Microbiology, Vol. 73, No. 3, 2004, pp. 267–270. Translated from Mikrobiologiya, Vol. 73, No. 3, 2004, pp. 320–325.

EXPERIMENTAL ARTICLES

The Effect of the Extracellular Bacteriolytic Enzymes of *Lysobacter* sp. on Gram-Negative Bacteria

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Received July 14, 2003

Abstract—The effect of the extracellular bacteriolytic enzymes of *Lysobacter* sp. on gram-negative bacteria was studied. These enzymes were found to be able to hydrolyze the peptidoglycan that was isolated from the gram-negative bacteria, the hydrolysis being completely inhibited by the cell wall lipopolysaccharide of these bacteria. The native cells of the gram-negative bacteria became susceptible to the bacteriolytic enzymes after the permeability of the outer membrane of the cells was altered by treating them with polymyxin B.

Key words: lysoamidase, bacteriolytic enzymes, cell wall, peptidoglycan, lipopolysaccharide, polymyxin B.

The extracellular bacteriolytic enzymes of some microorganisms are able to lyse a wide range of gram-positive bacteria [1]. As a rule, intact gram-negative bacteria are resistant to these enzymes, although the peptidoglycan isolated from the cell wall of such bacteria is hydrolyzed [2, 3].

It is known that the cell wall of gram-negative bacteria consists of a thin peptidoglycan layer covered with an outer membrane, which is made up of a lipid A–containing lipopolysaccharide, core, oligosaccharide (or O-antigen), phospholipids, lipoproteins, and porin proteins. The cell surface peptidoglycan of gram-positive bacteria is composed of many layers and is covalently linked to anionic polymers (teichoic and teichuronic acids).

The peptidoglycan of gram-negative bacteria usually contains diaminopimelic acid and has bonds directly between peptide subunits. In contrast, the peptidoglycan molecules of gram-positive bacteria rarely contain diaminopimelic acid and are linked via peptide bridges [4]. It is evident that the outer membrane of gram-negative bacteria protects the peptidoglycan layer from bacteriolytic enzymes. Only some highly purified enzymes are able to lyse intact gram-negative bacteria [5]. The mechanism of action of such enzymes on microbial cells remains largely unknown.

The bacterium *Lysobacter* sp. XL 1 [6] secretes into the medium three enzymes with bacteriolytic activity (muramidase, peptidase L1 and peptidase L2) [7–9], as well as a high-molecular-weight polysaccharide [10]. The positively charged bacteriolytic enzymes of *Lysobacter* sp. interact with the negatively charged polysaccharide, forming an extracellular enzyme–polysaccharide complex, known as lysoamidase. Lysoamidase lyses a wide range of gram-positive bacteria but only two gram-negative bacteria, *Fusobacterium necroforum* and *Prevotella melaninogenica* [1, 11].

The extracellular bacteriolytic enzymes of *Lysobacter* sp. become able to hydrolyze the peptidoglycan of gram-positive bacteria only after the enzymes have come in contact with the cell wall anionic polymers, teichoic or teichuronic acids [1]. In this case, the negative charge of these acids rather than the specific chemical nature plays the key role.

The aim of this work was to study the lysis of gram-negative bacteria by the extracellular bacteriolytic enzymes of *Lysobacter* sp. and to understand the role of lipopolysaccharide in this process.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. Experiments were carried out with active and relatively inactive strains of *Lysobacter* sp. (*Lysobacter* sp. XL 1 and *Lysobacter* sp. XL 2, respectively [9]), *Escherichia coli, Pseudomonas putida, Pseudomonas aeruginosa,* and *Salmonella marcescens, Proteus vulgaris, Proteus mirabilis* (all from the collection at the Tarasevich State Institute of Standardization and Control of Medical and Biological Preparations), as well as *Fusobacterium necroforum* and *Prevotella melaninogenica* from the collection of the Moscow Institute of Stomatology.

The bacteria *E. coli, P. putida, P. aeruginosa, S. marcescens, F. necroforum, P. melaninogenica, Proteus vulgaris*, and *Proteus mirabilis* were grown at 37°C in 5/5 medium containing (%) aminopeptide, 6; tryptone, 0.5; yeast extract, 0.1; and soybean extract, 3 (pH 7.2). The medium was devised at the Skryabin Institute of Biochemistry and Physiology of Microorganisms. *Lysobacter* sp. XL 1 and XL 2 were grown in the same medium at 29°C.

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Table 1. The effect of the extracellular bacteriolytic enzymes of *Lysobacter* sp. on the peptidoglycan and the peptidoglycan–lipopolysaccharide complex of *E. coli* and *P. putida*

Substrate	Bacteriolytic activity, LU/ml			
	lysoamidase	L1 peptidase	L2 peptidase	muramidase
S. aureus cells (the positive control)	27.9	16.2	6.7	1.5
Peptidoglycan from *E. coli*	25.5	7.9	5.8	1.2
Peptidoglycan from *P. putida*	15.2	3.3	1.3	0
The peptidoglycan–lipopolysaccharide complex of *E. coli*	0	0	0	0
The peptidoglycan–lipopolysaccharide complex of *P. putida*	0	0	0	0
A mixture of *E. coli* peptidoglycan and *E. coli* lipopolysaccharide	0	0	0	0

Table 2. The effect of polymyxin B on the action of lysoamidase on gram-negative bacteria

Bacterium	Number of strains	Bacteriolytic activity		
		lysoamidase	polymyxin B	lysoamidase + polymyxin B
Fusobacterium necroforum	1	+	–	+
Prevotella melaninogenica	1	+	–	+
Escherichia coli	2	–	–	+
Pseudomonas putida	1	–	–	+
Pseudomonas aeruginosa	6	–	–	+
Salmonella marcescens	1	–	–	+
Proteus vulgaris	1	–	–	–
Proteus mirabilis	1	–	–	–

Note: The signs "+" and "–" stand for "lysis" and "no lysis," respectively.

Enzyme isolation and purification. Lysoamidase was isolated from the culture liquid of *Lysobacter* sp. XL 1, which was grown in a pilot fermentor at the Skryabin Institute of Biochemistry and Physiology of Microorganisms [11]. Bacteriolytic endopeptidase L1 was isolated from the same culture liquid and purified by gel filtration on Sephacryl S-200 and ion-exchange chromatography on CM-Sephadex and an FPLC Mono S column [8]. Bacteriolytic endopeptidase L2 was isolated from the culture liquid of *Lysobacter* sp. XL 2 and purified by ion-exchange chromatography on CM-Sephadex and DEAE-Toyopearl and gel filtration on Toyopearl HW-50F [9].

Determination of the bacteriolytic activity of *Lysobacter* sp. enzymes in vitro. Bacteriolytic activity was assayed with lyophilized preparations of autoclaved cells, cell walls, or peptidoglycan, which were suspended in 10 mM Tris–HCl buffer (pH 8.0) to a concentration of 1 mg/ml (OD_{540} = 0.8). An aliquot (0.5 ml) of the suspension was mixed with 100 μl of a solution of lysoamidase (1 mg/ml), peptidase L1 (10 μg/ml), peptidase L2 (4 μg/ml), or muramidase (50 μg/ml) in the buffer and incubated at 37°C for 15–30 min. One unit of bacteriolytic activity (LU) was defined as the amount of enzyme that diminished the optical density OD_{540} of bacterial suspensions by 0.01 in minute. The effect of the *E. coli* lipopolysaccharide on bacteriolytic activity was studied as follows: Fifty microliters of enzyme preparation (3 mg/ml) in the buffer was mixed with 0.5 ml of the *Lysobacter* sp. peptidoglycan suspension (OD_{540} = 0.8) and 0.2 ml of the *E. coli* lipopolysaccharide solution (20 mg/ml), and the mixture was incubated at 52°C for 15 min. Then activity was measured as described above.

Determination of the bacteriolytic activity of *Lysobacter* sp. enzymes against pathogenic gram-negative bacteria. The sensitivity of *P. aeruginosa, S. marcescens, F. necroforum*, and *P. melaninogenica* cells to lysoamidase was determined in the presence of polymyxin B. One milliliter of a suspension of test bacterial cells in the buffer was placed aseptically in a sterile test tube and mixed with 1.5 ml of a solution containing 1 mg/ml (≈10 LU/ml) lysoamidase and 40 μg/ml (≈312 U/ml) polymyxin B. The mixture was incubated at 37°C for 24 h and analyzed for the number of viable test bacterial cells by the serial dilution technique. Aliquots (0.1 ml) of all dilutions were plated on LB agar, the plates were incubated at 30°C, and the colonies grown were enumerated. The effect of a preparation on a test bacterium was considered to be positive

(marked by the sign "+" in Table 2) if the plating of bacterial cells from dilutions containing 10^7–10^9 cells/ml did not give rise to colonies. Otherwise, the effect of this preparation on the test bacterium was considered to be negative (marked by the sign "–" in Table 2). To determine the number of cells in the control cell suspension, it was mixed with 1.5 ml of the sterile buffer instead of the lysoamidase solution.

The preparation of the peptidoglycan–lipopolysaccharide complex of gram-negative bacteria. This complex was obtained by the methods of Braun and Sieglin [12] and Goodwin and Shedlarsk [13] with minor modifications. Cells (50 g) were suspended in 100 ml of the buffer, and the suspension was subjected to three cycles of freezing at –70°C and thawing. The cell homogenate was supplemented with 3 mg DNase and added dropwise to 150 ml of 4% SDS. The preparation was boiled for 30 min, kept at 4°C for 12 h, sonicated twice for 30 s, and centrifuged at 20000 *g* for 1 h. The pellet was suspended in 100 ml of water, and the suspension was added dropwise to 150 ml of 4% SDS. The preparation was boiled for 15 min, kept at 4°C for 12 h, and centrifuged at 20000 *g* for 1 h. The pellet was suspended in 125 ml of water. The suspension was sonicated for 30 s and centrifuged at 20000 *g* for 10 min. The precipitate was discarded, and the supernatant was centrifuged at 20000 *g* for 1 h. The pellet was washed three times with distilled water, suspended in the buffer, incubated at 37°C with pronase (0.1 mg/ml) for 3 h, boiled for 15 min, and centrifuged at 20000 *g* for 1 h. The precipitate was washed three times in water and lyophilized. The lyophilized preparation represented the peptidoglycan–lipopolysaccharide complex.

The preparation of the peptidoglycan of gram-negative bacteria. Peptidoglycan was prepared by the method of Streshinskaya *et al.* [14]. An amount (0.7 g) of the peptidoglycan–lipopolysaccharide complex was suspended in 70 ml of 0.5 N trichloroacetic acid (TCA). The suspension was stirred at 4°C for 48 h and then centrifuged at 15000 *g* for 30 min. The pellet was suspended in 70 ml of 0.5 N TCA. The suspension was stirred at 37°C for 48 h and then centrifuged at 15000 *g* for 30 min. The precipitate was washed five times in water and used as a peptidoglycan preparation.

RESULTS AND DISCUSSION

The extracellular bacteriolytic enzymes of *Lysobacter* sp. are able to lyse a wide range of gram-positive bacteria, but, to the best of our knowledge, only two gram-negative bacteria (*Fusobacterium necroforum* and *Prevotella melaninogenica*).

To prove the supposition that lysoamidase can hydrolyze the peptidoglycan of gram-negative bacteria, it was isolated from *E. coli* and *P. putida* cells. The data presented in Table 1 show that lysoamidase and individual bacteriolytic enzymes isolated from it can efficiently hydrolyze the peptidoglycan of *E. coli* and *P. putida.* For comparison, the table shows that the bacteriolytic enzymes of *Lysobacter* sp. efficiently lyse the gram-positive bacterium *Staphylococcus aureus.* These data suggest that the inability of the bacteriolytic enzymes of *Lysobacter* sp. to lyse native gram-negative bacteria may be due to the inability of these enzymes to penetrate to their substrate (peptidoglycan) through the outer membrane of the gram-negative cell wall.

The outer membrane of gram-negative bacteria consists of a lipopolysaccharide whose oligosaccharide moiety contains negatively charged phosphate and carboxylic groups [15]. The structure of the lipopolysaccharide is somewhat similar to that of the anionic polymers of gram-positive bacteria. For instance, the lipopolysaccharide of some gram-negative bacteria contains 1,5-poly(ribitol phosphate) chains, which are known to form the 1,5-poly(ribitol phosphate) teichoic acids of gram-positive bacteria [16].

To study the effect of the lipopolysaccharide of the outer membrane of gram-negative bacteria on the activity of the extracellular bacteriolytic enzymes of *Lysobacter* sp., we obtained preparations of the *E. coli* and *P. putida* peptidoglycan, which contained the outer membrane lipopolysaccharide of these bacteria. The study of the effect of the bacteriolytic enzymes of *Lysobacter* sp. on these preparations showed that neither lysoamidase nor the individual bacteriolytic enzymes of *Lysobacter* sp. were able to hydrolyze the peptidoglycan of gram-negative bacteria in the presence of the lipopolysaccharide (Table 1). This can be explained by the inhibition of these enzymes through the interaction of their positively charged groups with the negatively charged groups of the lipopolysaccharide. The addition of the purified lipopolysaccharide of *E. coli* to the peptidoglycan of this bacterium made it resistant to lysoamidase and the individual bacteriolytic enzymes (Table 1). All these data suggest that the negatively charged groups of the outer membrane lipopolysaccharide of gram-negative bacteria interact with the positively charged bacteriolytic enzymes, thereby preventing their penetration to the substrate (peptidoglycan) through the outer membrane.

It would be reasonable to suggest that the impairment of the barrier function of the outer membrane may facilitate the penetration of the bacteriolytic enzymes of *Lysobacter* sp. to the peptidoglycan of gram-negative bacteria. For instance, it is known that the heating of gram-negative cells or their treatment with EDTA alters the permeability of the outer membrane and makes the peptidoglycan of gram-negative bacteria susceptible to bacteriolytic enzymes [2]. It is also known that *B. subtilis* YT-25 cells secrete into the medium a native lytic factor (NLF), which alters the hydrophobic structure of the lipopolysaccharide of gram-negative bacteria in such a manner that the extracellular bacteriolytic enzymes of *B. subtilis* YT-25 become able to penetrate to the inner peptidoglycan layer and to hydrolyze it

[17]. The NLF consists of about ten amino acid residues and a long chain of fatty acids. Takahara *et al.* [18] suggested that it is the presence of fatty acids that is responsible for the activity of the NLF. The cyclic peptidolipid antibiotic polymyxin B is a structural analog of the NLF and, hence, may have a similar mechanism of action. In gram-negative bacteria, polymyxin B interacts mainly with the lipopolysaccharides of the outer membrane, thereby altering its normal structural organization and permeability [19]. The study of the effect of lysoamidase on the intact cells of gram-negative bacteria pretreated with polymyxin B showed that such cells, as a rule, become susceptible to lysoamidase (Table 2). Only two gram-negative bacteria (*Proteus vulgaris* and *Proteus mirabilis*) remained resistant to lysoamidase after their treatment with polymyxin B, which can be explained by the specific structure of the outer membrane or the peptidoglycan of these two bacteria. Thus, some gram-negative bacteria, including pathogenic ones, become susceptible to bacteriolytic enzymes after being treated with polymyxin B. This antibiotic interacts with the lipopolysaccharide of the outer membrane, thereby changing its permeability and preventing the interaction between bacteriolytic enzymes and the lipopolysaccharide. As a result, the peptidoglycan layer of the cell wall of gram-negative bacteria becomes susceptible to the bacteriolytic enzymes. In contrast, the bacteriolytic enzymes of *Lysobacter* sp. cannot hydrolyze the peptidoglycan of gram-positive bacteria without interacting with the negatively charged cell wall polymers (teichoic and teichuronic acids) [1].

To conclude, this study showed that the bacteriolytic enzymes of *Lysobacter* sp. are able to hydrolyze the peptidoglycan isolated from gram-negative bacteria. The lipopolysaccharide of the outer membrane may be involved in blocking the action of bacteriolytic enzymes on the gram-negative bacterial cell wall. Lysoamidase is able to lyse the native cells of gram-negative bacteria in the presence of polymyxin B.

ACKNOWLEDGMENTS

This work was supported by project nos. 96-04-48365, 00-04-48073, and 00-15-97851 from the Russian Foundation for Basic Research and by grant no. NSh-1382.2003.4 from the program "Leading Scientific Schools."

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