

## Characterization of *Lactobacillus helveticus* strains resistant to lysozyme

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**Summary** — Strains of *Lactobacillus helveticus* resistant to 100 µg/ml of lysozyme were obtained by 2–4 successive transfers in milk containing lysozyme at this concentration. The non-growing cells exhibited a greater resistance to loss of viability and to lysis. Resistant strains were similar to sensitive strains in regard to growth in milk as measured by the conductimetric method, carbohydrate fermentation and enzymatic activity patterns, NaCl and antibiotic resistance. Lysozyme resistance in *L. helveticus* exhibited stability in the absence of lysozyme. Acquisition of resistance was apparently obtained by strain adaptation and not by selection of spontaneous mutants.

### *Lactobacillus helveticus* / lysozyme resistance

**Résumé** — Caractérisation des souches de *Lactobacillus helveticus* résistantes au lysozyme. Des souches de *L. helveticus* résistantes à 100 µg/ml de lysozyme ont été obtenues par 2–4 repiquages successifs dans du lait contenant du lysozyme à cette concentration. Les cellules non-proliférantes des souches résistantes ont une plus grande résistance vis-à-vis de la perte de viabilité et vis-à-vis de la lyse. Les souches résistantes ont les mêmes propriétés que les souches sensibles en ce qui concerne la croissance dans le lait (mesurée par conductimétrie), les profils de fermentation des sucres et des activités enzymatiques, la résistance au NaCl et aux antibiotiques. La résistance au lysozyme chez *L. helveticus* reste stable en l'absence de lysozyme. L'acquisition de la résistance se fait apparemment par adaptation de la souche et non par sélection de mutants spontanés.

### *Lactococcus helveticus* / résistance au lysozyme

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## INTRODUCTION

Lysozyme can be defined as a 1,4 *N*-acetylmuramidase which cleaves the glycosidic bonds of peptidoglycan of the bacterial cell wall. Because of the lytic activity of the enzyme on several species of Gram-positive and Gram-negative bacteria, it has been extensively used to prevent late blowing faults caused by *Clostridium tyrobutyricum* in cheese (Carini *et al*, 1985; Anonymous, 1987). Literature is available on this subject, describing the use of lysozyme in cheesemaking and its action on bacteria (Carini *et al*, 1985).

The wide spectrum of lysozyme activity has led to the study of the relationship between the enzyme and lactic acid bacteria. In particular, its interference with ripening of hard cheeses by inhibition of metabolite production and/or proteolytic activity by dairy species has been investigated (Carini *et al*, 1985). More importantly, further studies have shown that the presence of lysozyme might represent a serious problem in some cheese (such as Grana Padano, Parmesan, Emmentaler, Saint Paulin and Gouda) production technologies, where lactobacilli present in natural whey cultures or used as starters might be inhibited by the enzyme (Bottazzi *et al*, 1978; Lodi *et al*, 1983; Grazia *et al*, 1984; Anonymous, 1987).

Previous studies have shown that lysozyme sensitivity is species and strain-dependent and that 100 µg/ml of the enzyme is the best threshold level to determine the sensitivity of the strains (Neviani *et al*, 1988a). It has been well established that lactic streptococci in general are more resistant to lysozyme than lactobacilli. Furthermore, among lactobacilli, *Lactobacillus helveticus* has shown a higher sensitivity to the lytic enzyme than other lactobacilli (Neviani *et al*, 1988a, 1988b). In view of the widespread presence of *L. helveticus* in

thermophilic cultures used in cheese-making, it seemed particularly interesting to study lysozyme resistance in this species with the eventual application as starters. The ability to obtain *L. helveticus*-resistant variants was shown in a preliminary study (Neviani *et al*, 1988b) by growing 8 or 12 serial subcultures in autoclaved milk containing 100 µg/ml of lysozyme.

The present work specifies the optimal conditions for obtaining lysozyme resistant derivatives from *L. helveticus* lysozyme sensitive strains and describes the properties of these resistant derivatives.

## MATERIALS AND METHODS

### *Strains, media and growth conditions*

*L. helveticus* strains 1, 3, 4, 11, 13, 14, 27 and 31 were obtained from the stock collection of the Istituto Sperimentale Lattiero Caseario (Lodi). They were maintained as frozen stocks at -20 °C in litmus milk. The resistant strains, labelled "R", were maintained under selective pressure at -20 °C in litmus milk containing 100 or 500 µg/ml of lysozyme.

In preparation for experiments, *L. helveticus* strains were grown in MRS broth (De Man *et al*, 1960) at 42 °C for 15–18 h.

### *Microbiological growth monitoring*

Bacterial growth was evaluated by conductance measurements using a Malthus instrument growth analyzer (Baynes *et al*, 1983; Jason, 1983; Firstenberg-Eden and Eden, 1984; Owens, 1985). The system is able to detect changes in conductance of the growth medium caused by the bacterial metabolism and the consequent production of new electrically charged molecules. Changes (expressed in microsiemens, µS) are shown graphically as conductance curves that are strictly related to the microbial increase (Baynes *et al*, 1983; Neviani *et al*, 1988a, 1988b). Furthermore, 2 parameters

can be considered to study the microbial metabolism: i), the conductance detection time (*DT*) defined as the point (expressed in h) where the baseline ends and the region of acceleration of the curve begins; the *DT* is related to the initial concentration of bacteria in the sample; ii), the generation time (*GT*) calculated for each strain by using a regression line referred to the correlation between the bacterial concentration and the *DT* of different sample dilutions. The *GT* of a strain is related to the slope of its regression line (Firstenberg-Eden and Eden, 1984).

### **Sensitivity to lysozyme**

*L. helveticus* strains 4, 11, 31 were grown in autoclaved (115 °C for 10 min) skim milk at 42 °C for 15–18 h. Autoclaved skim milk was also dispensed in 9-ml amounts in 10-ml capacity growth tubes of the Malthus growth analyzer containing 100 µg/ml of hydrochloride lysozyme (SPA, Milano, Italy). The cultures were then inoculated into the tubes so as to obtain a cell concentration of  $\approx 10^4$ /ml. Control tubes were similarly inoculated and included autoclaved skim milk without lysozyme. For the evaluation of *GT*, different initial cell concentrations (from  $10^6$ /ml to 1/ml) were used. The sensitivity to the enzyme was observed after evaluation of the differences on *GT*, *DT*, slope and final conductivity of the curves among the cultures with and without lysozyme.

### **Acquisition of lysozyme resistance**

*L. helveticus* cultures were successively transferred to 10 ml of autoclaved milk containing 100 mg/ml of lysozyme so as to obtain  $\approx 10^5$  cells/ml and incubated at 42 °C for 18 h. The transfers were carried out in the Malthus growth analyzer according to the following scheme: control cultures were inoculated with (L) and without (LC) 100 µg/ml of lysozyme. After growth, L subcultures were inoculated again with (2 L) and with-out (1 LC) 100 µg/ml of lysozyme and so on, to reach the superposition of the conductance curves between  $xL$  and ( $x-$

1)LC cultures, where  $x$  represents the number of transfers.

### **Stability of lysozyme resistance character**

Resistant variants 4 R, 11 R and 31 R of *L. helveticus* strains 4, 11 and 31 were subcultured in sterile milk in the absence of lysozyme and incubated at 42 °C for 15–18 h. After successive transfers, the resulting cultures were tested for lysozyme resistance after growth in sterile skim milk with and without 100 µg/ml of lysozyme by the Malthus growth analyzer. The subcultures grown in presence of lysozyme compared to the corresponding cultures in its absence and showing similar conductance curve and *DT* were considered still resistant to 100 µg/ml of lysozyme. Lysozyme sensitive strains 4, 11 and 31 were similarly grown in the Malthus growth analyzer and considered as control cultures.

### **Sensitivity to lysozyme of non-growing cells**

Overnight cultures were harvested by centrifugation at 4 °C then washed twice in 50 mmol/l phosphate buffer, pH 7.0. The pellet was resuspended in the same buffer and incubated at 37 °C with or without 100 µg/ml of lysozyme. At different times, samples were taken and were enumerated in MRS agar plates.

### **Lysis of cells**

Overnight cultures were harvested by centrifugation at 4 °C then washed once in distilled water. The pellet was resuspended in 100 mmol/l phosphate buffer, pH 7.0, to obtain a bacterial suspension at  $OD_{650} = 1$  unit. Several concentrations of lysozyme were added to study lysis. Duplicate tubes containing no lysozyme were prepared as controls. Incubations were carried out at 37 °C, and changes in optical density were followed by using a model UV120602 Shi-

madzu spectrophotometer at OD<sub>650</sub> after different periods of incubation.

### Physiological and biochemical tests

Phenotypic changes among resistant variants were studied using cells harvested from overnight cultures on MRS broth. They were examined for carbohydrate fermentation and enzymatic activity patterns using the API 50CH and API ZYM identification kits (API Biomerieux, France) according to the manufacturer's instructions. The minimum inhibitory concentration (MIC) of lysozyme, nisin (K & K Laboratories, 10<sup>6</sup> UR/g) and different antibiotics (Sigma Chemical Co) were calculated after growth in MRS broth. The antibiotics were: ampicillin (No A-9393), polymyxin B sulfate (No P-1004), chloramphenicol (No C-0378), tetracycline (No T-3258), novobiocin (No N-1628), kanamycin monosulfate (No K4000), erythromycin (No E-6376), rifamycin SV (No R8626) and streptomycin sulfate (No S6501). Inhibition of growth in MRS broth + 0, 1, 2.5, 5 and 10% NaCl was also detected. Antibiotics and NaCl containing tubes were inoculated so as to obtain a final cell concentration of  $\approx 10^5$ /ml. Tubes were incubated at 42 °C and checked for growth after 24 and 48 h. Acid production in sterile skim milk during 24 h of incubation at 42 °C was evaluated by pH measurements with a Metrohm 654 pH meter and expressed as pH curves.

## RESULTS

### Sensitivity to lysozyme

The growth in milk, as measured by the conductimetric method (fig 1, *L. helveticus* 4 as example), was inhibited by 100 µg/ml of lysozyme for the 3 strains tested 4, 11 and 31. As expected, by decreasing the initial cell concentration the inhibition by lysozyme was higher, as shown by the delay of DT (table I). This observation was also confirmed by the study of the GT (table II): in fact, the doubling time of the cul-

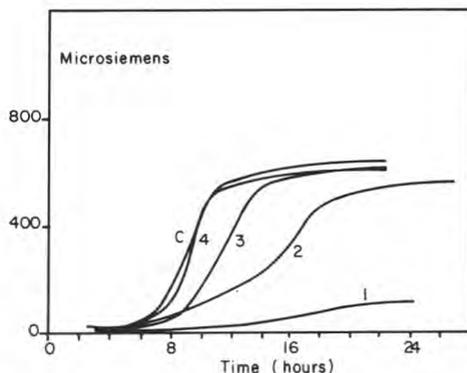


Fig 1. Acquisition of resistance to lysozyme in *Lactobacillus helveticus* strain 4 by successive transfers. Growth in milk with 100 µg/ml of lysozyme. 1-4: transfer number. C: control in milk without lysozyme.

Acquisition de la résistance au lysozyme chez *Lactobacillus helveticus* souche 4, par repiquages successifs. Croissance dans du lait avec 100 µg/ml de lysozyme. 1-4 : numéro du repiquage. C : contrôle dans du lait sans lysozyme.

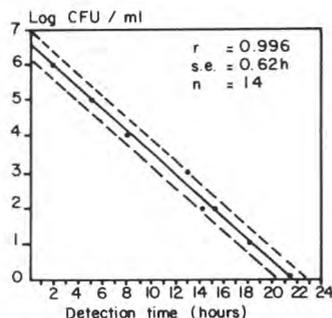


Fig 2. Regression line of the microbial count versus the detection time (DT) of *Lactobacillus helveticus* strain 4. --- : 95% confidence interval ( $\pm 1.96$  standard error).

Droite de régression de la concentration cellulaire en fonction du temps de détection (TD) de la souche 4 de *Lactobacillus helveticus*. --- : intervalle de confiance à 95% ( $\pm 1,96$  erreur standard).

**Table I.** Inhibition of *Lactobacillus helveticus* by lysozyme, measured as changes in detection time (DT). Growth in milk with (+ lys) or without (- lys) 100 µg/ml of lysozyme. nd: non determined. *Inhibition de Lactobacillus helveticus par le lysozyme, mesurée par la modification du temps de détection (DT). Croissance dans du lait avec (+ lys) ou sans (- lys) 100 µg/ml de lysozyme. nd: non mesuré.*

No of bacteria/ml	Detection time (h)					
	Strain 4		Strain 11		Strain 31	
	- lys	+ lys	- lys	+ lys	- lys	+ lys
10 <sup>6</sup>	1.7	3.5	1.2	8.0	1.7	4.5
	1.7	3.7	1.8	8.3	2.2	6.4
10 <sup>5</sup>	5.0	12.0	4.4	12.2	4.8	14.0
	5.0	12.5	4.4	12.5	5.2	16.6
10 <sup>4</sup>	7.8	16.0	7.8	15.9	9.6	21.8
	7.9	16.1	8.0	19.0	10.0	22.2
10 <sup>3</sup>	13.2	23.9	13.7	23.2	12.4	29.8
	12.9	24.9	14.0	24.1	12.6	31.3
10 <sup>2</sup>	14.2	33.6	16.2	28.0	15.1	39.2
	15.3	35.6	16.6	29.7	15.2	40.0
10 <sup>1</sup>	18.1	39.3	18.8	33.1	17.0	nd
	18.4	42.1	19.1	35.1	17.6	nd
10 <sup>0</sup>	21.0	nd	22.7	38.6	20.5	nd
	21.7	nd	23.3	40.7	20.7	nd

tures in the presence of lysozyme was strongly increased.

The regression coefficients (table II) of the regression lines (fig 2) used to calculate the *GT* for the cultures with and without lysozyme confirmed the accuracy of the method.

#### Acquisition of lysozyme resistance

*L. helveticus* strains 4, 11 and 31 became progressively resistant to lysozyme during successive transfers in milk containing 100 µg/ml of lysozyme (fig 1). The conductance curves obtained at each transfer showed that strains 4, 11 and 31 needed 3, 4 and 3 transfers respectively to become complete-

ly resistant, as shown by the superposition of the curves with (L) or without (LC) lysozyme (fig 1). Similar results were obtained with the 5 other strains tested (1, 3, 13, 14, 27): they needed 2 (strain 27) to 4 transfers. Therefore, it is not necessary to extend the selection protocol to 8 or 12 serial subcultures as previously recommended (Neviani *et al*, 1988b).

As heterogeneity in initial populations could be expected, the experiments on the successive transfers were performed with clones freshly isolated from the initial population of *L. helveticus* strains 4, 11 and 31. A similar acquisition of lysozyme resistance was obtained with the progressive reduction of growth inhibition in milk with lysozyme.

**Table II.** Inhibition of *Lactobacillus helveticus* by lysozyme, measured as changes in generation time (GT). Growth in milk with (+ lys) or without (- lys) 100 µg/ml of lysozyme. *r* = correlation coefficient of the regression line used to calculate GT.

*Inhibition de Lactobacillus helveticus par le lysozyme, mesurée par la modification du temps de génération (GT). Croissance dans du lait avec (+ lys) ou sans (- lys) 100 µg/ml de lysozyme. r = coefficient de corrélation de la droite de régression utilisée pour calculer GT.*

	Strain 4		Strain 11		Strain 31	
	- lys	+ lys	- lys	+ lys	- lys	+ lys
GT	60	136	67	97	56	151
<i>r</i>	0.996	0.993	0.994	0.996	0.994	0.996

### Stability of lysozyme resistance character

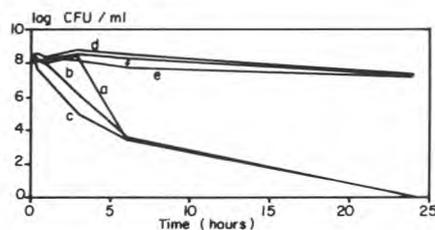
Conductimetric tests confirmed that after 5 subcultures in milk, *L. helveticus* resistant variants 4R, 11R and 31R were still resistant to 100 µg/ml of lysozyme whereas sensitive strains 4, 11 and 31 remained sensitive as expected. These data indicated that it is not necessary to maintain the strains under selective pressure to preserve the phenotypic stability of the lysozyme resistance character.

### Sensitivity to lysozyme of non-growing cells

Sensitivity to lysozyme of *L. helveticus* strains 4, 11 and 31 was also confirmed for non-growing cells (fig 3); in fact, lysozyme already caused a sharp decrease in the number of the viable cells after 1 to 2 h of incubation. The remaining viable cell population for all 3 strains was  $\approx 10^3$ /ml after 6 h and decreased to 10/ml after 24 h of incubation.

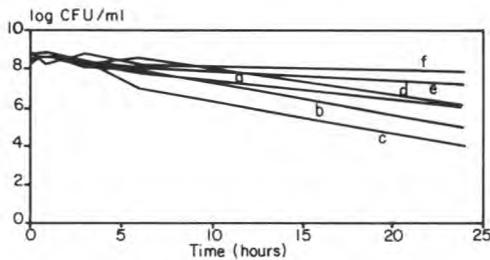
However, resistant variants 4R, 11R and 31R showed no difference in viability for the first 4 h when compared to controls without the enzyme (fig 4); after 6 h, de-

crease in the number of viable cells was observed for the 3 strains (fig 4), but at lower rate if compared to that of strains 4, 11 and 31 (fig 3). In the absence of lysozyme, sensitive strains 4, 11 and 31 and their resistant variants 4R, 11R and 31R showed a similar decrease in the number of viable cells (figs 3 and 4).



**Fig 3.** Sensitivity to lysozyme of non-growing cells of *Lactobacillus helveticus*. Incubation at 37 °C for 24 h in 50 mmol/l phosphate buffer, pH 7.0 with (curves a, b and c : strains 4, 11 and 31 respectively) or without (curves d, e and f : strains 4, 11 and 31 respectively) 100 µg/ml of lysozyme.

*Sensibilité au lysozyme de cellules non proliférantes de Lactobacillus helveticus. Incubation à 37 °C pendant 24 h en tampon phosphate (50 mmol/l, pH 7,0) avec (courbes a, b et c : souches 4, 11 et 31 respectivement) ou sans (courbes d, e et f : souches 4, 11 et 31 respectivement) 100 µg/ml de lysozyme.*



**Fig 4.** Sensitivity to lysozyme of non-growing cells of *Lactobacillus helveticus* resistant strains. Incubation at 37 °C for 24 h in 50 mmol/l phosphate buffer, pH 7.0 with (curves a, b and c : strains 4R, 11R and 31R respectively) or without (curves d, e and f : strains 4R, 11R and 31R respectively) 100 µg/ml of lysozyme.

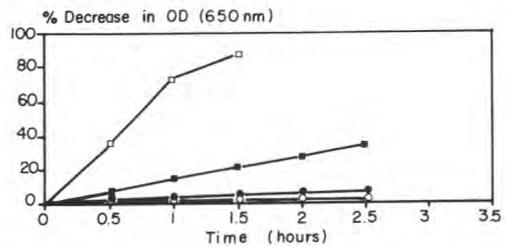
*Sensibilité au lysozyme de cellules non proliférantes de souches résistantes de Lactobacillus helveticus. Incubation à 37 °C pendant 24 h en tampon phosphate (50 mmol/l, pH 7,0) avec (courbes a, b et c : souches 4 R, 11 R et 31 R respectivement) ou sans (courbes d, e et f : souches 4 R, 11 R et 31 R respectivement) 100 µg/ml de lysozyme.*

### Cell lysis by lysozyme

Rapid lysis of sensitive strain *L. helveticus* 31 occurred in phosphate buffer containing 100 µg/ml of lysozyme (fig 5). This lysis was also observed with lysozyme resistant variant 31R but at a reduced rate. Cell autolysis in absence of lysozyme was negligible for both the sensitive and the resistant strains (fig 5). Similar differences were observed for strains 4 and 11 and their resistant variants 4R and 11R.

### Physiological and biochemical tests

No differences were observed in carbohydrate fermentation and enzymatic activity patterns, NaCl resistance, MIC of different



**Fig 5.** Lysis by lysozyme of cells of *Lactobacillus helveticus* strains 31 et 31R. Lysis determined by decrease in optical density at 37 °C of cells suspension in 100 mmol/l phosphate buffer at pH 7.0 with (—□—, —■—) or without (—○—, —●—) 100 µg/ml of lysozyme. Strains: sensitive 31 (—□—, —○—) or resistant 31 R (—■—, —●—).

*Lyse par le lysozyme de Lactobacillus helveticus souche 31 et 31R. Lyse mesurée par la diminution de densité optique à 37 °C d'une suspension cellulaire en tampon phosphate 100 mmol/l à pH 7,0 avec (—□—, —■—) ou sans (—○—, —●—) 100 µg/ml de lysozyme. Souches : sensible 31 (—□—, —○—) au résistante 31 R (—■—, —●—).*

antibiotics (ampicillin, chloramphenicol, erythromycin, kanamycin, novobiocin, polymyxin, rifamycin, streptomycin, tetracycline and nisin) or acid production, among *L. helveticus* strains 4, 11 and 31 and their resistant variants 4R, 11R and 31R. An exception is that strain 4 R showed an increase in nisin resistance (table III). As expected, the MIC of lysozyme strongly increased for the resistant variants, particularly strain 31R (table III).

### DISCUSSION

The determination of growth by conductance measurements was a very effective tool in this study. This technique has allowed the measurement of the acquisition of resistance to lysozyme until the growth

parameters in milk with lysozyme were stabilized.

The results showed that it was easy to obtain *L. helveticus* variants whose growth in milk was not disrupted by a lysozyme concentration equivalent to that (20–50 µg/ml) used in hard and semi-hard cheese technology (Lodi, 1990). This observation probably explains why lysozyme does not apparently affect the acidification of milk and curd formation in industrial fabrication. It should be mentioned that the contribution of *L. helveticus* has been reduced in modern cheese technology for Emmental cheese. On the other hand, one can reasonably assume that industrial strains of *L. helveticus* have been spontaneously adapted or have been modified to growth in milk with lysozyme.

The isolation and characterization of lysozyme-resistant derivatives (Litwack, 1958; Brumfitt, 1959) have been described in some Gram-positive bacteria. In *Micrococcus lysodeikticus* there was a difference in the rate of growth between the sensitive and resistant strains (Litwack, 1958; Brumfitt, 1959) whereas no difference was observed in *L. helveticus* in the present study. These results are consis-

tent with the loss of resistance (by back-mutation) in *M. lysodeikticus* and the stability of resistance in *L. helveticus*.

The growth of sensitive strains of *L. helveticus* was not completely inhibited by 100 µg/ml of lysozyme. Therefore, cell populations of these strains comprise a small percentage of resistant cells that will be the origin of new cell populations completely resistant to lysozyme. Similar heterogeneity in lysozyme resistant was observed in cell populations obtained from clones isolated from initial sensitive cell populations. These observations suggest that the phenotypic expression of lysozyme resistance is extremely variable among individual cells of *L. helveticus* grown in milk without lysozyme.

The antibacterial properties of lysozyme were characterized by 3 effects (Iacono *et al*, 1980; i), a lytic effect, by lysis of non-growing cells; ii), a bactericidal effect, by loss of viability in a non-nutritive medium; iii), a bacteriostatic effect, by partial or total inhibition of growth in a culture medium. All of these effects were not necessarily detected simultaneously in the same strain (Brumfitt, 1959; Iacono *et al*, 1980). Concerning *L. helveticus*, an increase of resistance to lysozyme was observed for the lytic, bactericidal and bacteriostatic effects. Therefore, one can reasonably assume that the hydrolytic activity of lysozyme is the major mechanism of cell death. Consequently, an increase in lysozyme resistance would be caused by some modifications in the chemical composition of the cell wall, as previously observed in *M. lysodeikticus* and *Bacillus megatherium* (Brumfitt, 1959). However, some cell lysis in *L. helveticus* resistant strains occurred at bacteriostatic concentrations of lysozyme. Therefore it appears that the cell viability also depends on the rate of cell wall synthesis necessary to offset the destruction of cell wall material.

**Table III.** Minimum inhibitory concentration (MIC) of lysozyme and nisin for *Lactobacillus helveticus* strains 4, 11, 31, and their resistant variants 4R, 11R and 31R.

*Concentration minimale inhibitrice (CMI) du lysozyme et de la nisine chez les souches de Lactobacillus helveticus 4, 11, 31 et leurs variants résistants 4R, 11R et 31R.*

Strains	MIC of lysozyme (µg/ml)	MIC of nisin (µg/ml)
4	25	0.5
4R	100	2.0
11	50	1.0
11R	200	1.0
31	25	0.5
31R	400	0.5

The mechanism involved in the selection of resistant derivatives of *L. helveticus* is not clear. It appears that a cell population of the initial strain contained some cells that were spontaneously resistant to lysozyme. Consequently, by adding lysozyme to milk the resulting population should be composed of cells resistant to lysozyme and should give a normal growth during a subsequent transfer in milk containing lysozyme. In fact, a partial inhibition of growth was observed.

Therefore, acquisition of resistance in *L. helveticus* was apparently obtained by strain adaptation during growth in milk containing lysozyme. Clearly, further data are needed to explain the acquisition of resistance to lysozyme in *L. helveticus*.

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