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THE EFFECT OF OSMOTIC SHOCK ON THE ACCESSIBILITY OF THE MUREIN LAYER OF EXPONENTIALLY GROWING *ESCHERICHIA COLI* TO LYSOZYME

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Summary

The restricted access of lysozyme to the murein layer of exponential phase *Escherichia coli* is enhanced considerably by osmotic shock. When cells suspended in Tris/EDTA/sucrose are diluted 11-fold in water or 10 mM EDTA in the presence of lysozyme, their susceptibility to lysozyme increases by a factor of 50—100, for both *Escherichia coli* JC411 and W3110, grown to the early exponential phase in unsupplemented or supplemented minimal media, and in Brain Heart Infusion.

Since an 11-fold dilution causes lysis of lysozyme spheroplasts, the effects of a 2-fold dilution have also been investigated. A 2-fold dilution of cells suspended in Tris/EDTA/sucrose still increases their susceptibility to lysozyme by a factor of 10—50, but the resulting spheroplasts remain intact. EDTA is necessary to permit lysozyme access to the murein layer during the dilution, which is ineffective in the presence of 5 mM $MgCl_2$.

These results are discussed in terms of the formation of lysozyme spheroplasts from young *Escherichia coli*.

Introduction

Lysozyme has long been used to convert gram-negative cells to spheroplasts [1—8], which have been used primarily as sources of subcellular components such as plasmids, membranes, or the bacterial chromosome [9—14]. Although some attention has been focused on the spheroplasting process itself [4], not much is known about how lysozyme gains access to the murein of gram-negative bacteria. In these bacteria, murein is only a relatively minor layer of the cell wall; the major layer is the outer membrane, which is linked covalently to the murein [15,16]. The structure and interactions of these two cell wall layers

is such that lysozome access to the murein must be severely limited, as indicated by the following findings. First, it has recently been shown that the outer membrane is essentially impermeable to hydrophilic molecules larger than 600 daltons [17]. Second, lysozyme acts at the inner surface of the murein layer [18,19] and it must therefore penetrate through the murein layer as well as the outer membrane before it can hydrolyze murein polysaccharide chains. Third, the three-dimensional organization of murein is such that the interchain distance of adjacent polysaccharide chains is 0.4 nm [20]; such closely packed chains do not fit into the lysozyme cleft [21].

In spite of these barriers, lysozyme can be made to reach and degrade the murein layer. In the case of exponentially growing cells, this is accomplished as follows. First, lysozyme is added in considerable excess relative to the total cell murein *. Second, cells are suspended in a buffer which generally contains dilute Tris or Tris/EDTA to destabilize the outer membrane [1,3,4,7,8,10—13]. Third, the suspension buffer contains sucrose [4,5,10—13].

While the advantages of excess lysozyme and agents to destabilize the outer membrane can be easily understood, this is not so for the sucrose requirement. In the case of exponential phase *Escherichia coli*, this requirement has been interpreted to mean that cell plasmolysis is necessary for spheroplast formation [4,22], but it is not clear why and how plasmolysis increases the susceptibility of the murein layer to lysozyme action. In fact, we have recently investigated the access of lysozyme to the murein of stationary phase cells, and found that for these cells there is no relation between plasmolysis and lysozyme access to the murein layer [19]. Lysozyme access to the murein layer was minimal, even in the presence of a plasmolyzing buffer containing Tris/EDTA, but could be enhanced considerably by exposing the cells to an osmotic shock [19].

It is possible that these differences (i.e. lysozyme has access to the murein of plasmolyzed exponential phase cells but not to the murein of plasmolyzed stationary phase cells) are real and intrinsic to exponential and stationary phase cells. This seemed unlikely however, and we have therefore examined the effect of osmotic shock on the access of lysozyme to the murein of exponential phase *E. coli*.

Materials and Methods

(*A*) *Cell growth. Escherichia coli* JC411 (*leu*$^-$ *his*$^-$ *arg*$^-$ *met*$^-$ *lac*$^-$ *mal*$^-$ *mtl*$^-$ *xyl*$^-$ *str*^{r}) and *E. coli* W3110 were grown in minimal medium as described previously [23]; early exponential phase cultures were harvested when they reached a density of 0.1 mg/ml ($2.3 \cdot 10^8$ viable cells/ml).

Since it was sometimes necessary to carry out lysozyme treatments on a single batch of harvested cells over a period of several hours or days, cells had to be stored under conditions such that their response to the lysozyme treatment remained invariant. It was essential to include 1 mM Mg^{2+} in the first suspension buffer to prevent autolysis. Accordingly, harvested cells were sus-

* Many treatments employ a lysozome/cell ratio (w/w) of 10—50% [3,4,6—8,10,11,13]; since gram-negative cells contain about 1—1.5% murein [30], this implies a lysozyme/murein ratio (w/w) of 500—5000%.

pended in 1 mM $MgCl_2$/200 mM Tris · HCl (pH 8.0) (Tris/Mg^{2+} buffer) to a density of about 17 mg/ml, kept at 0°C, and used in all experiments, unless stated otherwise. Under these conditions, the lysozyme treatment of Fig. 1 gave similar results when carried out after 0, 1 or 3 days. Pelleted cells were also stable to lysozyme treatment after storage at 0°C for several days. Washing had little effect on the susceptibility of exponential phase cells to lysozyme.

Cell densities were expressed as mg cells (dry mass)/ml [24].

(*B*) *Tris/EDTA/sucrose lysozyme treatment* [19]. Buffer components were added separately at fixed times (see Fig. 1a) to the cells described above, resulting in a suspension which contained 200 mM Tris · HCl, 0.5 mM EDTA, 0.5 M sucrose (pH 8.0), and which is referred to as a Tris/EDTA/sucrose suspension. Egg white lysozyme (EC 3.2.1.17, Boehringer und Soehne GmbH, Mannheim, W. Germany), dissolved to 11 mg/ml in 200 mM Tris · HCl (pH 8.0), was added at 3.5 min (see Fig. 1a), at final concentrations indicated for each experiment. All lysozyme treatments were carried out at 23°C.

To measure osmotic sensitivity, 0.2-ml portions of lysozyme-treated cell suspensions were added to 2 ml diluent (either water, 10 mM EDTA (pH 7.6—8.0) in water, or 20 mM $MgCl_2$ in water as stated in text) and mixed rapidly, after which the absorbance of the diluted suspension was recorded for 3—4 min at 399 nm with a Vitatron photometer (Vitatron, The Netherlands). The resulting recordings are referred to as lysis curves.

(*C*) *Determination of the rates at which cells become osmotically sensitive.* When it is added to bacteria, lysozyme starts degrading the murein layer if it binds to and cleaves an appropriate murein polysaccharide segment. If no other layers contribute to the stability of the cell wall (see below) cells will become osmotically sensitive, and the rate r_{os}^{bd} at which lysozyme renders cells osmotically sensitive * can therefore be used as a probe for lysozyme access to the murein polysaccharides **.

The rate r_{os}^{bd} is determined by measuring the osmotic sensitivity of a lysozyme-treated cell suspension as a function of time. This is done by measuring the extent of cell lysis after cells are exposed to a vigorous osmotic shock, which causes osmotically sensitive cells to lyse virtually instantaneously. However, there may be a significant increase in lysozyme activity after a vigorous osmotic chock, such as that caused by an 11-fold dilution [19]. As a result, cells which are not osmotically sensitive before dilution may become osmotically sensitive at a rate r_{os}^{ad} after 11-fold dilution.

(1) Determination of r_{os}^{ad}. Since cells which become osmotically sensitive after 11-fold dilution are not protected by sucrose they also lyse, at a rate r_l^{ad} *

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* The different rates r_{os} at which cells are rendered osmotically sensitive by lysozyme are distinguished by superscripts. Thus, r_{os}^{bd} and r_{os}^{ad} refer to the rates at which cells are rendered osmotically sensitive before dilution and after the 11-fold dilution used to lyse osmotically sensitive cells, respectively. These rates should not be confused with the rate r_l^{ad} at which cells lyse after 11-fold dilution.

** For the purposes of this paper "murein polysaccharide accessibility to lysozyme" or "lysozyme access to murein" implies that lysozyme can (a) penetrate through the cell wall layers, (b) bind properly to polysaccharide chains, and (c) hydrolyze murein polysaccharide chains. Thus, in this context, if there is no murein degradation there is no "lysozyme access to the murein" as defined here. This may either be because lysozyme cannot get through the cell wall layers, or it may be because it cannot bind to single polysaccharide chains.

which will be limited by the rate r_{os}^{ad} at which they become osmotically sensitive. Thus r_{os}^{ad} equals r_1^{ad}, and r_{os}^{ad} can therefore be determined directly from lysis curves by following the time course of lysis after 11-fold dilution.

To a first approximation cell lysis after 11-fold dilution follows an exponential decay curve, as can be seen when the lysis curves of Figs. 1 and 2 are replotted as log A versus time. Thus, the rate of lysis at time t after dilution depends on the cell density at time t; this rate can be expressed as the percentage of the available cells which lyse in one minute, or

$$r_{os}^{ad} = r_1^{ad} = \frac{\Delta A_t}{\min} \cdot \frac{1}{A_t} \tag{1}$$

where $\Delta A_t/\min$ is the change in the absorbance at time t and A_t is the absorbance at time t.

Lysis rates due to lysozyme activity after dilution were determined at $t < 30$ s; the lysis rate is relatively independent of t during the early portions of a lysis curve.

(2) Determination of r_{os}^{bd}. The determination of r_{os}^{bd} is complicated by the fact that cells may become osmotically sensitive after as well as before dilution. If r_{os}^{ad} is high relative to r_{os}^{bd} the absolute size of the cell fraction which lyses immediately upon dilution cannot be determined reliably (see for instance Figs. 1 and 2). Nevertheless, the rate at which this fraction increases can be obtained by comparing the absolute position of successive lysis curves, as illustrated in Fig. 3a. This is done by plotting the absorbance observed for each lysis curve at a fixed time shortly after dilution (for instance after 12 s) against the time at which the dilution is carried out (solid circles in Fig. 3b). This plot shows the increase in the percentage of cells which were osmotically sensitive before dilution. Since to a first approximation such a plot also follows an exponential decay curve, the rate r_{os}^{bd} at which this increase occurs can be calculated from this plot with Eqn. 1. Similar results are obtained when this procedure is based on the absorbancies measured 24 s after dilution (open squares in Fig. 3), indicating that the choice of a particular time shortly after dilution is not important. In our earlier experiments, the calculation of r_{os}^{bd} was based on plots constructed from four or five lysis curves, such as shown in Fig. 3b. However, in later experiments r_{os}^{bd} was calculated from only two lysis curves, obtained immediately and about 20 min after the addition of lysozyme, respectively.

Results

(*A*) *Effect of dilution on the lysozyme susceptibility of young cells*

When young *E. coli* are suspended in Tris/EDTA/sucrose and exposed to lysozyme they are rendered osmotically sensitive at a low rate r_{os}^{bd} before 11-fold dilution, and at a high rate r_{os}^{ad} after 11-fold dilution, as illustrated by Figs. 1 and 2.

Fig. 1 shows that when 11-fold dilutions were made in 10 mM EDTA (in water) at various times after the addition of lysozyme the lysis curves were identical. The simplest explanation for this result is that lysozyme did not significantly degrade murein before, but only after 11-fold dilution in EDTA. This was tested by exposing cells to lysozyme for the first time during rather than

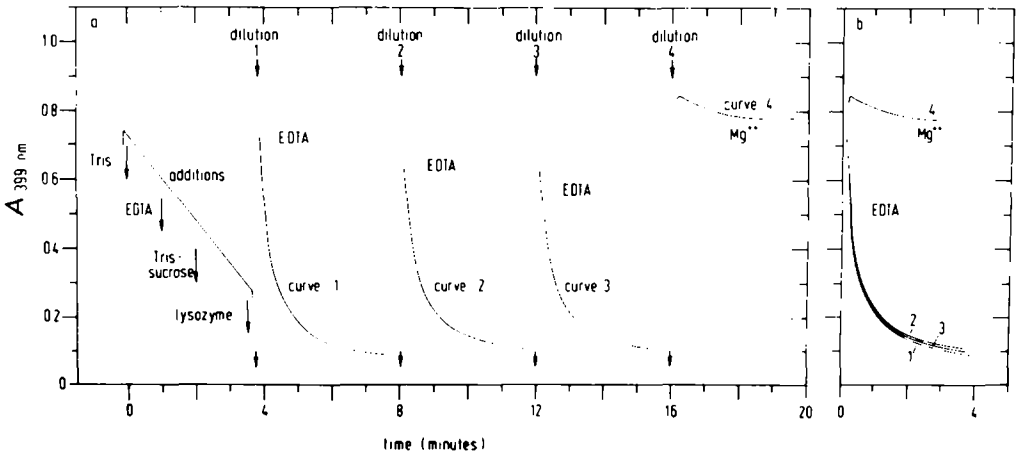

Fig. 1. Effect of lysozyme on young *E. coli* JC411. Buffer components and lysozyme were added to cells at the times indicated by arrows. Portions of the cell suspension were diluted 11-fold into 10 mM EDTA or 20 mM $MgCl_2$ at the times indicated by arrows, (final concentration of lysozyme = 2 μg/ml). The lysis curves have been superimposed in panel b to facilitate comparisons; zero time in panel b is the time at which the dilutions were initiated. Due to the time required for mixing and pouring the diluted cell suspensions into the spectrophotometer cell (always less than 10 s), recordings begin 5—10 s after the onset of mixing.

before the dilution. In the control experiment (Fig. 2a), cells were exposed to several concentrations of lysozyme prior to dilution, as in Fig. 1. In a parallel experiment (Fig. 2b), also carried out according to the schedule of Fig. 1a, lysozyme was not added prior to dilution, but was present instead in the diluent. The conditions of these experiments were such that the final lysozyme

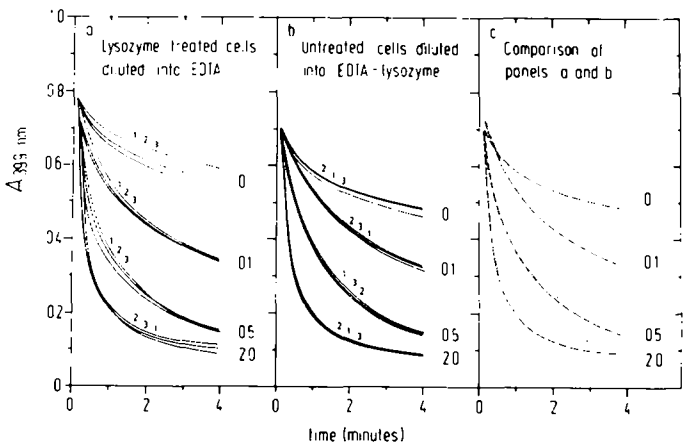

Fig. 2. Effect of adding lysozyme to young *E. coli* JC411 before and during dilution. Panel a: Lysozyme was added to the Tris/EDTA/sucrose suspension to concentrations of 0, 1.1, 5.5 and 22 μg/ml at 3.5 min; the suspensions were then diluted 11-fold into 10 mM EDTA at 3.75, 8 and 12 min (curves 1, 2 and 3, respectively). Panel b: Cells suspended in Tris/EDTA/sucrose were diluted 11-fold in 10 mM EDTA containing lysozyme at concentrations of 0, 0.11, 0.55 and 2.2 μg/ml, at 3.75, 8 and 12 min (curves 1, 2 and 3, respectively). Panel c: Comparison of panels a and b: ....., curves 2 of panel a; - - - - -, curves 2 from panel b. Final lysozyme concentrations (μg/ml) after 11-fold dilution are indicated next to each set of EDTA or EDTA-lysozyme dilutions. Upper left, middle and lower right numbers refer to the upper, middle, and lower curves of each set, respectively.

concentration after dilution of cells in 10 mM EDTA (Fig. 2a) or 10 mM EDTA-lysozyme (Fig. 2b) was the same for comparable sets of lysis curves. Since the lysis observed when cells were only exposed to lysozyme during dilution (Fig. 2b) was identical to the lysis observed when cells were preincubated with 11 times higher lysozyme concentrations for up to 8.5 min prior to dilution (Fig. 2a), the lysis observed in Figs. 1 and 2a must have been due to lysozyme activity after dilution.

The time elapsed after cells are suspended in Tris/EDTA/sucrose and before lysozyme is added is not important. Cells suspended in Tris/EDTA/sucrose and kept at 22°C behaved as illustrated in Fig. 1 when lysozyme was added at various times up to 7 h later. That is, they were relatively resistant to lysozyme and became susceptible only after 11-fold dilution into 10 mM EDTA. Since cells remain plasmolyzed for only a few minutes after suspension in sucrose [22], plasmolysis may be less important than has been assumed [4,22]. The results of Figs. 1 and 2 (*E. coli* JC411) were obtained with all of the strains tested so far, which include several K-12 strains (the lipopolysaccharides of these rough strains do not have detectable O-antigens (van Heerikhuizen, H., unpublished)), *E. coli* 0111 : K58 (a smooth strain with complete lipopolysaccharide), its mutant J5, the lipopolysaccharides of which lack O-antigen chains when the cells are grown in the absence of galactose [25], and several smooth strains of porcine origin with serotypes 08 : K'200' : H31 and 08 : K'200' : NM [26]. Thus, the state of the outer membrane lipopolysaccharides does not appear to be important in determining the access of lysozyme to the murein during 11-fold dilution.

(B) Determination of r_{os}^{bd} and r_{os}^{ad}

The rate r_{os}^{bd} was determined as described in Materials and Methods and illustrated in Fig. 3. Fig. 4 shows that r_{os}^{bd} increased approximately linearly with the lyso-

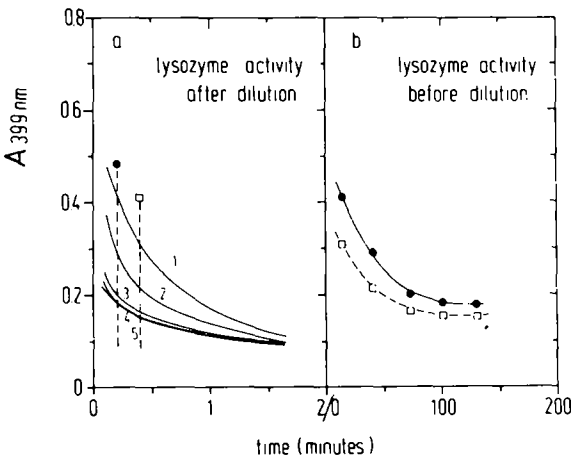

Fig. 3. Determination of r_{os}^{bd}. Exponential phase *E. coli* JC411, suspended in Tris/EDTA/sucrose to 1.7 mg/ml, were exposed to 8.8 μg/ml lysozyme (Fig. 1). Panel a: Lysis curves obtained after 11-fold dilution in 10 mM EDTA at 4, 30, 60, 90 and 120 min (curves 1 to 5, respectively). Panel b: The absorbancies observed in panel a, 12 s (•) or 24 s (□) after dilution, were plotted against the time of dilution. Eqn. 1 was used to calculate that $r_{os}^{bd} \approx 1.35\%$/min, based on the first half of either of these two curves.

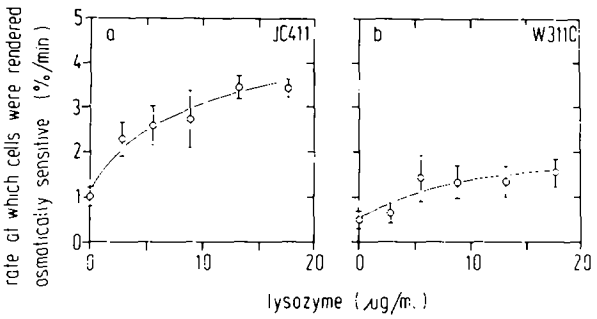

Fig. 4. r_{os}^{bd} as a function of the lysozyme concentration before dilution. Exponential phase *E. coli* were exposed to lysozyme concentrations varying from 0 to 18 μg/ml, which rendered the cells osmotically sensitive at the rates shown. Data points are averages of four rates, determined for two separate cultures. Vertical bars represent ± one standard deviation.

zyme concentration to 3.5%/min for *E. coli* JC411 and 1.5%/min for *E. coli* W3110, at the maximum lysozyme concentrations used (17.6 μg/ml). The rates of Fig. 4 were determined as soon as possible after the cells had been harvested; similar rates were obtained 5—6 h after the cells had been harvested.

Fig. 5 shows that the rate r_{os}^{ad} increased linearly with the lysozyme concentration up to about 1 μg/ml lysozyme (after 11-fold dilution), at which point it was about 150%/min for both *E. coli* JC411 (Fig. 5a) and W3110 (Fig. 5b). There were no systematic differences in the lysis rates when cells were tested immediately or 5—6 h after harvesting. There were small differences however at various times after the addition of lysozyme. Fig. 5 shows the results obtained immediately after the addition of lysozyme; the lysis rates obtained 22.5 min after the addition of lysozyme, were systematically lower and amounted to about 110%/min for both *E. coli* JC411 and *E. coli* W3110.

The ratio r_{os}^{ad}/r_{os}^{bd} was relatively constant at all lysozyme concentrations tested; the rate at which lysozyme rendered cells osmotically sensitive increased about 40—50-fold for *E. coli* JC411 and about 70—120-fold for *E. coli* W3110 upon 11-fold dilution in 10 mM EDTA, in spite of the fact that the lysozyme concentration after dilution was 11-fold lower than before dilution.

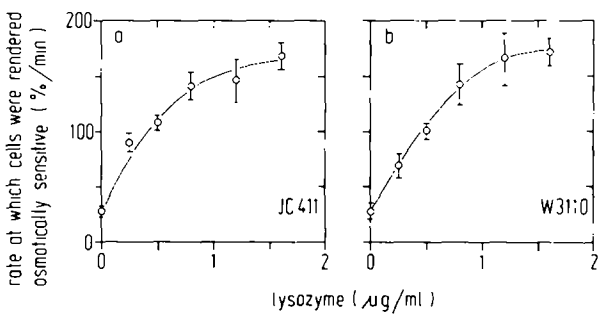

Fig. 5. r_{os}^{ad} as a function of the lysozyme concentration after 11-fold dilution. The cell suspensions of Fig. 4 were diluted 11-fold into 10 mM EDTA, after which the cells were rendered osmotically sensitive at the rates shown. Data points are averages for two separate cultures, each of which was tested 1 and about 5 h after harvesting. Vertical bars indicate ± one standard deviation.

The experiments of Figs. 4 and 5 were carried out with 10 mM EDTA as the diluent to eliminate the potential contribution of the outer membrane to the stability of the cell wall. Table I shows that water and 10 mM EDTA had essentially identical effects on osmotically sensitive cells. Even though the concentration of EDTA was only 0.05 mM after dilution in water, this was evidently sufficient to insure rapid lysis. When EDTA was not present in the suspension buffer, however, water was less effective as a diluent than 10 mM EDTA (data not shown).

Table I also shows that 20 mM $MgCl_2$ in water was not effective as a lysis diluent. Cells which had been rendered osmotically sensitive before dilution (as shown by the fact that they lysed in 10 mM EDTA or water) failed to lyse in 20 mM $MgCl_2$, indicating that $MgCl_2$ stabilizes the cell wall of exponential phase cells even when the covalent murein structure has been degraded to the extent that it can no longer protect cells from lysis. Thus, while 10 mM EDTA or water can be used as diluents to follow the effect of lysozyme on the murein layer, 20 mM $MgCl_2$ cannot, because the lack of lysis after dilution in 20 mM $MgCl_2$ does not necessarily reflect an intact murein layer.

(C) Effect of a 2-fold dilution on the accessibility of murein to lysozyme

In the experiments described above, we used an 11-fold dilution to enhance the access of lysozyme to the murein layer. To maintain the resulting spheroplasts intact however, it is necessary to employ a less drastic dilution. It was found that, as was the case with stationary phase cells [19], a 2-fold dilution was sufficient to permit lysozyme access to the murein layer, but not sufficient to lyse the resulting spheroplasts, which were true spheroplasts in that they were not only osmotically sensitive in water or 10 mM EDTA, but also spherical as seen by freeze-fracture electron microscopy. The presence of EDTA in the suspension buffer was crucial in permitting enhanced lysozyme access to the murein following the 2-fold dilution. When EDTA was omitted or Mg^{2+} was added, lysozyme failed to significantly degrade the murein layer after 2-fold

TABLE I

RATE AT WHICH CELLS WERE RENDERED OSMOTICALLY SENSITIVE BEFORE AND AFTER 11-FOLD DILUTION

Exponential phase cells were exposed to 8.8 μg/ml lysozyme before and 0.8 μg/ml lysozyme after dilution for a lysozyme/cell ratio (w/w) of 0.52%. Rates were determined for two separate cultures, each of which was treated with lysozyme about 1 and about 6 h after harvesting and suspension in Tris/Mg^{2+} buffer. Four rates were generally determined after each lysozyme treatment. Rates are listed as average ± one standard deviation, with the total number of rate determinations in parentheses.

Strain and diluent	Before dilution r_{os}^{bd} (%/min)	After dilution r_{os}^{ad} (%/min)
JC411		
10 mM EDTA	3.37 ± 1.53 (15)	115 ± 12 (16)
water	3.91 ± 1.65 (16)	99 ± 17 (16)
20 mM $MgCl_2$	0.46 ± 0.43 (4)	2.3 ± 1.0 (8)
W3110		
10 mM EDTA	1.42 ± 0.57 (15)	124 ± 26 (16)
water	2.34 ± 0.70 (13)	113 ± 42 (16)
20 mM $MgCl_2$	0.11 ± 0.07 (4)	5.9 ± 1.8 (8)

dilution. Mg^{2+} simply prevented lysozyme access to the murein layer; it did not inhibit lysozyme. When Mg^{2+} was added to a cell suspension after, instead of before 2-fold silution, lysozyme degraded the murein layer rapidly, in agreement with similar results for stationary phase *E. coli* [19].

The accessibility of the murein layer before and after 2-fold dilution depended on the growth conditions. When *E. coli* JC411 and W3110 were exposed to 5.5 μg/ml lysozyme, r_{os}^{bd} varied from about 0.2—0.5%/min (stationary phase cells in Brain Heart Infusion (Difco)) to about 2—8%/min (exponential or stationary phase cells in minimal medium with or without casamino acids or Nutrient Broth (both Difco)). When these cells were exposed to 2-fold dilution there generally was a considerable increase in their lysozyme sensitivity; the rate at which lysozyme rendered these cells osmotically sensitive after 2-fold dilution varied from about 5—35%/min (cells grown in variously supplemented minimal media) up to 45%/min (stationary phase cells in Brain Heart Infusion).

Discussion

It has long been known that the presence of sucrose in the suspension buffer considerably improves lysozyme access to the murein layer and it has been assumed therefore that gram-negative cells should be plasmolyzed for successful lysozyme treatment [4,22].

This conclusion may be incorrect, however, since plasmolysis and lysozyme sensitivity can easily be separated. After the addition of sucrose, cells remain plasmolyzed for only a few minutes; after this time, the cytoplasmic membrane expands again [22]. While such cells are no longer plasmolyzed, they still become sensitive to lysozyme whenever they are diluted during the next 7 h. Conversely, it is also possible to plasmolyze cells without rendering them sensitive to lysozyme; when Mg^{2+} is added to cells either before or after plasmolysis, osmotic shock fails to result in penetration lysozyme through the outer membrane.

The fact that dilution can enhance lysozyme activity implies that it is difficult to interpret the result of an osmotic sensitivity determination in which the extent of cell lysis is determined after cells are exposed to a 5—20-fold dilution in a hypotonic buffer. Even though most cells in a suspension may be osmotically insensitive before dilution, they may well become osmotically sensitive during the osmotic sensitivity test itself, and the osmotic sensitivity of the cells being tested may therefore be overestimated considerably.

Such overestimation could perhaps explain the paradoxical observation that lysozyme-treated cells often remain rodshaped, despite the fact that they appear to be osmotically sensitive [2,4,11,27—29]. If in fact these cells were not yet, or only partially, osmotically sensitive at the time of microscopy, and then became fully osmotically sensitive during the osmotic sensitivity determination, they would appear to have been rodshaped as well as osmotically sensitive. When osmotic sensitivity is determined as described in this paper, this relationship is not found. Instead, the development of osmotic sensitivity is always correlated with the conversion of rodshaped cells to spheres.

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