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Inhibitory Effect of 1-Methyldodecyldimethylamine Oxide and N, N'-Bis(dodecyldimethyl)-1,2-ethanediammonium Dibromide on the Spores of *Bacillus cereus*

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ABSTRACT. 1-Methyldodecyldimethylamine oxide (MDDO) and N,N'-bis(dodecyldimethyl)-1,2-ethanediammonium dibromide (BDED) exhibit a significant affinity for the surface of *Bacillus cereus* spores and adsorb very rapidly to the cells; they have a pronounced inhibitory effect on spore outgrowth. In order to alter the affinity of the spore surface for these inhibitors, the spores were pretreated with sodium dodecyl sulfate (SDS), and with an electronegative (Tween 80) and electropositive (histone) compound. In SDS-pretreated spores the inhibitory effect of MDDO and BDED was abolished to a considerable extent. Whereas the development of intact spores was inhibited already after germination, in SDS-pretreated spores the postgermination development continued but was not completed. In Tween-80-pretreated spores the addition of BDED led only to a retardation of outgrowth and division; BDED added only during the division stage interrupted further development completely. Histone-pretreated spores stopped their development instantaneously after the addition of BDED at any phase of the postgermination development. The possible mechanisms of the interaction of the compounds used with spore surface or rather with the state of its structures are discussed.

Aminooxides belong to compounds exhibiting a good antimicrobial activity (Čupková *et al.* 1981; Mlynarčík *et al.* 1981), the optimal length of their aliphatic chain being C_9—C_{15}. These compounds act as uncouplers of respiration and ATP synthesis (Mlynarčík and Hugo 1981; Miko *et al.* 1980). Organic ammonium salts are commonly applied as disinfectants. The plasma membrane is the main site of their action; some other cell functions can be impaired at higher concentrations (Mlynarčík *et al.* 1981). The antimicrobial effects of 1-methyldodecyldimethylamine oxide (MDDO) and of N,N'-bis(dodecyldimethyl)-1,2-ethanediammonium dibromide (BDED) are comparable with the commonly used disinfectants of the ammonium salts type. Both compounds also inhibit the developmental cycle of sporulating bacteria (Čupková *et al.* 1981). It was the aim of the present study to explain the

effect of these compounds on the germination and outgrowth of *Bacillus cereus* spores in connection with their primary binding to a dormant or outgrowing spore.

MATERIAL AND METHODS

Strain. Bacillus cereus NCIB 8122.

Cultivation medium. Bactopeptone medium containing trace elements (Vinter 1956).

Preparation of spores. Free spores were prepared on the bactopeptone medium with agar. Cultivation proceeded at 20 °C for 6 d. The sporulated culture was washed off with physiological saline, washed three times with the same physiological saline and three times with distilled water. No vegetative cells or cell debris were found after this treatment. The spore suspension was kept at 4 °C.

Germination and postgerminative development of spores. Heat-activated spores (65 °C, 15 min) were cultivated on a rotary shaker at 30 °C. The culture development was followed by measuring absorbance changes (Spekol, A_{600}) and by phase contrast microscopy.

Inhibitors. 1-Methyldodecyldimethylamine oxide was synthesized by Devínsky *et al.* (1977), N,N′-bis(dodecylmethyl)-1,2-ethanediammonium dibromide by Lacko *et al.* (1979).

Adsorption of inhibitors to spore surface. Spore suspension in physiological saline (cell concentration 74/nL) was subjected to increasing concentrations of inhibitors and the unbound fraction of the inhibitor was determined in the supernatant. MDDO was determined according to Oelschläger *et al.* (1971), BDED according to Weiss (1970).

Pretreatment of spore surface. After a 1-h contact of spores with sodium dodecyl sulfate (SDS) at 10 mg/L and Tween or histone (100 mg/L) the spore suspension was centrifuged and washed twice with distilled water. The spores were suspended in distilled water and stored at 4 °C. Spore germination in the bactopeptone medium had the same characteristics during 5 d and did not change even after a two-fold heat activation (65 °C, 15 min).

RESULTS AND DISCUSSION

Adsorption of MDDO and BDED to spores and the effect of inhibitors on spore germination and outgrowth

Adsorption isotherms of MDDO are of the Langmuir type according to Giles' classification (Giles *et al.* 1974). The main fraction of the inhibitor is bound during the first 5 min; the adsorbed amount of the inhibitors does not practically increase during 20—120 min (Fig. 1 *top*). The compound apparently exhibits a high affinity for the spore surface. When the spores germinated in the presence of MDDO (10—15 mg/L) they lost their refractility but postgerminative development was not observed before 120 min. BDED appears to have a similar high affinity for the spore surface but its adsorption curves at low concentrations of the compound are different. The interaction is slower even if the cell surface adsorbs high amounts of the compound already at the beginning (Fig. 1 *bottom*). The inhibition of spore germination in the presence of BDED was pronounced. The spores remained refractile

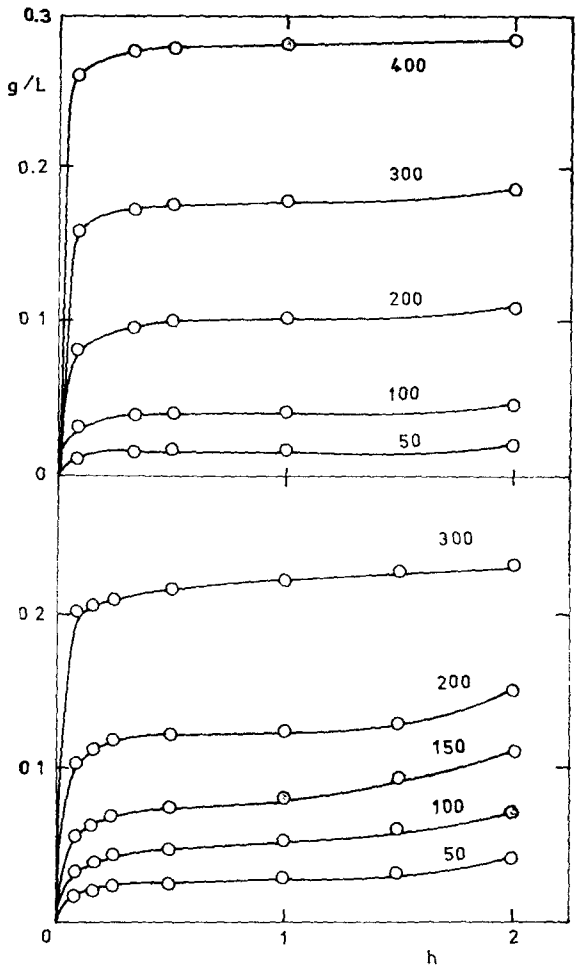

FIG. 1. Quantity of MDDO (*top*) or BDED (*bottom*) adsorbed by *B. cereus* spores as dependent on time; *numbers at curves* — concentrations of compounds (mg/L).

and only their rims darkened. Germination was completely prevented even at 2 mg/L BDED.

Changes in the inhibition of germination and postgerminative development after pretreatment of the spore surface

Both inhibitors contain aliphatic chains of identical length (C_{12}) and it can be assumed that this is one of the conditions of their effect and/or of their adsorption to the spores. Spore germination was followed in the presence of inhibitors and after SDS pretreatment in order to establish whether SDS (containing an identical C_{12} chain) can compete with the effect of inhibitors. The treatment of spores with SDS influenced the development of the culture in the presence of MDDO (10 mg/L) added either at the beginning or during various stages of the postgerminative development. Absorbance values of the cultures are presented in Fig. 2. It can be seen that postgerminative development could be detected in all cases. However, phase-contrast microscopy indicated that the development was atypical as the culture con-

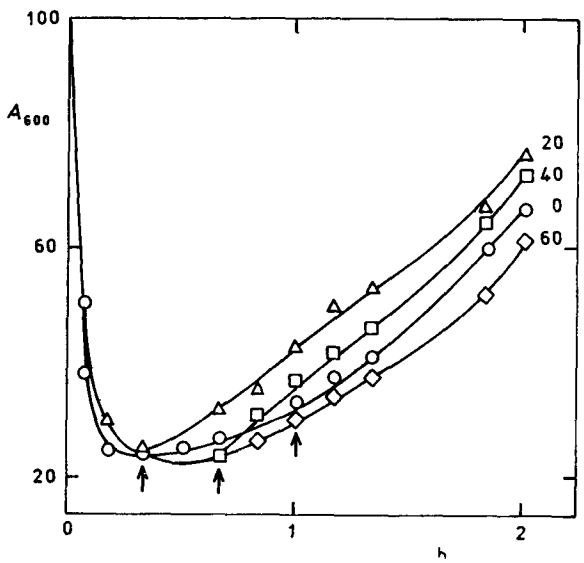

FIG. 2. Germination and postgerminative development of *B. cereus* spores (absorbance A_{600}, %) treated with SDS after addition of MDDO (10 mg/L) at various stages of development (*arrows*); *numbers at curves* — time of MDDO addition.

tained after 2 h only elongated undivided cells. The treatment of spores with SDS thus reversed the inhibitory effect of MDDO to a certain extent, but division was prevented.

In order to obtain a similar effect with BDED it was necessary to saturate the spore surface with SDS at 1 g/L. Germination of SDS-pretreated spores and of intact spores at a lower inhibitor concentration (2 mg/L) were practically identical; however, as compared with SDS-pretreated spores, the intact spores interrupted their development. A concentration of 5 mg/L decreased significantly the germination proper of SDS spores but postgerminative development could be observed. In intact spores at a higher concentration of BDED (5 mg/L) the spore germination was incomplete and their postgerminative development stopped completely. At a lower concentration (2 mg/L) spore germination in intact spores was completed but their postgerminative development was stopped.

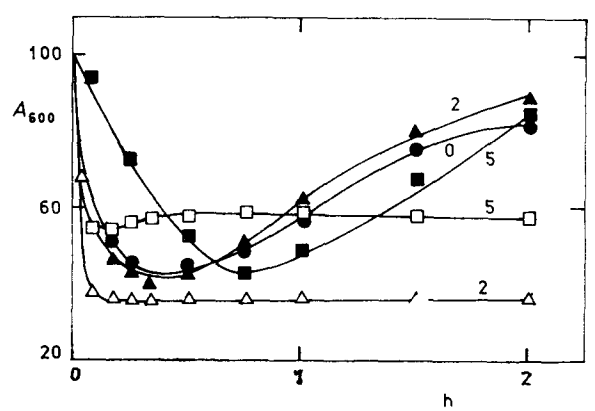

FIG. 3. Germination and postgerminative development (absorbance A_{600}, %) of intact spores (*open symbols*) and spores treated with SDS (*closed symbols*) after treatment with BDED; *numbers at curves* — BDED concentration (mg/L).

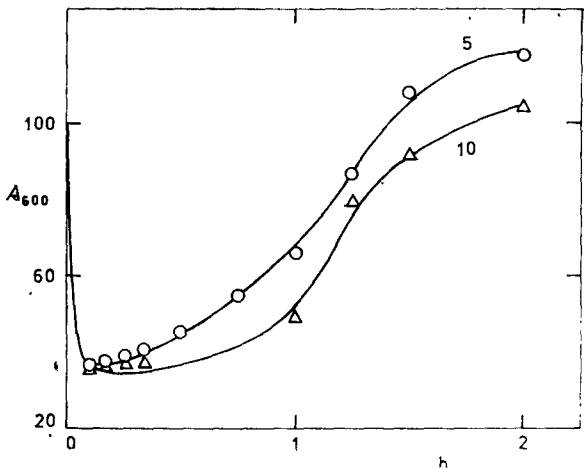

FIG. 4. Germination and post-germinative development of *B. cereus* spores (absorbance A_{600}, %) after addition of MDDO and SDS to the germination medium at 5 and 10 mg/L.

The decreased inhibitory effect of BDED in SDS-pretreated spores is significant but it cannot be unambiguously explained. If we assume that the hydrophobic interaction with the spore surface is the primary mechanism of the antimicrobial activity it would indicate that a simple competition of C_{12}-aliphatic chains is involved. MDDO, BDED and even SDS would then orient toward the negatively charged spore surface by the lipophilic moiety of the molecule. However, a mutual interaction of the inhibitor with the bound SDS or their competition cannot be excluded. This possibility is also supported by the relatively identical course of the germination and further development of intact spores if MDDO and SDS were added simultaneously to the germination medium (Fig. 4).

During the interaction of SDS with BDED an ionic compound $[BDED]^+$ $[SDS]^-$ could arise in which the cation $[BDED]^+$ could retain the antimicrobial efficiency. This possibility appears to be confirmed by spore germination and further spore development after a simultaneous addition of both compounds to the medium before inoculation (Fig. 5).

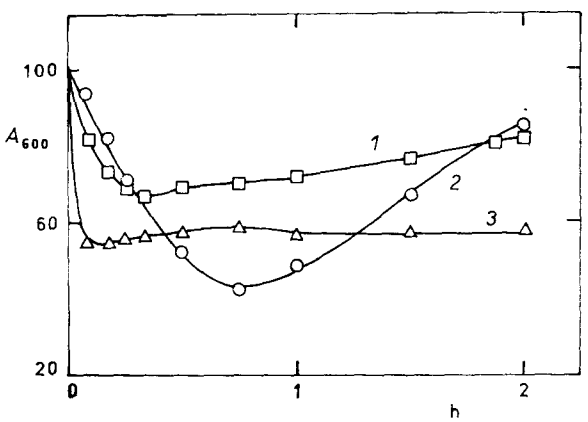

FIG. 5. Germination and post-germinative development of *B. cereus* spores (absorbance A_{600}, %) after treatment with BDED and SDS; *1* — intact spores in the presence of BDED (5 mg/L) and SDS (1000 mg/L); *2* — spores pretreated with SDS in the presence of BDED (5 mg/L); *3* — intact spores in the presence of BDED (5 mg/L).

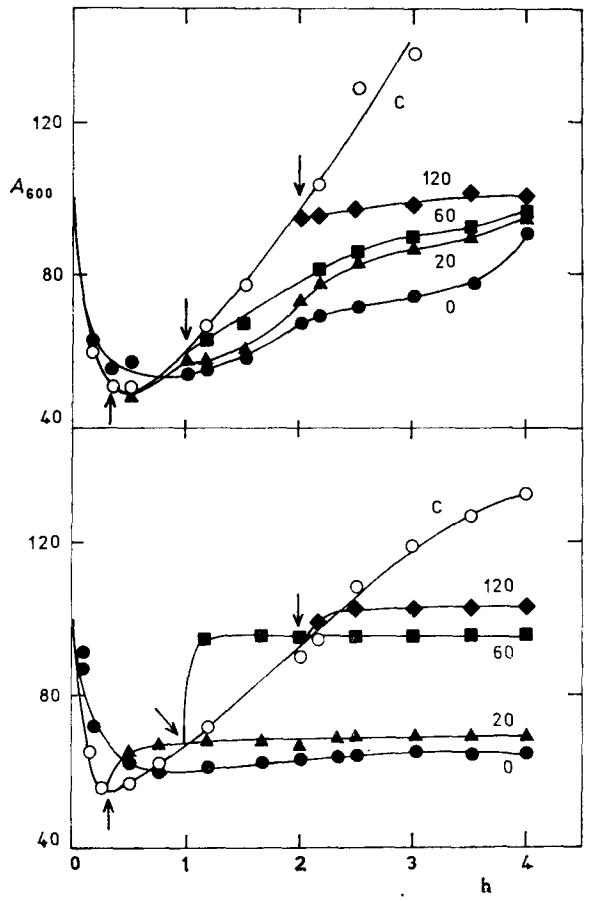

FIG. 6. Changes in the development of *B. cereus* spores (absorbance A_{600}, %) treated with Tween 80 (*top*) or histone (*bottom*) after treatment with BDED (2 mg/L) at various stages of development (*arrows*); *numbers at curves* — time of addition of the compounds; C — control.

Waehnelt (1975) described conformational changes of proteins after treatment with SDS. The spore surface is also formed primarily by proteins; SDS may thus change the affinity of the spore surface towards other compounds, in this case to the above-mentioned inhibitors. This possibility is also indicated by our experience that the abolition of the inhibitory effect was more pronounced when the SDS-pretreated spores were used in the experiments as late as after 18—20 h.

Tween 80 is another compound interacting with the spore surface. In experiments in which germination of bacterial spores is studied it is used at low concentrations for wetting of spores and their homogeneous distribution in the medium (Dobiáš and Vinter 1966). Tween 80 was hence used for pretreatment of spores at 100 mg/L as an electronegative compound. Histone, a positively charged compound exhibiting an affinity for the peripheral cell structures in the postgerminative spore stage (Vinter and Šťastná 1967) was used at an identical concentration. The effect of BDED (as a more efficient inhibitor) was studied in both types of pretreated spores.

Adsorption of Tween 80 to the spore surface retarded spore germination but did not influence their postgerminative development. The addition of

BDED (2 mg/L) after a 20-min and 60-min cultivation slowed down their development and divided cells occurred only after a 4-h cultivation. The addition of BDED after a 2-h cultivation (when the cells already divided) led to an instantaneous interruption of further development. The cell surface was hence accessible to the inhibitor at this stage (Fig. 6 *top*).

Pretreatment of the spores with histone did not influence either the germination or the postgerminative stage. In histone-pretreated spores BDED exhibited a fast inhibitory effect and development was interrupted (Fig. 6 *bottom*). In such an experiment the sharp increase of culture absorbance after the addition of BDED cannot be explained. However, it is known that the addition of histone (or of some other basic compounds) to germinating *B. cereus* spores increases significantly the absorbance of the culture. Refractility of the cells increases but, in the case of histone, a slow further development is observed (Vinter and Šťastná 1967). It is possible that BDED stimulates this alteration of peripheral spore structures caused in fact by the bound histone. A high inhibitory effect of BDED is manifested here and development is interrupted.

On the spore surface hydrophobic interactions and interactions determined by polarity of the interacting components may occur. Interaction of the polar moiety of the molecule with surface receptors is probably the primary mechanism of the antimicrobial effect of compounds with positively charged molecules, whereas the hydrophobic chain plays a secondary role. The character of the polar group is probably important for the mechanism of action whereas the hydrophobic chain is probably responsible for its intensity.

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