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Effect of Minimal Bactericidal Doses of Chlorhexidine and Other Antiseptics on *Bacillus subtilis* 720 Ribosomes

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The bactericidal doses (BD_{99}) of antiseptics which reduce the number of viable *Bacillus subtilis* 720 by 2-3 orders of magnitude in 15 min have been determined. A new method for prelysis treatment of bacilli has been developed. Chlorhexidine is shown to be harmless for *B. subtilis* ribosomes and to act upon their cell wall and membrane. The studied antiseptics clearly fall into two groups in terms of their capacity to facilitate the lysis of bacilli.

Key Words: *Bacillus subtilis; chlorhexidine; antiseptics; ribosomes; degradation*

In contrast to the case with antibiotics, the mechanisms of the bactericidal effects of antiseptic agents, despite their long and broad history of use, are still little understood. One of the principal components of the cell is the ribosomal system, the destruction of which may lead to the loss of cell viability, and therefore, accumulation of data on its degradation will help unravel the subcellular underpinnings of the bactericidal action of antiseptics. This study was aimed at assessing the damage inflicted on ribosomes of a representative of the family Bacillaceae under the effect of BD_{99} (bactericidal dose) of chlorhexidine and some other antiseptics.

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MATERIALS AND METHODS

Bacillus subtilis 720 was cultured with aeration up to the exponential phase of growth (OD_{650}=1.0) in an original medium rich in yeast, pH 7.3, containing mineral salts from medium M-9 [3], 0.5% glucose, 0.2% yeast autolysate, and 0.2% casein hydrolysate. In order to assess the BD_{99}, 4 ml of bacterial culture were added to the mother liquor of antiseptic; after a 15-min incubation at 37°C the exposure was stopped by diluting the mixture with cold buffered normal saline, pH 7.0, and the cells were inoculated in meat-peptone agar. For detecting the damaged rRNA, the culture exposed to one or several BD_{99} of antiseptics was diluted 10-fold with normal saline at 0°C, sedimented by centrifugation, and washed once. The pellet was suspended in the same solution or TKM buffer (0.01 M Tris-

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HCl, 0.1 M KCl, and 0.01 M magnesium acetate, pH 7.4) to attain OD_{650}=5.0 and treated either using an original technology (see below) or with an equal volume of "lytic mixture" [5] containing 20 mM Tris-HCl, 20 mM EDTA, 1.2% sodium dodecylsulfate, 40% sucrose, 0.04% bromophenol blue, and NaOH to attain a pH of 7.4. The lysate was layered in wells with 3% polyacrylamide gel (90×1× 140 mm) prepared on 0.05 M Tris-borate buffer, pH 8.3 [4] and subjected to electrophoresis. The gel was colored with ethidium bromide and photographed. The intensity of rRNA bands on the electrophoregrams was assessed visually. The ribosomes were isolated from *B. subtilis* 720 by differentiated ultracentrifugation as described elsewhere [6].

RESULTS

The concentrations of antiseptics and the temperature which lower the number of viable *B. subtilis* cells by 2-3 orders of magnitude in 15 min were determined: iodine (I_2) — 0.05%, richlocaine — 0.2%, salicylic acid — 0.2%, mercuric chloride — 0.003%, phenol — 0.3%; formaldehyde — 0.02%, chlorhexidine — 0.002%, and chloroform — 0.3%; heating to 50°C.

Analysis of lysates showed that the methods used in Apirion's laboratory [5] do not permit the detection of rRNA bands in intact *B. subtilis* cells (Fig. 1, *a*). *B. subtilis* were found to form no extracellular nuclease which might destroy rRNA (data not presented). This conclusion was confirmed by adding exogenous ribosomes to the lysed mixture (Fig. 1, *b*, *c*). We assumed that the lytic mixture used, despite the clarification of the suspension, does not lyse *B. subtilis*. This prompted us to develop a new method of treatment. We investigated the effects of lysozyme, ultrasound, and 4 nonionic detergents, and of various compounds thereof, on a suspension of intact bacilli (Fig. 1, *d-f*). The optimal variant was chosen, which visualizes intensive bands of large (23S and 16S) rRNA on electrophoregrams. This procedure of prelysis preparation of bacilli was named TLT technology (Tris-Lysozyme-Triton). It involved suspending the cells in TKM buffer to attain OD_{650}=5.0 and their treatment with lysozyme (2 mg/ml at 20°C, 5 min) and then with Triton X-100 (1%, 0°C, 10 min). Before the suspension was layered on the gel it was mixed with an equal volume of lytic mixture [5].

Using TLT technology, we found that, similarly as in another gram-positive microorganism, *Bifidobacterium bifidum* 791 [2], chlorhexidine in doses of 1 to 5 BD_{99} does not cause rRNA degradation in *B. subtilis* (Fig. 1, *g*, *i*).

Intact cells of *B. subtilis* 720 could not be lysed without additional measures; in contrast to this, the same cells treated with chlorhexidine alone yielded clear bands of 23S and 16S rRNA (Fig. 1, *h*). Chlorhexidine facilitated the lysis of hay bacillus only when added to a growing culture heated to 37°C, and its cooling to 0°C abolished the effect of chlorhexidine (Fig. 1, *j*). Chlorhexidine was effective in a wide range of concentrations — from 0.002 to 0.01% (Fig. 1, *i*), provided that the lysis was stimulated very rapidly, in less than 1 min (Fig. 1, *k*).

In terms of their capacity to stimulate the lysis of bacilli the tested agents fall clearly into two groups. One group of substances, including formaldehyde, iodine, mercuric chloride, and salicylic acid, when used in doses of 1 to 5 BD_{99}, and high

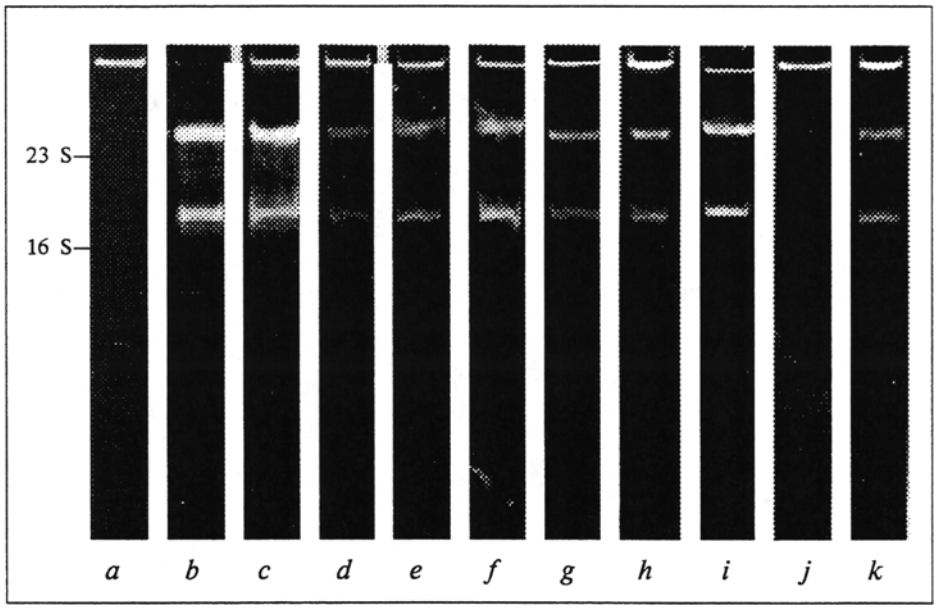

Fig. 1. Electrophoregrams of *B. subtilis* 720 rRNA, obtained by various methods of prelysis treatment of the cells. *a*) control cells; *b*) suspension of *B. subtilis* 720 ribosomes in TKM buffer with 0.01% chlorhexidine; c) mixture of cells and isolated ribosomes; *d*) cells treated with 1% Triton X-100 solution at 0°C for 10 min; *e*) cells treated with lysozyme in a dose of 1 mg/ml at 20°C for 5 min and then with Triton X-100 at 0°C for 10 min; *f*) cells treated as in e and then exposed to ultrasound (28,500 Hz, 0°C, 6 sec); *g*) culture after exposure to 1 BD_{99} chlorhexidine (0.002%, 37°C, 15 min) treated by TLT technology; *h*) the same, but after TLT treatment; *i*) culture with 0.01% chlorhexidine (5 BD_{99}, 37°C, 15 min); *j*) culture after exposure to 1 BD_{99} chlo- rhexidine in a concentration of 0.002% at 0°C for 15 min; *k*) culture treated with 2 BD_{99} chlorhexidine at a concentration of 0.005%, 37°C, for 1 min. Here and in Fig. 2: before being layered on gels, each sample was mixed with lytic mixture [5].

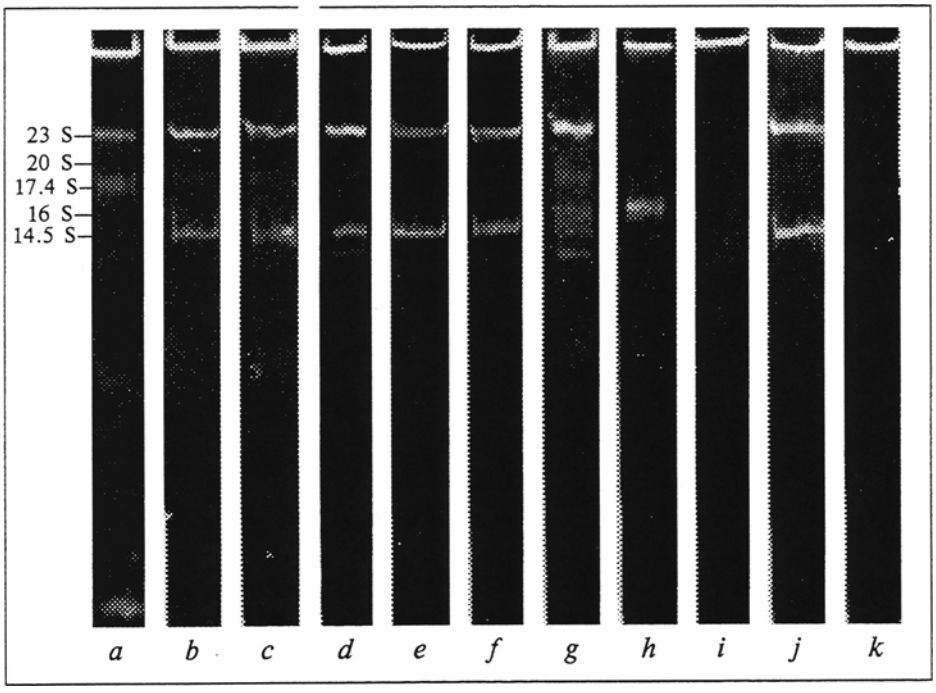

Fig. 2. Effects of some antiseptics and high temperature on the rRNA status in a *B. subtilis* 720 culture as shown by electrophoresis in 3% polyacrylamide gel. Effects on the culture at 37°C during 15 min of: *a*, *e*) phenol (0.3%); *c*) chloroform (0.3%); *d*) chlorhexidine (0.002%); *f*) richlocaine (0.2%). After phenol (*e*) and richlocaine (*f*) the cells were treated by TLT technology; *g*) after heating at 50°C for 15 min the cells were treated with chlorhexidine (0.005%, 37°C, 1 min); *h*) culture treated with chlorhexidine (0.005%, 37°C, 1 min) and heated with it at 50°C for 15 min; *i*–*k*) culture exposed to 0.02% formaldehyde solution in a dose of 1 BD_{99} at 37°C for 15 min, then to chlorhexidine (*j*), and then treated by TLT technology (*k*).

temperature, is devoid of such a capacity: rRNA bands are absent on the electrophoregrams (Fig. 2, *i*). The substances of the other group, namely, chlorhexidine, phenol, chloroform, and richlocaine, were able to stimulate the effect of the lytic mixture when used in doses of 1 to 5 BD_{99} (Fig. 2, *a-d*). The novel local anesthetic richlocaine (benzoic ester of 1-allyl-2,5-dimethylpiperidol-4 hydrochloride) exhibits this property particularly strongly (Fig. 2, *b*, *f*).

The substances of group 2 differ from each other in some specific effects on ribosomes. Phenol leads to almost complete degradation of 16S and partial degradation of 23S molecules with the formation of 20S fragments (Fig. 2, *a*). Sometimes they may be formed (in small quantities) in exposure to chlorhexidine as well (Fig. 2, *b-d*). If the same cells exposed to phenol are then treated by TLT technology, no damage to rRNA will be detected (Fig. 2, *e*). The reason for such a difference is still to be fathomed. Despite the nature of the bactericidal factors, similar products of rRNA degradation are formed in *B. subtilis* cells: 20S, 17.4S, and 14.5S fragments. Specifically, in the cells killed by heating alone, mainly the 16S rRNA fragment is destroyed and all three fragments are formed. In combined exposure to chlorhexidine and heating all the rRNA is destroyed with the formation of the sole stable 17.4S product (Fig. 2, *g*, *h*).

We tried to use chlorhexidine treatment as an adjunct in studies of the effects of antiseptics from the first group on ribosomes. However, chlorhexidine did not remain inert and often radically altered the status of rRNA (Fig. 2, *g*, *h*). Still, since some substances, for example, formaldehyde (Fig. 2, *i-k*) prevent the lysis of bacilli treated by TLT technology, possibly suppressing the activity of lysozyme, the use of chlorhexidine may prove to be an alternative method for analysis of rRNA.

Hence, the mechanism of the effect of chlorhexidine on gram-positive bacteria does not include damage to ribosomes. One component of the bactericidal effect of some antiseptics (chlorhexidine, richlocaine, chloroform, and phenol), maybe the primary one, is injury to the bacillus cell wall. The changes developing in this are detected by stimulation of cell lysis with sodium dodecylsulfate. These compounds evidently alter the fluidity of the cytoplasmic membrane [1]. This creates a signal triggering the mechanism of rRNA degradation by cell nucleases. Since the products of rRNA degradation are in many cases the same, different exposures of the cell may induce such a "signal." This indicates the existence of a universal mechanism of ribosome degradation in bacteria.

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