corresponding to $E_{540} = 0.165$ and 0.21 for crystal violet, respectively (a). Inhibition of biofilm formation, with 100% for *B. cenocepacia* and *A. xylosoxidans* corresponding to $E_{540} = 0.26$ and 0.21 for crystal violet, respectively (b).

comitans and was able to disrupt the biofilms formed by different species of pathogenic microorganisms (to a various degree, depending on microbial species). In the future we are planning to test the ability of recombinant dispersin to increase the effects of other antimicrobial agents, which have normally low activity against pathogenic microorganisms existing in biofilms, as was shown previously for the combined effect of dispersin and tobramycin on bacteria of the *B. cepacia* complex [12].

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