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The Effect of Unbalanced Cell Wall Synthesis on the Protoplast Formation in Cells of *Staphylococcus aureus* "Oxford"

Wpływ niezrównoważonej syntezy ściany komórkowej na tworzenie się protoplastów z komórek *Staphylococcus aureus* „Oxford"

Влияние неуравновешенного синтеза клеточной стенки на скорость образования протопластов из клеток *Staphylococcus aureus „Oxford"*

The synthesis of cell wall polymers in bacteria can be dissociated from protein synthesis. Hancock and Park (2), and Mandelstam and Rogers (4) showed that mucopeptide synthesis could occur in *Staphylococcus aureus* cells in the presence of growth-inhibiting concentration of chloramphenicol, an inhibitor of protein synthesis. Hash et al. (3) concluded that the cells of *Staphylococcus aureus* pretreated with tetracycline also continued to synthesize cell wall mucopeptide and nucleic acids, while the synthesis of protein was suppressed. Shockman (8) observed that valine or threonine starvation could also result in the inhibition of protein synthesis and continuation of the synthesis of cell wall mucopeptide.

According to Shockman (8) there are several alternative mechanisms to explain the behaviour of the additional cell wall substance synthesized. The presence of more wall material could result in: 1) thicker wall structure, 2) more dense wall structure and 3) the same thickness or density of cell wall but a larger surface area per culture. Davies (1) presented electron micrographs of *Staphylococcus aureus* H cells which showed thicker walls during unbalanced cell wall synthesis, i.e. when the protein synthesis was inhibited by tetracycline. Following biochemical and morphological studies Shockman (8) proved that unbalanced cell wall synthesis in *Streptococcus faecalis* 9790 resulted in wall thickening.

The purpose of this paper was to investigate the effect of unbalanced cell wall synthesis on the formation of protoplasts in cells of *Staphylococcus aureus* "Oxford".

MATERIALS AND METHODS

Strains

Staphylococcus aureus "Oxford" strain was used in all experiments reported here.

Media

The culture medium used was Headley-Wright broth containing 1% of 10% glucose.

Chemicals and enzyme

The staphylolytic enzyme prepared from the culture of *Streptomyces griseus* according to the method of Ward and Perkins (10) was a gift from Dr H. R. Perkins from National Institute for Medical Research, London.

All chemicals used were of Analar grade.

Exponential growth of cells

Lyophillized cells of *Staphylococcus aureus* were suspended in 10 ml of Headley-Wright broth containing glucose and allowed to grow overnight at 35° on a shaker. Next day 200 ml of the same medium were inoculated with 1 ml of the overnight culture and shaken for about 3 hrs at 35° to reach optical density of 0.290—0.300 (diluted 1:10) on a Spekker photocolorimeter, that was equivalent to 1 mg/ml dry weight of bacteria. The culture was rapidly cooled in ice and allowed to stand overnight at 2—4°. The contents were mixed with 200 ml of fresh Headley-Wright broth, heated up to 35° and shaken for about 30—60 min. at 35° to attain optical density of 1 mg/ml dry weight of bacteria. The cells were cooled in ice, centrifuged, washed three times with water and resuspended in water to obtain turbidity equivalent to 3 mg/ml dry weight of bacteria.

Unbalanced cell wall synthesis

The cells grown according to the above method were resuspended in cold (2°) double strenght buffered glucose medium: 37.5 ml of 18% KH_2PO_4, 12.5 ml of 40% glucose and 30 ml of 1 N NaOH were made up to 500 ml with cold distilled water. The washed staphylococcal cells were resuspended in 100 ml of buffered glucose medium to reach optical density equivalent to 2 mg/ml dry weight of bacteria, warmed up to 35° and mixed with 100 ml of cell wall medium that was also warmed up to the same temperature. 100 ml of the wall medium contained: 4 ml of 10 mg/ml DL-alanine, 4 ml of 10 mg/ml glycine, 4 ml of 10 mg/ml DL-glutamate, 4 ml of 10 mg/ml L-lysine and 10 ml of 1 mg/ml chloramphenicol. The mixture was shaken at 35° for 90 min., the cells were centrifuged, washed three times with water and resuspended in water to reach optical density corresponding to 3 mg/ml dry weight of bacteria.

Formation of protoplasts

Three lots of 5 ml of 3 mg/ml test- and control-suspensions in water were centrifuged and resuspended in:

1) 4 ml of 30% poliethylene glicol (PEG v/v) in 0.005 M tris-HCl buffer, pH 7.9, 0.01 M $MgSO_4$, and 100 µl of the staphylolytic enzyme.

2) 4 ml of 0.005 M tris-HCl buffer, pH 7.9.

3) 4 ml of 0.005 M tris-HCl buffer, pH 7.9, and 100 µl of the enzyme.

All mixtures were incubated in a water bath at 37° and the optical density was checked at 0, 30, 60, 120, 180, 240 and 300 min. Samples were diluted (1:10) in respective solution (buffer or 30% PEG). The rate of protoplast formation at various incubation times was estimated by reduction of optical density, after osmotic shock of cells in tubes No. 1. The Spekker readings were converted to the per cent reduction of the zero-time turbidity for plotting a graph. The mixtures in tubes No 1 were controlled under a phase contrast microscope throughout incubation.

RESULTS

Table 1. Optical density of control cell suspension at various times of incubation with staphylolytic enzyme

Sample No		Time (minutes)						
		0	30	60	120	180	240	300
1	Suspension in 30% PEG, 100 µl of enzyme Mg^{2+} (0.01 Mf. conc.)	0.600	0.480	0.450	0.380	0.380	0.365	0.360
2	Suspension in buffer, 100 µl of enzyme	0.590	0.410	0.225	0.060	0.040	0.020	0.000
3	Suspension in buffer	0.595	0.580	0.572	0.565	0.555	0.550	0.550

Table 2. Optical density of test cell suspension at various times of incubation with staphylolytic enzyme

Sample No		Time (minutes)						
		0	30	60	120	180	249	300
1	Suspension in 30% PEG, 100 µl of enzyme Mg^{2+} (0.01 Mf. conc.)	0.580	0.560	0.530	0.520	0.500	0.490	0.460
2	Suspension in buffer, 100 µl of enzyme	0.575	0.500	0.460	0.400	0.320	0.250	0.100
3	Suspension in buffer	0.582	0.560	0.545	0.530	0.530	0.530	0.525

Tables 1 and 2 present a drop in optical density of the test- and control-cell suspensions diluted 1:10 in corresponding solutions (buffer or 30% PEG). They show that the reduction of turbidity of the test cell suspension (in PEG + enzyme) is much smaller than that of the control

Table 3. Reduction of turbidity of cell suspensions after osmotic shock

Time (min.)	Test cells			Control cells		
	optical density in 30% PEG (dil. 1:10)	optical density in buffer (dil. 1:10)	% lysis	optical density in 30% PEG (dil. 1:10)	optical density in buffer (dil. 1:10)	% lysis
0	0.580	0.580	—	0.600	0.600	—
30	0.560	0.535	(7.8)	0.480	0.420	(27.6)
60	0.530	0.500	(13.8)	0.450	0.140	(75.9)
120	0.520	0.420	(27.6)	0.380	0.060	(89.7)
180	0.500	0.380	(34.9)	0.380	0.020	(96.6)
240	0.490	0.350	(39.7)	0.365	0.015	(97.5)
300	0.460	0.250	(56.9)	0.360	0.005	(99.3)

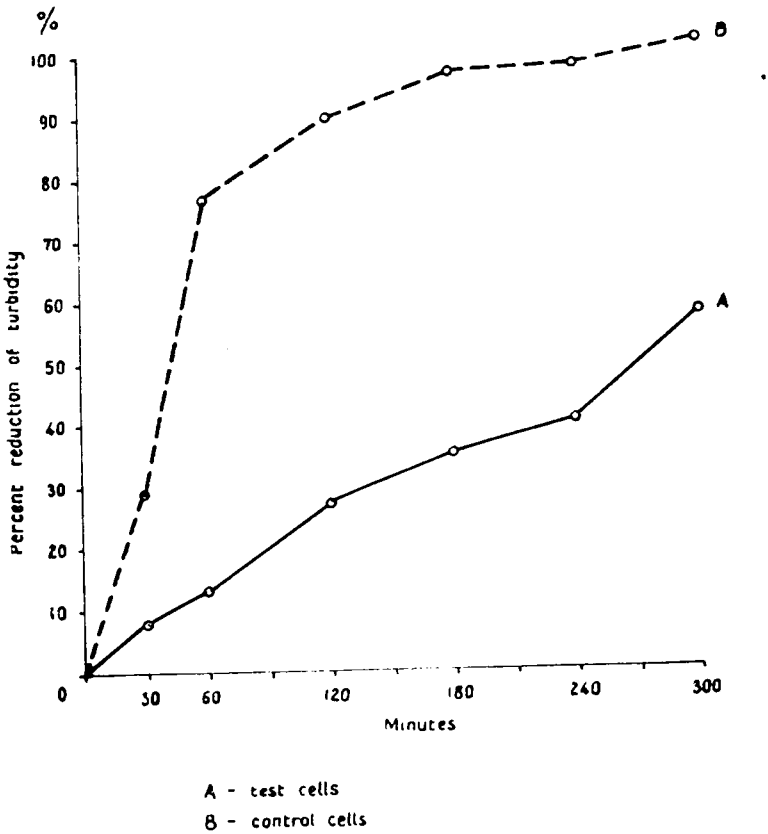

Fig. 1. Effect of time of exposure to staphylolytic enzyme on the change in optical density of *Staphylococcus aureus* suspensions subjected to osmotic shock

cell suspension. The same difference was observed in tubes containing only suspension in buffer and the lytic enzyme. The lysis of control cells occurred promptly in comparison with the test cells. Within 120 min. about 90% of the control cells were lysed, while at the same time only 40% of the cells were degraded. These results suggest that the test cells with thicker walls required a longer exposure to the activity of the lytic enzyme.

Table 3 and Fig. 1 illustrate the per cent reduction of turbidity of the test- and control-cell suspension (in 30% PEG + enzyme) after osmotic shock, i.e. after dilution 1:10 in buffer. After 1 hour incubation the control cell suspension showed a 75% reduction in turbidity following dilution in buffer, while the test cell suspension — only 13%. After 3 hours of incubation nearly all the control cells turned into osmotically fragile forms, while the test cell suspension showed only a 56.9% reduction in turbidity after 5 hours. These experiments indicate that the cells in which unbalanced cell wall synthesis occurred prior to protoplast formation did not convert into osmotically fragile bodies as readily as did the control cells. This phenomenon may be explained by the thickening of the cell walls in the test cells.

DISCUSSION

The osmotically fragile forms of bacteria, obtained by the substances which degrade cell walls in the presence of osmotic protection, are called protoplasts (with or without quotation marks), spheroplasts, protoplast-like bodies or L-forms. The term protoplast is widely used to describe osmotically fragile forms of bacterial cells which are deprived of their cell walls and are unable to regenerate them and to replicate. Mitchell and Moyle (7) were the first to report the osmotic properties of *Staphylococcus aureus* "protoplasts" obtained as a result of autolytic changes in a hypertonic environment. Hash (3) obtained "protoplasts" by treating *Staphylococcus aureus* cells with a fungal N-acetylohexosaminidase in 0.5 M sucrose. The literature dealing with osmotically fragile forms of bacteria was reviewed by Mc Quillen (6), Martin (5), and Weibul (11). Schuhardt (9) noted that duration of exposure to a lytic enzyme was a very important factor for obtaining revertible or nonrevertible forms of osmotically fragile bodies of the *Staphylococcus aureus* cells. He observed that the rate of formation of the revertible forms, which he called spheroplasts, obtained after 5 to 10 min. of exposure to staphylolytic enzyme — lysostaphin — in the presence of 24% NaCl, fell off rapidly with prolonged exposure. In view of the residual turbidity of 30 or even 60 min. exposure preparations,

it seemed probable that most of the osmotically fragile cells were finally turned into protoplasts by prolonged lysostaphin treatment.

The present results demonstrate that the *Streptomyces* enzyme degrades the cell walls of *Staphylococcus* "Oxford" in the presence of hypertonic environment of PEG, and that this environment protects the majority of fragile bodies against osmotic shock. The time required for maximal rate of protoplast formation from control cells was markedly shorter than that necessary for the same process in the test cells in which the unbalanced cell wall synthesis resulted in the thickening of the cell walls. Thus it can be concluded that the rate of protoplast formation from bacterial cells depends on the thickness of a cross-linked mucopeptide layer. This phenomenon explains the greater readiness to spheroplast conversion of gram negative cells which contained a very small proportion of mucopeptide (5—10%) in comparison with gram positive cells in which mucopeptide is a basic component of cell walls (50—90%).

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STRESZCZENIE

Przebadano wpływ niezrównoważonej syntezy ściany komórkowej, odbywającej się w obecności chloramfenikolu — inhibitora syntezy białka, na szybkość tworzenia się protoplastów z komórek *Staphylococcus aureus* "Oxford". Do degradacji ściany komórkowej używano enzym stafilolityczny N-acetylohexozaminidazę, otrzymaną ze szczepu *Streptomyces griseus*. Zdolność ochronną polietylenoglikolu, jak również aktywność enzymu kontrolowano za pomocą mierzenia gęstości optycznej zawiesiny komórek badanych i kontrolnych, inkubowanych w środowisku hipertonicznym w obecności enzymu, oraz zawiesiny komórek traktowanych enzymem bez osłony osmotycznej. Stopień tworzenia się protoplastów

określano na podstawie spadku gęstości optycznej zawiesiny komórek inkubowanych w obecności enzymu i polietylenoglikolu poddanych szokowi osmotycznemu przez rozcieńczenie w buforze.

Z doświadczeń wynika, że komórki, w których odbywała się niezrównoważona synteza ściany, wymagały znacznie dłuższego kontaktu z enzymem litycznym dla przekształcenia się w protoplasty w porównaniu z komórkami kontrolnymi. Przyczyną tego może być zgrubienie ściany komórkowej, które najprawdopodobniej nastąpiło w komórkach testowych podczas inkubacji w obecności aminokwasów ściany i chloramfenikolu.

Tab. 1. Optyczna gęstość zawiesiny komórek kontrolnych w różnym czasie inkubacji z enzymem stafilolitycznym.

Tab. 2. Optyczna gęstość zawiesiny komórek badanych w różnym czasie inkubacji z enzymem stafilolitycznym.

Tab. 3. Spadek gęstości optycznej zawiesiny komórkowej po szoku osmotycznym.

Ryc. 1. Wpływ czasu działania enzymu stafilolitycznego na spadek gęstości optycznej zawiesiny komórek badanych i kontrolnych *Staphylococcus aureus*, poddanych szokowi osmotycznemu.

РЕЗЮМЕ

Исследовалось влияние неуравновешенного синтеза клеточной стенки, протекающего при наличии хлорамфеникола — ингибитора синтеза белка, на скорость образования протопластов из клеток *Staphylococcus aureus* „Oxford". Для деградации клеточной стенки применен стафилолитический энзим, полученный из штамма *Streptomyces griseus*. Защитную способность полиэтиленгликоля, а также активность энзима контролировали с помощью измерения оптической густоты суспензии исследованных и контрольных клеток, инкубированных в гипертонической среде в присутствии энзима, а также суспензии клеток, подвергнутых обработке энзимом без осмотической защиты.

Степень образования протопластов определялась на основании понижения оптической густоты суспензии инкубированных клеток в присутствии энзима и полиэтиленгликоля, подвергнутых осмотическому шоку путем разбавления в буфере.

Из опытов следует, что клетки, в которых протекал неуравновешенный синтез стенки, в сравнении с контрольными клетками требовали значительно длительного контакта с литическим энзимом для

их преобразования в протопласты. Причиной этого явления может быть утолщение клеточной стенки, которое вероятно наступило в тестовых клетках во время инкубации при наличии аминокислот стенки и хлорамфеникола.

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