The Effect of Sublethal Doses of Rifampin on the Sporulation of *Clostridium botulinum*

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SUMMARY

Sublethal doses of rifampin (0.005 µg/ml), added to vegetatively growing cultures of a sporogenic mutant of *Clostridium botulinum* at inoculation time or after 4 h, resulted in a decrease of growth and in blockage of spore formation. But when rifampin was added 6 to 24 h after inoculation, normal growth and sporulation occurred, indicating that the time of addition was critical and that rifampin was most effective on rapidly dividing, exponential-phase cells. Ultrastructural studies showed that when rifampin was added at the time of inoculation, endospore development was blocked at stage III. During subsequent incubation (> 10 h) the cells lost their rigidity, and lysis of the mother cell was followed by that of the forespore. When the cultures were treated with rifampin at 4 h, about 40 % of the cells were blocked at stage III and about 60 % reached stages IV and V. Some showed excessive elongation and contained developing spores at each pole. They appeared to be derived from two daughter cells unable to form a division septum because of a specific inhibitory effect of rifampin on division. It would seem, therefore, that two daughter cells which are genetically coded to form endospores will do so irrespective of the development of a division septum, and the spores are formed at the 'old' polar regions.

INTRODUCTION

Rifampin is an antibiotic that inhibits bacterial DNA transcription by specifically binding to the RNA polymerase. Evidence from several investigators has indicated that DNA-dependent RNA polymerase may play an important role in the regulation of bacterial sporulation (Losick & Sonenshein, 1969; Doi *et al.* 1970). Spores of mutants of *Bacillus subtilis* which were selected for rifampin resistance were highly pleomorphic due to an alteration to the RNA polymerase (Doi *et al.* 1970; Korch & Doi, 1971). Leighton & Doi (1971) reported that the mRNA formed during sporulation of *B. subtilis* was unstable, but such data, obtained by using refractility of endospores as a parameter of sporulation, encompassed several spore-specific processes and may also have involved the deleterious side-effects of high concentrations of the drug, e.g. cytological damage (Coote, Wood & Mandelstam, 1973). We studied the effect of sublethal doses of rifampin on the growth and ultrastructure of *Clostridium botulinum* at various stages of spore formation.

METHODS

Organisms and growth conditions. The sporogenic mutant, m_{sp}^+ , which was derived from *Clostridium botulinum* type E, ATCC9564 (Emeruwa & Hawirko, 1972), yielded more than 80 % spores after 48 h at 30 °C. The active culture method was used for propagation in TPGY broth, which contained 5 % (w/v) Trypticase (Baltimore Biological Laboratories)* 0.5 % peptone (Difco), 0.4 % glucose, 0.5 % yeast extract and 0.2 % mercaptoacetate (Matheson, Coleman and Bell Ltd) as reducing agent. Initial cultures were derived from spores activated for 10 min at 65 °C and grown for 10 h at 30 °C in TPGY. A 10 % (v/v) inoculum of the initial culture was used to prepare the test cultures in tubes of TPGY which were sealed with rubber serum-caps to maintain anaerobic conditions. All cultures were incubated at 30 °C.

In test cultures, rifampin (1000 to 0.0008 µg/ml; Calbiochem) was added to the test cultures at zero time and after 6 h. In subsequent test cultures, rifampin (0.005 µg/ml) was added at zero time and at 4, 6, 8, 10, 12 and 14 h. Samples were withdrawn at 2 h intervals between 2 and 10 h, and periodically thereafter. Growth was measured by extinction at 600 nm and cells were examined by phase-contrast microscopy.

Electron microscopy. Cells were fixed by the method of Kellenberger, Ryter & Sechaud (1958). The pellet of cells was dehydrated in graded concentrations of ethanol and infiltrated with Araldite. Sections were mounted on 400-mesh uncoated grids, stained with uranyl acetate (25 %, w/v) and lead citrate (0.33 %) and examined with a Hitachi HU-11 electron microscope (Hawirko *et al.* 1973).

RESULTS AND DISCUSSION

The concentration of rifampin which inhibited growth, measured by E_{600} was determined empirically. The results showed that after 24 h incubation, growth was diminished by 70 % with 0.01 µg/ml and by 30 % with 0.005 µg/ml compared with untreated cultures, whereas 0.002 µg/ml had no effect (Table 1). The sublethal dose of 0.005 µg/ml, resulting in a high survival rate, was used to allow for selective inhibition of various processes of sporulation which could be followed morphologically. Other workers on prokaryotes have used doses ranging from 1 to 30 µg/ml and may have observed gross physical damage due to secondary lethal effects (Coote *et al.* 1973; Newton, 1972).

Growth curves of untreated cultures and cultures treated with 0.005 µg rifampin/ml at various times indicated that exponential growth ended at 6 h and that the stationary growth phase continued from 6 to 24 h (Fig. 1). The time of addition of rifampin was critical; when 0.005 µg/ml was added at inoculation time, the growth was diminished compared with when it was added between 4 and 24 h, indicating that rifampin was most effective on rapidly dividing, exponential-phase cells, in accord with the report of Coote *et al.* (1973).

Phase contrast studies of untreated cultures showed young vegetative cells at 0 h, and granulated vegetative cells and phase-dark forespores at 10 h. Refractile endospores first appeared at 12 h and were in most cells by 24 h. Cultures treated with rifampin at 0 h showed vegetative cells after 24 h. When the rifampin was added after 4 h most of the cells showed phase-dark forespores by 24 h, whereas when rifampin was added after at least 6 h refractile endospores predominated.

Within 10 h of the addition of rifampin to cultures at inoculation time, large numbers of cells, which appeared to be in a vegetative state by phase microscopy, showed the axial filament of stage I (Fig. 2*a*), forespore septum invagination of stage II (Fig. 2*b*), and fore-

Table 1. The effect of rifampin on the growth of the sporogenic mutant, *Msp*⁺, measured as E_{600}

Time of addition of rifampin (h)	0				6		
Incubation time (h)	2	6	10	24	12	16	24
Rifampin ($\mu\text{g/ml}$)							
0.01	0.19	0.21	0.16	0.14	0.39	0.47	0.37
0.005	0.19	0.29	0.30	0.30	0.45	0.53	0.45
0.002	0.24	0.47	0.42	0.45	0.47	0.50	0.46
0.001	0.27	0.50	0.46	0.51	0.50	0.51	0.51
0	0.24	0.43	0.41	0.42

Fig. 1. Growth of *C. botulinum* at 30 °C. Rifampin (0.005 $\mu\text{g/ml}$) added at (□) 0 h or (●) 4 h; ○, control (no rifampin added).

spore completion of stage III (Fig. 2c) by electron microscopy, and were characterized by many intracellular granules of poly- β -hydroxybutyrate (PHB). In these cultures spore development was arrested at stage III. Cells with an aberrant forespore septum or a spore knob were also observed (Figs. 2d and 3a). Since Korch & Doi (1971) noted the formation of a spore knob in 48 % of the spores of a rifampin-resistant mutant, the aberrant forms of sporulating cells of *C. botulinum* may also be rifampin-resistant mutants.

Fig. 2. Electron micrographs of sporogenic mutant *msp*⁺ treated with rifampin (0.005 μ g/ml) at 0 h. (a) Axial filament formation of stage I. (b) Forespore septum invagination of stage II. (c) Forespore completion of stage III. (d) Abnormal septation of stage II (note the double septa, arrowed).

Fig. 3. Electron micrographs of sporogenic mutant *msp*⁺ treated with rifampin (0.005 µg/ml) at 0 h. (a) A forespore with a spore-knob (k) at stage III. (b) Enlargement of localized segments of a cell after spore development was blocked at stage III. (c) Lysis of both forespore and sporangium after spore development was blocked at stage III. (d) An intact forespore in a lysed sporangium after spore development was blocked at stage III.

Fig. 4. Electron micrographs of sporogenic mutant *msp*⁺ treated with rifampin 4 h after inoculation, showing sporulation at each pole. (a) Spore development out of phase, with early septum invagination at one pole and complete septation at the other. Note the axial filaments (n), one in each half of the cell. (b) Septation complete at both poles (stage II). (c) Spore development out of phase, with one

After 12 h incubation, some of the cells were enlarged at local sites, forming several segments along their lengths (Fig. 3*b*). This change indicated that such cells had reached a stage at which they could no longer maintain their original shape, probably as a result of the loss of wall rigidity. The mechanism of the formation of these enlarged segments is unknown. At this stage of the sporulation cycle, rifampin may have triggered the release of lytic enzymes at a number of sites in the wall, thus creating weakened areas at which the cell became enlarged. By 24 h, the majority of the forespores also lysed during or after the lysis of the mother cells (Fig. 3*c*). A few intact forespores remained (Fig. 3*d*).

Cells treated with rifampin 4 h after inoculation were phase-dark by 24 h, and spore formation had proceeded to stages IV and V in about 60 % of the cells. About 5 to 8 % of the cells had become elongated, with a developing spore at each pole (Fig. 4*a, b, c*). Such cells could be considered as either filamentous single cells or two daughter cells still in continuity. Studies of normal sporulation have shown pairs of daughter cells, both at stage II (Remsen & Lundgren, 1965; Ryter, 1965; Rivier & Lecadet, 1973). Ryter (1965) even suggested that septation at both poles occurred naturally in *B. subtilis*. If the process responsible for the timing of cell division were inhibited by rifampin, and the information for spore formation were sequestered before division, the dividing cell would not form a division septum but each half would proceed to form a spore. Since sporulation is a prolonged process (6 to 8 h) compared with that of division (2 h) (Shaeffer, 1969), it would seem that the two daughters would be programmed to form endospores at the 'old' polar region of the new cells, which is in accord with the findings of Hitchins (1975). If a single cell were forming two spores, one at each pole, it is very likely that the synthesis of spore structures would be controlled by a central system; thus both spores would develop at relatively the same rate, and both would then reach a definite sporulation stage at any given time. However, Fig. 4(*a*) shows a cell containing two distinct axial filaments, with one pole of the cell just entering into stage II of spore formation whereas the other has completed this stage. Also, Fig. 4(*c*) shows a cell with a spore at one pole in stage V of development and showing segments of spore coat material, and a spore at the other pole between stages III and IV. Out-of-phase spore formation of these elongated cells may explain why it is difficult to synchronize cultures of *C. botulinum*.

On the basis of these findings, it was concluded that the elongated cells arose from two daughter cells that had failed to separate because synthesis of the division septum had been specifically inhibited by rifampin (Newton, 1972). We have observed that the addition of rifampin during vegetative growth of *C. botulinum* inhibits septum formation (unpublished observations).

The excessively long cells and spores (Fig. 4*b, c*) are reminiscent of the long narrow spores of rifampin-resistant mutants of *B. subtilis* reported by Korch & Doi (1971), who interpreted them as due to a specific alteration in RNA polymerase activity, which also resulted in some loss of control of the elongation process.

In summary, therefore, the addition of a sublethal dose of rifampin to exponentially-growing cells at, or 4 h after, inoculation resulted respectively in the blockage of spore formation at stage III or at stages IV and V, and in the inhibition of cell division with the production of excessively elongated cells which developed endospores at each pole.

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