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EXPERIMENTAL ARTICLES

The Accumulation of Proteins with Chitinase Activity in the Culture Liquids of the Parent and Mutant *Serratia marcescens* Strains Grown in the Presence of Mitomycin C

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Abstract—The study of the accumulation pattern of extracellular proteins with chitinase activity in the parent *Serratia marcescens* strain Bú 211 (ATCC 9986) grown in the presence of mitomycin C and its mutant strain with the constitutive synthesis of chitinases grown in the absence of the inducer showed that chitinase activity appeared in the culture liquids of both strains at the end of the exponential phase (4 h of growth) and reached a maximum in the stationary phase (18–20 h of growth). The analysis of the culture liquids (12 h of growth) by denaturing electrophoresis in PAAG followed by the protein renaturation step revealed the presence of four extracellular proteins with chitinase activity and molecular masses of 21, 38, 52, and 58 kDa.

Key words: *Serratia marcescens*, chitinase, mitomycin C, induction.

Chitinases (EC 3.2.1.14), i.e., enzymes hydrolyzing chitin, a persistent polymer of *N*-acetyl-D-glucosamine, attract researchers' interest due to their possible application in practice. Chitinases are considered to be potential fungicides, since they can hydrolyze chitin in the cell wall of phytopathogenic fungi [1]. Of great interest is the use of chitinases for obtaining the degradation products of chitin and its deacylated derivative chitosan, which are widely used in biotechnology, agriculture, medical and cosmetic practices, etc. [2].

The enterobacterium *Serratia marcescens* is one of the most potent producers of the extracellular chitinase complex, which contains from two to five proteins with chitinase activity. The biosynthesis of chitinases in *S. marcescens* is induced by their substrate, chitin [3], and by the SOS response inducer mitomycin C [4]. Recently, a mutant strain with the constitutive synthesis of chitinases had been obtained [5].

To the best of our knowledge, there is no information on the accumulation dynamics of chitinases in the culture liquid of *S. marcescens.* To fill this gap and to investigate the possibility of regulating and optimizing chitinase synthesis, we studied here the accumulation of extracellular proteins with chitinase activity in the culture liquids of the parent strain grown in the presence of mitomycin C and its mutant strain grown in the absence of the inducer.

MATERIALS AND METHODS

Experiments were carried out with the prototrophic pigmented *Serratia marcescens* strain Bú 211 (ATCC 9986) obtained from the collection of bacterial cultures at the Institute of Microbiology, Academy of Sciences of Armenia, and its D-5 mutant with the constitutive synthesis of chitinases [5]. The strains were grown in a modified Banting medium under the conditions described earlier [6]. Bacterial growth was monitored by measuring the optical density of cultures at 640 nm (OD_{640}) using 1-cm-pathlength cuvettes. Extracellular proteins were salted out from the culture liquid with sulfate ammonium at 90% saturation. Chitinase activity was assayed by measuring the amount of reducing sugars produced from chitin. The reducing sugars were determined with dinitrosalicylic acid (DNS) [7]. The reaction mixture for chitinase assay contained 0.5 ml of 0.1 M potassium phosphate buffer (pH 6.75), 0.4 ml of 12 mg/ml colloidal chitin, and 0.1 ml of the material tested. The mixture was incubated at 50°C for 30 min. One unit of activity was defined as the amount of enzyme liberating 1 mg of *N*-acetyl-D-glucosamine per hour. The colloidal chitin was prepared by the Chigaleichik and Pirieva method [8]. Glycol chitin was prepared by the Yamada and Imoto method [9]. Extracellular proteins were separated and their molecular masses were determined by denaturing electrophoresis in 12.5% PAAG [10]. The electrophoretically separated proteins were renatured, and their chitinase activity was determined with the fluorescent dye Calcofluor White M2R as described by Trudel and Asselin [11].

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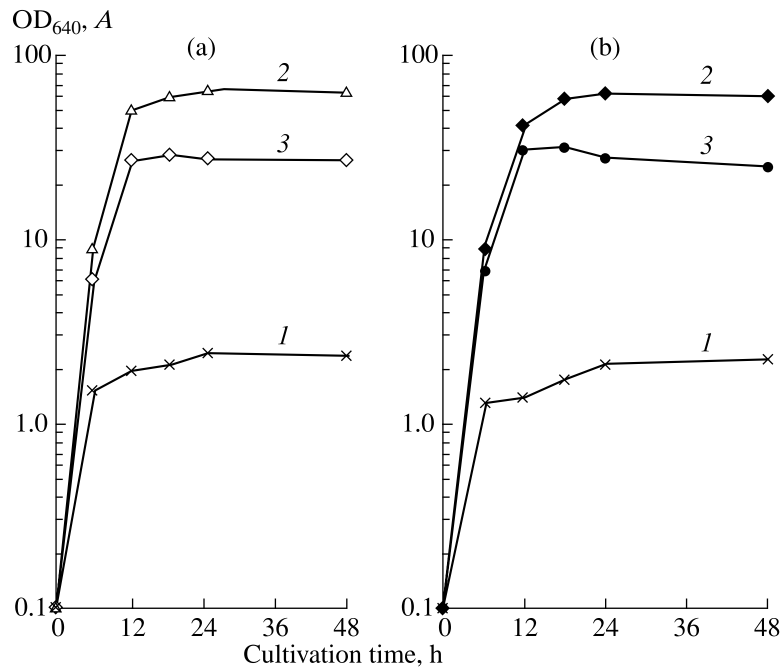

Fig. 1. Dynamics of chitinase activity in the culture liquids of (a) the mutant and (b) parent *S. marcescens* strains grown in the semisynthetic Banting medium in the presence (parent) or absence (mutant) of mitomycin C: (*1*) optical density at 640 nm; (*2*) chitinase activity per ml of the culture liquid; (*3*) chitinase activity per OD unit.

Mitomycin C and *N*-acetyl-D-glucosamine were purchased from Serva (Germany), Calcofluor White M2R was from Sigma (United States), chitin was manufactured in the Voikovskii plant of chemical reagents (Russia), and protein markers were obtained from the SP "Internauka" (Russia).

RESULTS AND DISCUSSION

Experiments showed that the culture liquid of the parent strain grown in Banting medium without inducers (colloidal chitin and mitomycin C) did not contain chitinases in amounts detectable by the assay method used. The addition of mitomycin C to the growth medium in an amount of 0.05 mg/l induced the synthesis of chitinases, which appeared in the culture liquid at the end of the logarithmic phase (4 h of growth). Then the amount of extracellular chitinases exponentially increased to reach a maximum in the stationary phase (18–20 h of growth). In the course of further cultivation (48 h of growth), the chitinase activity of the culture liquid virtually did not change (Fig. 1b). Similar dynamics of chitinase activity was observed in the culture liquid of the mutant strain grown in the semisynthetic Banting medium in the absence of the inducers (Fig. 1a).

The denaturing electrophoresis of extracellular proteins in PAAG with glycol chitin followed by the protein renaturation step and the assay of chitinase activity showed that the parent strain grown in the semisynthetic medium for 12 h produced one 38-kDa protein with chitinase activity (Figs. 2a, 3a). At the same time, this strain grown in Banting medium with mitomycin C for 12 h produced four extracellular proteins with chitinase activity, whose molecular masses were 21, 38, 52, and 58 kDa (Figs. 2b, 3b).

The mutant strain grown in Banting medium without the inducers for 12 h was found to produce the same four proteins (Figs. 2c, 3c). It should be noted that the chitinase activity of the 21-kDa protein was revealed only when the procedures of protein renaturation and chitinase assay after electrophoresis were optimized. Along with this, the molecular mass of one of the proteins of the chitinase complex, which had been determined as 45 kDa [4], was reestimated to be 38 kDa.

For chitinases to be detected by the procedure employed (denaturing electrophoresis followed by the protein renaturation step and chitinase assay), it was necessary that their content in the culture liquid reached a critical level. Thus, proteins with chitinase activity could not be detected electrophoretically in the culture liquids of 4-h-old parent and mutant strains, whereas analysis with DNS showed that these liquids possessed chitinase activity, and the standard denaturing electrophoresis of the culture liquids of 6-h-old strains followed by the protein band staining with Coomassie Brilliant Blue revealed the presence of proteins with molecular masses of 21, 52, and 58 kDa (Fig. 2). Mitomycin C either induced or enhances the synthesis of

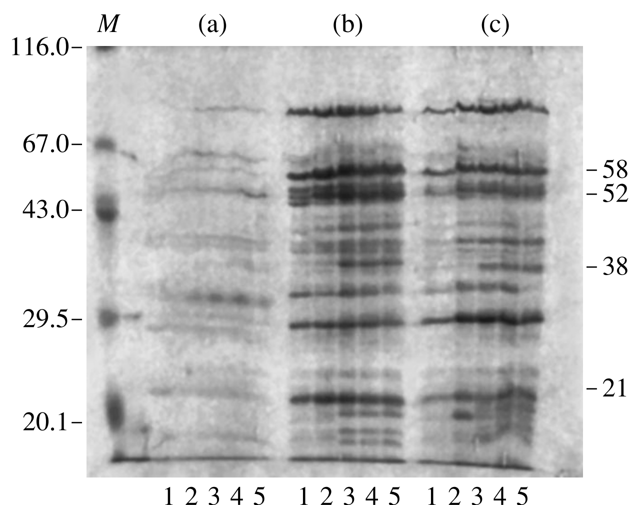

Fig. 2. Changes in the extracellular protein patterns of (a, b) the parent and (c) mutant *S. marcescens* strains as revealed by denaturing electrophoresis in 12.5% PAAG followed by the protein band staining with Coomassie Brilliant Blue R-250. Both strains were grown in Banting medium. The parent strain was grown either (b) in the presence or (a) absence of mitomycin C. Lanes 1–5 correspond to 6, 12, 18, 24, and 48 h of growth. M stands for marker proteins. The figures at the left of the gel indicate the molecular masses of the marker proteins (in kDa), and those at right of the gel the molecular masses of proteins with chitinase activity.

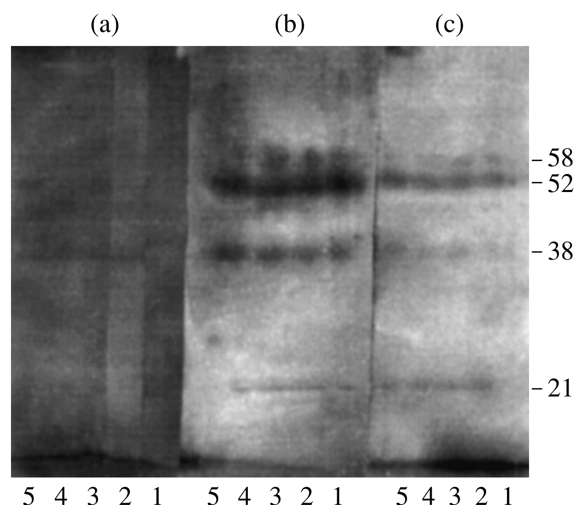

Fig. 3. Changes in the pattern of extracellular proteins with chitinase activity in the culture liquids of (a, b) the parent and (c) mutant *S. marcescens* strains as revealed by denaturing electrophoresis in 12.5% PAAG with glycol chitin followed by the protein renaturation step and determination of chitinase activity in the gel. The gel was stained with Calcofluor White M2R. Other conditions and designation as in the legend to Fig. 2.

extracellular proteins in *S. marcescens* (Fig. 2). The extracellular protein profile of the mutant strain grown in the semisynthetic Banting medium without mitomycin C (Fig. 2c) turned out to be close to that of the parent strain grown in Banting medium with the inducer (Fig. 1b).

In general, the synthesis of extracellular chitinases by *S. marcescens* grown without inducers is low. The employment of colloidal chitin as an inducer is inexpedient because of the tedious procedure of its preparation. Furthermore, the maximal level of chitinases in the *S. marcescens* culture grown in the presence of colloidal chitin is observed only after 6 to 8 days of growth [3], while it is observed after 18–20 h of growth in the presence of the SOS response inducer mitomycin C. In this case, mitomycin C induces the synthesis of all four extracellular proteins with chitinase activity. It is possible that other inducers of the SOS response, such as nalidixic acid and UV light, may also induce chitinase synthesis. The mutant *S. marcescens* strain D-5 [5] synthesizes the four proteins with chitinase activity in the semisynthetic Banting medium without any inducer, indicating the constitutive character of chitinase synthesis in this strain. Like the parent strain, the mutant strain accumulates the maximum amounts of the four chitinases after 18–20 h of growth.

The results obtained in this study may form the basis for developing an efficient biotechnological process of production of the chitinase complex, which will employ the mutant strain cultivated in the simple semisynthetic Banting medium [6] without any inducer. In this case, the cultivation time may be as low as 18–20 h.

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