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EXPERIMENTAL ARTICLES

The Mechanism of Action of the Extracellular Bacteriolytic Enzymes of *Lysobacter* sp. on Gram-Positive Bacteria: The Role of the Cell Wall Anionic Polymers of Target Bacteria

O. A. Stepnaya*, E. A. Begunova*, I. M. Tsfasman*, E. M. Tul'skaya, G. M. Streshinskaya**, I. B. Naumova†**, and I. S. Kulaev*,****

**Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

***Faculty of Biology, Moscow State University, Vorob'evy gory, Moscow, 119992 Russia*

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Abstract—The study of the extracellular bacteriolytic enzymes of *Lysobacter* sp. showed that they can efficiently hydrolyze the peptidoglycan of gram-positive bacteria provided that there is an electrostatic interaction of these enzymes with the cell wall anionic polymers, teichoic and teichuronic acids in particular. The hydrolytic action of bacteriolytic enzymes on the cell wall largely depends on the negative charge of the teichoic and teichuronic acids rather than on their chemical composition.

Key words: lysoamidase, bacteriolytic enzymes, cell wall, peptidoglycan, anionic polymers.

Bacteriolytic enzymes, or peptidoglycan hydrolases, are a group of enzymes that hydrolyze the chemical bonds of peptidoglycan, the major structural component of bacterial cell walls. The intracellular peptidoglycan hydrolases of bacteria (autolysins) are involved in the synthesis and hydrolysis of cell walls, spore germination, growth processes, and cell division. Extracellular bacteriolytic enzymes are necessary for supplying nutrients and energy to bacteria and for providing their competitiveness in nature [1, 2].

The bacterium *Lysobacter* sp. [3] secretes into the medium three bacteriolytic enzymes (muramidase, bacteriolytic peptidase L1, and bacteriolytic peptidase L2) [4–6], as well as a negatively charged high-molecular-weight polysaccharide. The positively charged bacteriolytic enzymes of the lysoamidase complex interact with the negatively charged polysaccharide, thereby stabilizing themselves [7]. Lysoamidase actively lyses a wide range of gram-positive bacteria and some gram-negative bacteria [8].

The main structural component of the cell walls of gram-positive bacteria is a multilayer peptidoglycan, which is covalently linked to teichoic and teichuronic acids (the anionic polymers of the bacterial cell wall) [1, 9]. Peptidoglycan is a complex polymer composed of glycan chains with alternating residues of *N*-acetylmuramic acid and *N*-acetylglucosamine. The glycan chains are linked to peptide subunits by the lactyl residue of muramic acid. The peptide subunits are linked to each other by peptide bridges. Teichoic acid is a polymer containing phosphodiester, polyol, and sugar residues. Teichuronic acid is a polymer containing uronic acid and sugar residues. Teichoic and teichuronic acids are linked to the cell wall peptidoglycan via the sixth carbon atom of muramic acid.

The complex and heterogeneous chemical composition and elaborate architectonics of the bacterial cell wall as the substrate of bacteriolytic enzymes is very likely to determine the mechanism of their interaction with the cell wall and, in the final analysis, their ability to hydrolyze the peptidoglycan. In our opinion, of great significance is the site of the initial enzymatic attack (the inner surface or the interior of the cell wall in the case of autolysins and the outer surface of the cell wall in the case of extracellular bacteriolytic enzymes).

The mechanism of action of the autolytic enzymes of some gram-positive bacteria has been discussed in a number of publications. For instance, Pooley *et al.* [10] showed that the electrostatic interaction of bacillar and staphylococcal autolysins with teichoic acids is a necessary condition of efficient enzymatic attack, whereas the efficient autolysis of pneumococcal and clostridial cells requires that teichoic acids contain phosphorylcholine residues [11, 12]. The replacement of ethanolamine for choline in teichoic acids makes bacterial cells resistant to autolysis, genetic transformation, and bactericidal antibiotics, as well as makes the daughter cells unable to detach from each other [13].

The autolytic *N*-acetylmuramyl-L-alanine amidase of *Bacillus subtilis* W-23 hydrolyzes its native cells and

† Deceased.

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cell walls (which contain ribitol teichoic acids) but is unable to degrade the cells of the *B. subtilis* strain whose cell walls contain glycerol teichoic acids [14].

The mechanism of action of extracellular bacteriolytic enzymes on the cell walls of susceptible bacteria is as yet poorly understood, although it is known that the bacteriolytic enzymes of the lysoamidase complex efficiently degrade the cell walls of the gram-positive bacteria *Staphylococcus aureus* and *Streptomyces chrysomallus*, which contain ribitol teichoic acids. The peptidoglycan isolated from the cell walls of these bacteria is resistant to the lysoamidase enzymes [1].

The aim of this work was to study the role of the cell wall anionic polymers of gram-positive bacteria in the mechanism of action of the bacteriolytic enzymes of lysoamidase.

MATERIALS AND METHODS

Microorganisms. The bacteria used in this work were the active and relatively inactive producers of lysoamidase (*Lysobacter* sp. XL 1 and *Lysobacter* sp. XL 2, respectively [4]), *Staphylococcus aureus* 209-P, *Micrococcus luteus* VKM B-1314, *Streptomyces chrysomallus* VKM Ac-628, *Bacillus subtilis* W-23, and *B. subtilis* 168 from the collection of the Laboratory of Regulation of Biochemical Processes, Skryabin Institute of Biochemistry and Physiology of Microorganisms; *Staphylococcus epidermidis, Streptococcus mutans*, and *Streptococcus pyogenes* from the collection at the Tarasevich State Institute of Standardization and Control of Medical and Biological Preparations; and *B. subtilis* SA 398 and *Bacillus subtilis* L 5706, which were obtained from Prof. Yang (the University of Wales).

Preparation of test bacterial cells for bacteriolytic assay. *St. aureus* 209-P cells were grown in peptone–yeast extract medium at 37°C for 24 h, autoclaved at 2 atm for 30 min, harvested by centrifugation at 5000 *g* for 20 min, washed three times in 10 mM Tris–HCl buffer (pH 8.0), and lyophilized.

M. luteus VKM B-1314 cells were grown in the peptone–yeast extract medium at 37°C for 16 h, harvested by centrifugation, washed three times in the buffer, and lyophilized.

B. subtilis W-23, *B. subtilis* 168, *B. subtilis* SA 398, and *B. subtilis* L 5706 were grown at 37°C for 16 h 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. The cell walls of these strains grown in 5/5 medium were proved to contain teichoic acids.

B. subtilis W-23 and *B. subtilis* 168 were also grown at 37°C for 48 h in a medium with a reduced phosphate content (mM): $NaH_2PO_4 \cdot 2H_2O$, 1.5; $(NH_4)_2SO_4$, 50; K_2SO_4, 30; citrate, 1.0; $MgCl_2 \cdot 6H_2O$, 1.25; $CaCl_2$, 0.1; $FeCl_3$, 0.1; $ZnCl_2$, 25; $MnCl_2 \cdot 4H_2O$, 0.025; $CuCl_2$, 0.005; $CoCl_2 \cdot 6H_2O$, 0.005; $Na_2MoO_4 \cdot 2H_2O$, 0.005; and glucose, 30 g/l. The cell walls of these two strains grown in the low-phosphate medium (arbitrarily designated *B. subtilis* W-23* and *B. subtilis* 168*) contain teichuronic acids [15]. Cells were harvested by centrifugation at 5000 *g* for 20 min, washed three times in the buffer, and lyophilized.

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 [8]. Bacteriolytic endopeptidase L1 was isolated from the lysoamidase preparation and purified by gel filtration on Sephacryl S-200 and ion-exchange chromatography on CM-Sephadex and an FPLC Mono S column [5]. Bacteriolytic endopeptidase L2 and muramidase were 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 [4].

Assay of the bacteriolytic activity of the lysoamidase enzymes. Bacteriolytic activity was assayed with the lyophilized preparations of cells, cell walls, and peptidoglycan as the substrates, which were suspended in the buffer to a concentration of 1 mg/ml ($OD_{540} = 0.8$). An aliquot (0.5 ml) of the particular substrate 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 (U) was defined as the amount of enzyme that diminished the optical density OD_{540} of the substrate suspension by 0.01 in 1 min.

The preparation of the cell walls of *B. subtilis* W-23* according to Shaw *et al.* [16]. Cells grown as described above were harvested by centrifugation at 5000 *g* for 15 min, washed three times in 10 volumes of 0.1 M phosphate buffer (pH 6.8), frozen at –42°C, and disrupted in a French press. The cell homogenate was supplemented with DNase and RNase at concentrations of 20 µg/ml each. Unbroken cells were removed by centrifugation at 1200 *g* for 15 min. Cell walls were precipitated by centrifugation at 10000 *g* for 15 min and washed four times with a cold 10 mM Tris–HCl buffer (pH 7.2) by centrifugation under the same conditions. To inactivate autolytic enzymes, the fraction of cell walls was incubated at 100°C for 15 min, suspended in 50 ml of 10 mM Tris–HCl buffer (pH 8.0), and incubated with 100 µg/ml trypsin at 37°C for 3 h. Then the cell wall preparation was washed three times with water, suspended in 30 ml of a chloroform–methanol mixture (2 : 1) to extract lipids, and evaporated under a vacuum in the presence of $CaCl_2$.

The preparation of the anionic polymers and peptidoglycan of *B. subtilis* W-23* by the method of Schleifer and Kandler [17]. Lyophilized cells (10 g) were suspended in 10% trichloroacetic acid (TCA) and

Table 1. The effect of lysoamidase on gram-positive bacterial cells with different types of cell wall anionic polymers

Bacteria	Number of strains	Cell wall anionic polymer	Lysoamidase
Staphylococcus aureus 209-P	1	Ribitol teichoic acid [19]	+
Streptomyces chrysomallus Ac-628	1	Ribitol teichoic acid [20]	+
Bacillus subtilis W-23	1	Ribitol teichoic acid [20]	+
Staphylococcus epidermidis	3	Glycerol teichoic acid [20]	+
Streptococcus mutans	2	Glycerol teichoic acid [19]	+
Bacillus subtilis 168	1	Glycerol teichoic acid [10]	+
Streptococcus pyogenes	2	Teichuronic acid [20]	+
Micrococcus luteus	1	Teichuronic acid [20]	+

Note: The sign "+" indicates the lysis of bacterial cells by lysoamidase.

Table 2. The effect of lysoamidase and the constituent bacteriolytic enzymes on *B. subtilis* cells with different types of cell wall anionic polymers

Bacteria	Cell wall anionic polymer	Bacteriolytic activity, U/ml			
		Lysoamidase	L1 peptidase	L2 peptidase	Muramidase
Bacillus subtilis W-23	Ribitol teichoic acid 1,5-poly-(Rib-ol*P*)	188	7.5	1.32	0.5
Bacillus subtilis 168	Glycerol teichoic acid 1,5-poly(Gro-*P*)	31	5.4	2.4	0
Bacillus subtilis SA 398	Ribitol and glycerol teichoic acids	90	5.4	2.44	1.6
Bacillus subtilis L 5706	Ribitol and glycerol teichoic acids	80	11	2.2	2.0
Bacillus subtilis W-23*	Teichuronic acid -3)-α-D-Gal*p*NAc--(1-4)-α-D-Glc*p*A-(1-	159	6.24	2.3	1.5
Bacillus subtilis W-168*	Teichuronic acid -3)-α-D-Gal*p*NAc--(1-4)-α-D-Glc*p*A-(1-	158.7	5.5	2.1	1.1

extracted by incubation at 4°C for 48 h. The extract was mixed with ethanol. The precipitated anionic polymers were dissolved in water, and the solution was dialyzed and lyophilized.

The cells that remained after the extraction of anionic polymers were incubated in 10% TCA at 100°C for 20 min. The homogenate was cooled and centrifuged at 5000 *g* for 30 min. The precipitate was washed with distilled water and then with 10 mM Tris–HCl buffer (pH 7.8), incubated at 37°C for 18 h in a solution of trypsin (1 mg/ml) in the same buffer, and again washed with distilled water and the buffer. Then the preparation was incubated in 2.4% SDS at 100°C. The resulting peptidoglycan preparation was lyophilized.

The acid hydrolysis of the cell wall anionic polymers of *B. subtilis* W-23*. The anionic polymers were hydrolyzed in 2 N HCl at 100°C for 3 h. The hydrolysate was analyzed for ribitol phosphate esters, polyols, and aminosugars [18].

The acid hydrolysis of the peptidoglycan of *B. subtilis* W-23*. The peptidoglycan was hydrolyzed in 6 N HCl at 100°C for 18 h, and the hydrolysate was analyzed using a Hitachi amino acid analyzer (Japan).

The periodate oxidation of the cell wall teichoic acids. An aliquot (104 mg) of the cell wall preparation was suspended in 5 ml of 0.25 M $NaIO_4$ in 20 mM Tris–HCl buffer (pH 6.0). The suspension was incubated at room temperature for 72 h and centrifuged. The precipitate was washed with 200 ml of distilled water, incubated in 0.5 N HCl at room temperature for 8 h, and washed with distilled water to a neutral reaction of the washings. Then the preparation was dehydrated with ethanol and acetone.

RESULTS AND DISCUSSION

It is well known that the bacteriolytic enzymes of lysoamidase can efficiently lyse the intact cells and cell walls of gram-positive bacteria that contain ribitol teichoic acids [1]. It was of interest to study if these enzymes are also able to lyse the cell walls of gram-positive bacteria with a different type of anionic polymers. For this purpose, we investigated the effect of lysoamidase on test bacterial cells with different teichoic and teichuronic acids in their cell walls. As is evident from the data summarized in Table 1, lysoamidase can lyse gram-positive bacterial cells whose walls contain ribitol teichoic acids, glycerol teichoic acids, or teichuronic acids. The cell walls of the bacteria studied differed not only in the type of anionic polymers but

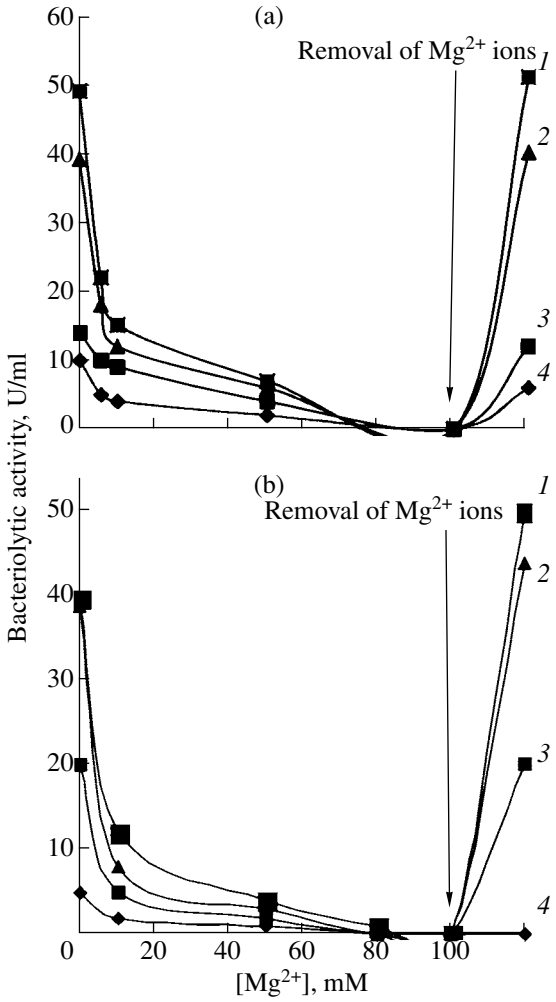

Fig. 1. The effect of different concentrations of Mg^{2+} on the activity of (*1*) lysoamidase, (*2*) L1 peptidase, (*3*) L2 peptidase, and (*4*) muramidase with respect to (a) *St. aureus* 209-P and (b) *M. luteus* cells.

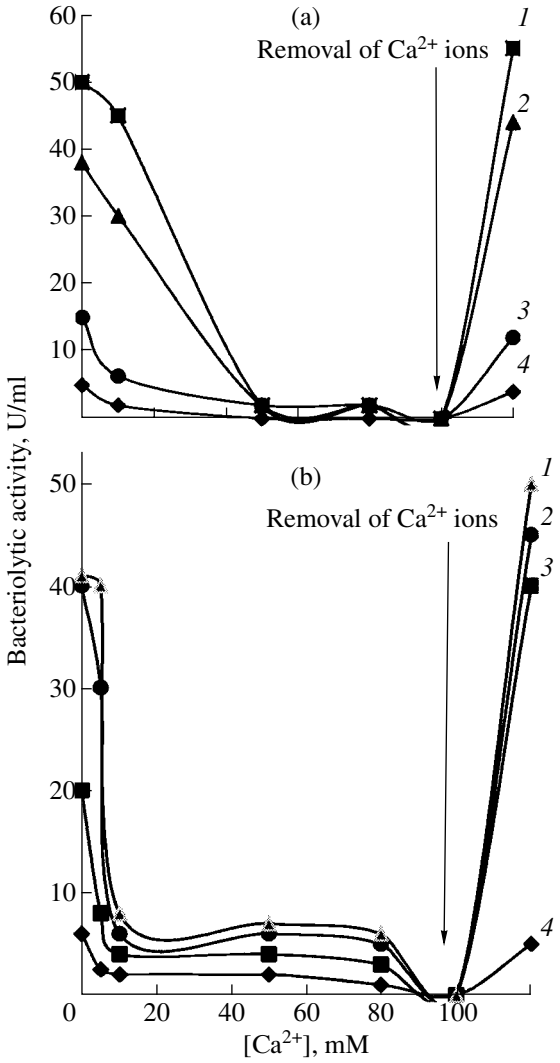

Fig. 2. The effect of different concentrations of Ca^{2+} on the activity of (*1*) lysoamidase, (*2*) L1 peptidase, (*3*) L2 peptidase, and (*4*) muramidase with respect to (a) *St. aureus* 209-P and (b) *M. luteus* cells.

also in the structure of peptidoglycans. For instance, the cell walls of *St. aureus* 209-P, *M. luteus* VKM B-1314, and *Str. chrysomallus* VKM Ac-628 were found to contain A3α-, A2α-, and A3γ-type peptidoglycans, respectively.

Further studies were performed with four *B. subtilis* strains that differed in the composition of their cell walls. The cell walls of *B. subtilis* SA 3989 and *B. subtilis* L 5706 contain both ribitol and glycerol teichoic acids [10]. The cell walls of *B. subtilis* W-23 and *B. subtilis* 168 contain the same A1α-type peptidoglycan [19] but different teichoic acids (ribitol and glycerol teichoic acids, respectively). When grown in a medium deficient in phosphate, these strains (designated in this case *B. subtilis* W-23* and *B. subtilis* 168*) synthesize cell walls that contain teichuronic acids instead of teichoic acids [15]. To make sure that this was really the case, the cell walls of *B. subtilis* W-23* were subjected to acid hydrolysis and then analyzed for sugars. The hydrolysate was found to contain galactose, glucose, mannose, glucosamine, galactosamine, and unidentified ninhydrin-reactive compounds but not polyols or ribitol phosphates. The absence of both of the latter compounds indicated that the cell walls of *B. subtilis* W-23* do not contain ribitol teichoic acid, whereas the presence of galactosamine indicated that these cell walls contain teichuronic acids. The hydrolysate of the peptidoglycan isolated from *B. subtilis* W-23* contained diaminopimelic acid, glutamic acid, and alanine but not the degradation products of teichoic acid. All these data conclusively showed that the cell walls of *B. subtilis* W-23* contained teichuronic acids and no teichoic acids.

Data on the bacteriolytic activity of lysoamidase and the constituent individual enzymes are summarized in Table 2. As can be seen from this table, the constituent

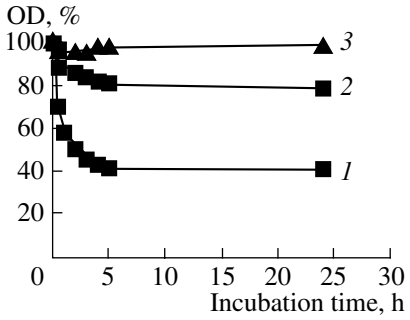

Fig. 3. The effect of lysoamidase on (*1*) the native cell walls, (*2*) the cell walls oxidized with $NaIO_4$, and (*3*) the isolated peptidoglycan of *B. subtilis* W-23*.

bacteriolytic enzymes of lysoamidase did not show any significant dependence of their activity on the type of the cell wall of test bacteria. At the same time, lysoamidase was the most active with the bacterial cells whose walls contained only ribitol teichoic acids (*B. subtilis* W-23), moderately active with the cells containing both ribitol and glycerol teichoic acids (*B. subtilis* SA 3989 and L 5706), and the least active with the cells containing only glycerol teichoic acids (*B. subtilis* 168). The replacement of teichuronic acids for glycerol teichoic acids in the cell walls of *B. subtilis* 168* considerably (by five times) enhanced the activity of lysoamidase with the cells of strain 168. These data could suggest that the efficiency of lysoamidase depends either on the chemical composition or on the negative charge (and its topography) of the anionic polymers constituting the cell walls of target bacterial cells.

To decide which of these suggestions is true, we investigated the effect of the bivalent cations Mg^{2+} and Ca^{2+} (which screen the negative charge of anionic polymers) on the activity of lysoamidase and the constituent bacteriolytic enzymes. For this purpose, dried cells (20 mg) of *St. aureus* 209-P (containing ribitol teichoic acids) and *M. luteus* (containing teichuronic acids) were incubated at 25°C for 2 h in 10 ml of 10 mM Tris–HCl buffer (pH 8.0) containing either $MgCl_2$ or $CaCl_2$ at concentrations of 6, 10, 50, and 100 mM and then used for assaying bacteriolytic activity. In the control experiments, bacteriolytic activity was assayed with bacterial cells that were first incubated in the presence of Mg^{2+} or Ca^{2+} ions and then washed three times with distilled water. As can be seen from Figs. 1 and 2, lysoamidase and the constituent enzymes lost bacteriolytic activity against the *St. aureus* and *M. luteus* cells that were incubated in the presence of Mg^{2+} or Ca^{2+} ions at concentrations higher than 50 mM. After these ions were washed off, the bacterial cells again became susceptible to the bacteriolytic enzymes (Figs. 1, 2).

To verify the possibility that the described effect of the bivalent cations was due to their ability to alter the rigidity of the peptidoglycan layer rather than to screen its negative charges, we investigated the effect of periodate oxidation (which affects the structure of teichuronic acids but not their charge) on the activity of lysoamidase with the cell walls of *B. subtilis* W-23*. Measurements showed that lysoamidase actively hydrolyzed the native cell walls of *B. subtilis* W-23* but not the peptidoglycan isolated from this strain (Fig. 3). The periodate oxidation of the cell walls decreased—but only slightly (by about 20%)—their susceptibility to lysoamidase, indicating that the chemical composition of the cell wall anionic polymers is not of crucial importance for the lytic activity of lysoamidase and the constituent enzymes.

Thus, the interaction of the bacteriolytic lysoamidase complex with the negative charges of the cell wall anionic polymers of gram-positive bacteria is a necessary condition of the efficient lysis of these bacteria.

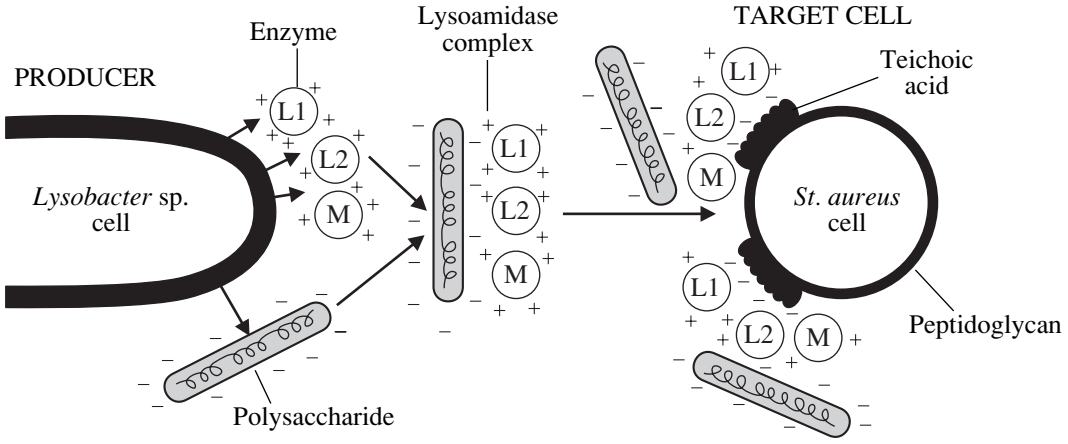

Fig. 4. A putative scheme showing the formation and the functioning of the lysoamidase enzyme complex: L1, endopeptidase; L2, *N*-acetylmuramyl-L-alanine amidase; M, muramidase.

Figure 4 schematically shows the interaction of positively charged bacteriolytic enzymes with a negatively charged extracellular polysaccharide, which gives rise to an enzyme–polysaccharide complex known as lysoamidase. Due to electrostatic interactions between the positive charges of the bacteriolytic enzymes and the negative charges of the cell wall anionic polymers, the bacteriolytic complex is fixed on the surface of target bacterial cells. This fixing is a necessary condition of the efficient hydrolysis of the cell wall peptidoglycan and eventually the lysis of the target bacterial cells.

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