Selective Inhibition of *Bacillus subtilis* Sporulation By Acridine Orange and Promethazine

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Two structurally similar compounds were found to inhibit sporulation in *Bacillus subtilis* 168. A dye, acridine orange, and an antischizophrenic drug, promethazine, blocked sporulation at concentrations subinhibitory to vegetative growth, while allowing synthesis of serine protease, antibiotic, and certain catabolite-repressed enzymes. The sporulation process was sensitive to promethazine through $T_2$, whereas acridine orange was inhibitory until $T_4$. The drug-treated cells were able to support the replication of phages $\phi e$ and $\phi 29$, although the lytic cycles were altered slightly. The selective inhibition of sporulation by these compounds may be related to the affinity of some sporulation-specific genes to intercalating compounds.

As the *Bacillus subtilis* cell approaches its stationary phase of growth, an ordered sequence of events is triggered that results in the construction of a complex intracellular structure, the bacterial endospore. Early sporulation events are believed to be initiated by various mechanisms, including the derepression of catabolite-sensitive promotor genes (15). The initiation of sporulation also coincides with the preferential synthesis of several enzymes (2, 16). It is difficult to distinguish between events in the primary sequence specifically concerned in sporulation, i.e., events triggered by the occurrence of some activities in the primary dependence sequence, but not themselves part of that sequence, and the events due to changes in vegetative functions occurring at the end of exponential growth. Utilization of inhibitors specific for sporulation that permit normal growth and catabolite derepression should assist in defining those events unique to sporulation.

Acridine dyes profoundly affect living organisms by their interaction with nucleic acids; they inhibit transcription (5) and selectively eliminate cytoplasmic elements containing deoxyribonucleic acid (DNA) (1, 19). Another group of compounds, the phenothiazines, have a somewhat similar structure. Phenothiazines have received much attention recently because of their apparent usefulness in alleviating schizophrenic symptoms. At sub-bacteriocidal concentrations, these compounds were shown to effect curing of plasmids from strains of *Escherichia coli* (10, 11). The purpose of this investigation was to examine the effect of one of these phenothiazines, promethazine, and another curing agent, acridine orange, on sporulation in *B. subtilis*.

**MATERIALS AND METHODS**

Organism. *B. subtilis* 168 (TrpC2) was used as the wild-type strain, and SR22 (SpoA12 TrpC2) was used as an early-blocked asporogenous mutant. *B. amylo liquefaciens* H was the indicator strain for the sporulation-related antibiotic, and *B. subtilis* W22 was the indicator strain for the defective phage PBSX.

Preparation of spores. *B. subtilis* 168 spores were prepared and purified as described by Ito et al. (6). Purified spores were stored in water at 4°C.

Sporulation medium. Sporulation medium was essentially the same as the medium of Schaeffer as modified by Leighton and Doi (9). The medium consists of 16 mg of Difco nutrient broth per ml, 25 mM KCl, 2 mM CaCl$_2$, 10 $\mu$M FeSO$_4$, 10 $\mu$M MnSO$_4$, 0.1 mM MgSO$_4$, and 5 mM glucose.

Antibiotic test. The ability of colonies to produce antibiotic was determined by an overlay of *B. amyloliquefaciens* H in soft agar by the procedure of J. Spizizen (Spores III, p. 126-137, American Society for Microbiology, Ann Arbor, Mich., 1965). *B. subtilis* SR22 was included on all plates as a negative control. The drug acridine orange (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) or promethazine (Wyeth Laboratories Inc., Philadelphia, Pa.) was incorporated in the sporulation agar base (Difco Laboratories, Detroit, Mich.) on which the cultures were grown.

Sporulation test. Cultures were spread on AK agar no. 2 (Baltimore Biological Laboratory, Cockeysville, Md.) plates, with or without acridine orange or promethazine, and incubated at 37°C. After 48 h, 5 ml of Penassay broth (Difco) was added to each plate, and the cells were suspended by using sterile glass spreaders. The cell suspensions were counted for viability by plating on the sporulation agar base; spore viability was determined by plating
on the tryptose blood agar base after heating the suspension for 10 min at 80°C.

Protease assays. Two methods were utilized to measure protease production. Production of extracellular proteases was determined on plates as described by Keilman et al. (8).

Protease excreted into the liquid sporulation medium was measured by employing radioactive B. subtilis protein as substrate. The radioactive substrate was prepared by the method of Ito and Spizizen (Spores V, p. 107-112, American Society for Microbiology, Washington, D. C. 1972). Protease reaction mixtures contained 30 µg of radioactive protein (1.8 × 10⁶ cpm), 100 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.3), 20 mM MgCl₂, 0.4 mM CaCl₂, and 50 µl of enzyme preparation in a total volume of 250 µl. Reaction mixtures were incubated at 37°C for 20 min and stopped by adding 0.2 ml of 50% trichloroacetic acid followed by 0.2 ml of bovine serum albumin (2 mg/ml). After 15 min at 0°C, the mixtures were centrifuged at 6,000 × g for 5 min at room temperature. Samples (50 µl each) of the supernatant fractions were placed on 2.1-cm disks of Whatman glass filter paper and dried. Radioactivity was determined in a Packard scintillation spectrometer with a toluene-based scintillation fluid.

One unit of protease is defined as the amount of enzyme that solubilizes 1 µg of ¹⁴C-labeled protein in 20 min at 37°C. Total protein was determined by the method of Waddell (18). To distinguish between the two major types of protease, culture supernatant fluids were assayed with or without 10 mM ethylenediaminetetraacetate or 1 mM phenylmethylsulfonylfluoride.

Treatment with acridine orange and promethazine. All manipulations were carried out in subdued light, and all incubations were performed in the dark. B. subtilis cells were grown at 37°C in sporulation medium in a Nephelo culture flask until the stationary phase of growth, or stage 0, of spore formation. The culture was then divided into 20-ml portions in 250-ml Nephelo culture flasks containing either 6 µg of acridine orange per ml, 50 µg of promethazine per ml, or no additions (control). Treated and untreated organisms were examined periodically under a phase-contrast microscope for their ability to complete spore formation.

Bacteriophage development in treated cells. The bacteriophage strains φ29 and φe were prepared as described by Kawamura and Ito (7). Either B. subtilis 168 spores or vegetative cells of SR22 were inoculated into 10 ml of sporulation medium in a 250-ml Nephelo culture flask, incubated at 37°C, and centrifuged at 250 rpm. Cells were infected in the midlog or late log phase at multiplicities of infection of 5 for φ29 and 1 for φe, both in the presence and absence of the sporulation inhibitors.

Induction of PBSX. B. subtilis 168 was grown in antibiotic medium no. 3 at 37°C with shaking. The turbidity was followed by a Klett-Summmner colorimeter equipped with a no. 66 filter. When the turbidity reached approximately 30 units, the culture was treated with mitomycin C at a concentration of 1 µg/ml for 20 min. The antibiotic was removed by centrifugation at 10,400 × g for 10 min at 4°C, and the cells were resuspended in the same volume of fresh medium. The culture was incubated for an additional 3 h, at which time lysis was complete.

Promethazine, at a concentration of 100 µg/ml, was substituted for the mitomycin C in an otherwise duplicate culture to test the ability of this compound to induce PBSX.

RESULTS

Growth of B. subtilis in acridine orange and promethazine. The effect of acridine orange was tested by culturing cells at various concentrations of the dye (Fig. 1A). Normal growth of B. subtilis 168 was modified only slightly by the presence of acridine orange. As the dye concentration was increased, the lag phase was some-

![Fig. 1. Growth of B. subtilis 168 in sporulation medium containing various concentrations of acridine orange (A) and promethazine (B). Klett units were determined with a no. 66 filter. Symbols: (A) ●, no acridine orange; ○, 6 µg/ml; ●, 8 µg/ml; ○, 10 µg/ml; (B) ●, no promethazine; ○, 50 µg/ml; ○, 100 µg/ml.](http://jb.asm.org/)
what increased, and the growth rate was slightly reduced. Similar results were obtained with promethazine, and the final cell yields were reduced (Fig. 1B). At concentrations of 100 μg/ml or more, extremely long lag phases were observed before the onset of exponential growth. However, the growth rates at these concentrations were not affected to any marked extent. Incubation of *B. subtilis* 168 in the presence of these compounds for extended periods (16 h or more) tended to promote clumping of cells and cellular debris, which accumulated on the sides of the flasks. As a result, direct measurement of turbidity during these periods was not reproducible.

Effect of acridine orange and promethazine on spore formation. Refractile sporangia were observed in sporulating cells by T₆ (T₆ = hours after the end of exponential growth). Acridine orange- and promethazine-treated cells lost the ability to form refractile bodies when they were grown in the presence of acridine orange at concentrations greater than 6 μg/ml or with promethazine at concentrations greater than 50 μg/ml. Appreciable cell lysis occurred after 24 h of incubation in the presence of either of these compounds (Fig. 1).

To determine the extent to which sporulation was inhibited by these compounds, *B. subtilis* 168 was spread on AK plates with or without the inhibitors and incubated at 37°C for 48 h. The percentage of heat-resistant spores was determined for the control and inhibitor-treated cultures, as described above (Table 1). The acridine orange-treated cells produced less than 3% heat-stable spores. Note that the viable cell count in this treated culture was 100-fold lower than the control viable count. Much of this decrease in viable cells was due to lysis of the sporulation-inhibited cells during the 48-h incubation. At an acridine orange concentration of 16 μg/ml, the viable cell count was down to 10⁹/ml. The cell lysis, which was previously observed with inhibited cells in liquid culture (Fig. 1), was apparent in the suspensions of treated cells from the AK plates. The suspensions contained considerable cell debris and cell "ghosts" along with intact cells. The suspension from the control plates was comprised of free refractile spores and some cellular debris. Similar results were obtained with promethazine (Table 1). It should be noted that some cells escaped the inhibition produced by the drugs and successfully sporulated.

To ascertain if the lysis observed in sporulation-inhibited cultures was due to some cellular mechanism that functions during stationary phase, the onset of spore formation was delayed by the addition of 100 mM glucose to the cultures. Sporulation was delayed for 48 h in the control cultures by the additional glucose (Table 2). In the acridine orange-treated cultures, lysis was not observed until the 72-h reading in the 100 mM glucose medium, whereas complete lysis was seen in the 5 mM glucose medium after the normal growth period. Promethazine-treated cultures gave similar results, although some lysis was noted in the 100 mM glucose medium after only 48 h of incubation. However, this cell breakdown was insignificant when compared with the lysis in the treated culture with only 5 mM glucose, in which normal growth was observed. These data indicate that the lysis seen in the sporulation-inhibited cul-

### Table 1. Effect of sporulation-inhibiting compounds on the production of heat-resistant spores of *B. subtilis* 168

<table>
<thead>
<tr>
<th>Inhibitor (μg/ml)</th>
<th>No. of viable cells (V)</th>
<th>No. of heat-stable cells (S)</th>
<th>S/V x 10⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.3 x 10⁹</td>
<td>8.8 x 10⁹</td>
<td>94</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>1.0 x 10⁹</td>
<td>2.4 x 10⁹</td>
<td>2.4</td>
</tr>
<tr>
<td>Promethazine</td>
<td>4.4 x 10⁹</td>
<td>2.2 x 10⁹</td>
<td>50</td>
</tr>
<tr>
<td>40</td>
<td>3.1 x 10⁹</td>
<td>1.4 x 10⁹</td>
<td>0.44</td>
</tr>
<tr>
<td>60</td>
<td>9.1 x 10⁹</td>
<td>3.4 x 10⁹</td>
<td>0.04</td>
</tr>
</tbody>
</table>

### Table 2. Effect of glucose concentration on cell lysis

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control 5 mM</th>
<th>Control 100 mM</th>
<th>Acridine orange* 5 mM</th>
<th>Acridine orange* 100 mM</th>
<th>Promethazine* 5 mM</th>
<th>Promethazine* 100 mM</th>
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</thead>
<tbody>
<tr>
<td>24</td>
<td>286</td>
<td>445</td>
<td>78</td>
<td>468</td>
<td>67</td>
<td>452</td>
</tr>
<tr>
<td>48</td>
<td>230</td>
<td>410</td>
<td>0</td>
<td>450</td>
<td>24</td>
<td>310</td>
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<tr>
<td>72</td>
<td>187</td>
<td>294</td>
<td>0</td>
<td>133</td>
<td>6</td>
<td>55</td>
</tr>
</tbody>
</table>

*a* Acridine orange was present at a concentration of 6 μg/ml.

*b* Promethazine was present at a concentration of 50 μg/ml.

*c* Concentration of glucose in the sporulation medium.

*d* Refractile bodies present in the culture.
tures probably results from a sporulation-related event rather than a stationary-phase phenomenon.

To determine the periods during which the cells were sensitive to the sporulation-inhibiting capabilities of acridine orange and promethazine, the compounds were added to cultures at various times during sporulation. At $T_0$, the cultures were examined for the presence of refractile spores. With the inhibitor acridine orange at 6 $\mu$g/ml, the cells were unable to sporulate, even if the addition of the dye were delayed to $T_4$. After $T_4$, the compound allowed some spore formation, probably due to asynchrony of sporulation, since these cultures showed mixtures of free spores and vegetative cells. However, 50 $\mu$g of promethazine per ml inhibited sporulation in cultures only when it was added before $T_4$. If the drug was added at $T_5$, no spores were observed at $T_{24}$.

Effect of inhibitors on catabolite derepression and extracellular protease and antibiotic syntheses. Experiments were performed to determine if acridine orange or promethazine inhibited the catabolite derepression normally observed in sporulating cells. The inhibitory compounds were added to cultures at stage 0 of sporulation, when there was maximum accumulation of pyruvate and acetate in the medium (3, 4). Normal utilization of the acidic products of exponential growth was observed both in the presence and absence of acridine orange and promethazine, as indicated by the increase in the pH of the culture media (Fig. 2).

Two of the early functions associated with sporulation are the production of proteolytic enzymes and peptide antibiotics. The effect of acridine orange and promethazine on the induction of these sporulation-related products was, therefore, examined. When the compounds were added to cultures at $T_0$, postlogarithmic protease activity was not inhibited (Fig. 2). However, the inhibitor-treated cells elaborated the proteases more slowly than the control culture. Cell-free culture medium obtained from the acridine orange-treated culture at $T_7$ contained only about 40% of the proteolytic activity found in the medium of the untreated culture. Both the acridine orange- and promethazine-treated cultures showed the same percentage of metal-requiring and serine proteases as did the control. When the protease activity was determined by the plate method of Keilman et al. (8), the zones of hydrolysis in the drug-containing plates had the same diameters as the zones in the control plates after 24 h of incubation. At 48 h, the acridine orange- and the promethazine-containing plates had colonies devoid of spores, whereas the colonies on the control plates were mainly free spores.

Antibiotic assay plates indicated that both drug-treated and untreated cells produced anti-
biotics. Zones of inhibition of B. amyloliquefaciens H were approximately the same in both control and drug-containing plates.

Effect of promethazine on PBSX induction. Because of the extensive lysis observed in B. subtilis cultures treated with high concentrations of promethazine (>50 $\mu$g/ml), an attempt was made to determine if the lysis was due to PBSX induction. Cells treated with 100 $\mu$g of promethazine per ml failed to lyse, but a duplicate culture treated with mitomycin C lysed due to the induction of PBSX (Fig. 3). The production of PBSX in the mitomycin C-induced culture was verified by the killing action of the lysate on a lawn of B. subtilis W23. Promethazine-treated cultures incubated for 24 h that had undergone extensive lysis had no killing activity on W23.
Bacteriophage development in acridine orange- and promethazine-treated cells. Control cultures of *B. subtilis* 168 infected in the midlog growth phase with φ29 were seen to lyse between 1.5 and 2 h later (Fig. 4). Promethazine-treated or acridine orange-treated cells infected with φ29 at the same point in the growth cycle also began to lyse between 1.5 and 2 h postinfection, but they lysed more slowly.

The situation was much different if the cells were infected with φ29 in the late log phase. Only the untreated, control cells were able to support the infection. No lysis was observed in the acridine orange-treated or in the promethazine-treated cells infected with the phage. If the early-blocked asporogenous mutant SR22 was utilized as the host and infected in late log phase, lysis was observed in both untreated and treated cells. However, the onset of lysis was delayed in the drug-treated cultures.

Complete lysis was obtained in 2 h in *B. subtilis* 168 cells infected with φe during the midlog phase of growth (Fig. 5). Acridine orange-treated and promethazine-treated cells infected at the same phase of growth also lysed, although the rate of lysis was reduced in these cultures. The less efficient lysis observed in the φe-infected, drug-treated cells was very similar to that seen in treated cells infected with φ29 at the same stage of growth.

**DISCUSSION**

Sporulation in cultures of *B. subtilis* was prevented by the addition of either acridine orange or promethazine at concentrations that do not inhibit vegetative growth. Spore formation was blocked even though several of the early sporulation events were expressed. Proteolytic enzymes and spore-related antibiotics were produced in the presence of the inhibitors, and the cells were able to utilize excreted acidic compounds. These observations suggested that some sporulation-specific events were sensitive to the drugs.

Inhibitor-treated cells were also shown to support bacteriophage development. The lytic cycles of the phages φ29 and φe were slightly altered in the presence of the drugs. Cells infected in midlog phase lysed at approximately the same time as the control cells, but the rate of lysis was reduced. Late log-phase-treated cells infected with φ29 were not lysed. When the same experiment was performed with an early-blocked asporogenous mutant, lysis was observed in the inhibitor-treated cells, although the onset of lysis was delayed. These observations suggested that some sporulation-specific events were sensitive to the drugs.
Spores were inoculated into medium and incubated at 37°C. The acridine orange sporulation medium contained that drug at a final concentration of 6 μg/ml; the promethazine medium, at 50 μg/ml. At the times indicated by the arrows, φe was added (multiplicity of infection 1). Symbols: ●, uninfected; ○, φe infected.

Acridines are known to intercalate into DNA molecules (17). Phenothiazines, of which promethazine is a member, share a similar chemical structure and may also intercalate between the base pairs of DNA (10, 12). It has been suggested that the differential effects of intercalating compounds on cellular functions may be produced by an increased binding to localized regions of the DNA involved in transcription (14).

Acridine orange inhibits the derepression of catabolite-repressed enzymes in E. coli if added prior to their induction (14). Tricarboxylic acid cycle enzymes and proteolytic enzymes in B. subtilis are synthesized early in sporulation (3), but these catabolite-repressed enzymes were not sensitive to sporulation-inhibiting concentrations of acridine orange or promethazine. One possible explanation for these results is that the promoter sites for vegetative and catabolite-repressed genes may have low affinity for the intercalating compounds in comparison with certain sporulation-specific genes. Rogol-sky and Nakamura (13) have reported the preferential inhibition of sporulation by another intercalating compound, ethidium bromide. Their data, together with that of Keilman et al. (8), suggest that the DNA composition of sporulation-specific genes differs from those controlling the vegetative function of the cell.

Another possible explanation for the inhibition may be that these compounds affect one or more metabolic functions, such as direct inhibition of sporulation-specific enzymes or enzymes involved in the metabolism of early functions in sporulation.

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