

Morphology and Patterns of Protein Synthesis During Sporulation of *Bacillus subtilis* Ery^r Spo(Ts) Mutants

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Received for publication 27 November 1978

Erythromycin-resistant (Ery^r) mutants of *Bacillus subtilis* 168 fail to sporulate at high temperature (47°C) but sporulate normally at 30 to 35°C. They also fail to sporulate at any temperature in the presence of 2.5 µg of erythromycin per ml. Neither of these nonpermissive conditions appears to affect vegetative growth, and the periods of sensitivity to both conditions extend from 40 to 90% of the sporulation period. At 47°C, net incorporation of methionine and phenylalanine in postexponential Ery^r and 168 cells was similar, and fractionation of the labeled products by polyacrylamide gel electrophoresis gave patterns in which many of the bands produced by mutant and parental cells coincided. However, distinct differences were seen, and since no spore-specific morphogenesis occurred in the Ery^r cells at 47°C, a selective defect in spore gene expression was inferred. At 35°C plus erythromycin, spore morphogenesis proceeded normally until forespores were produced and then ceased, coincident with a marked increase in sensitivity of total protein synthesis to erythromycin. The effects seem to be nonspecific, therefore, and may indicate a change in cell permeability or ribosomal sensitivity to erythromycin.

Investigation of the effects, on sporulation in *Bacillus subtilis*, of mutations which cause structural modifications in components of the machinery of macromolecular synthesis may help define the role of natural modifications of this machinery in controlling the sporulation process (4). Certain rifampin-resistant (Rif^r) mutants are asporogenous (Spo⁻) or conditionally defective in sporulation but not in vegetative growth [e.g., Spo(Ts)] (11), and modifications in the structure of RNA polymerase, the target of rifampin, are known to occur during sporulation (6, 14). The existence of the rif^r spo(Ts) mutation implies sporulation-specific functions for RNA polymerase, since the apparent defect in RNA polymerase function is not involved in vegetative growth.

A similar class of streptomycin resistance (*str*^r), spo(Ts) mutations has been described (12), and we have demonstrated (24) that all erythromycin resistance (*ery*^r) mutations are also spo(Ts). These mutations result in discrete modifications of ribosomal proteins, and their existence implies that sporulation requires unique ribosomal functions not required for vegetative growth. A type of conditional ribosomal mutation was described earlier (5, 7, 23) in which resistance of vegetative growth to a variety of

antibiotics, including streptomycin and erythromycin, was accompanied by sensitivity of sporulation to the same antibiotics.

The Ery^r Spo(Ts) mutants, including many independent isolates, are remarkably homogeneous in phenotype, and all may arise from alterations of the same base pair in the structural gene for ribosomal protein L-17 (24). The mutations map near the *cysA515* locus among a cluster of ribosomal function markers. All phenotypes of the mutants cotransduce, cotransform, and corevert (24). The mutants sporulate well at 30 or 35°C but sporulate with less than 1% efficiency at 47°C. They also fail to form mature spores at 30 or 35°C in the presence of erythromycin concentrations greater than 0.3 µg/ml, although vegetative growth of the mutants is unaffected by concentrations of up to 10 µg/ml at either 30 or 47°C. Vegetative ribosomes from the Ery^s parent bind the drug rapidly and reversibly. Vegetative ribosomes from the Ery^r Spo(Ts) mutants bind erythromycin very slowly but irreversibly (24). Growth at 47°C or in the presence of 2.5 µg of the drug per ml at 30 or 47°C also fails to affect the production of phages SPO1 and 105C by vegetative cells of the mutants (A. O'Reilly, W. C. Johnson, and D. J. Tipper, unpublished data). Sensitivity of sporulation to both high temperature (24) and erythromycin (Tipper, unpublished data) extends from about 40% to about 90% of the sporulation

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period. This period is defined as the time from the end of exponential growth to the time of accumulation of 50% of the maximum number of phase-bright spores (24; see below).

There is no evidence suggesting that ribosomes are structurally altered during sporulation (10), although an altered ability of the initiation factors of sporulating cells to function *in vitro* has recently been reported (3). Alterations in ribosomal structure or properties need not occur during sporulation: sporulation-specific ribosomal functions may involve the interaction of ribosomes with novel nonribosomal proteins or enzyme products and may simply be quiescent during vegetative growth. Moreover, the new functions may not be directly involved in translation but could, for example, cause production of some of the hyperphosphorylated nucleotides detected in sporulating *B. subtilis* cells (18–20).

These hyperphosphorylated nucleotides, like the guanosine tetraphosphate involved in the stringent response to amino acid starvation, may regulate transcription. Clarification of these issues requires comparison of ribosomal functions during sporulation in mutant and wild-type cells.

Phage templates can be used as *in vitro* probes for RNA polymerase functions, but no equivalent purified *B. subtilis* sporulation messenger preparation is available as an assay for modifications in translational efficiency. Because of this, less specific tests of ribosome function during sporulation in the Ery^r Spo(Ts) mutants are necessary. This paper describes the effects of incubation, under nonpermissive conditions, on the morphology of Ery^r Spo(Ts) cells and on the one-dimensional polyacrylamide gel electrophoretic (PAGE) pattern of polypeptides synthesized in these cells. The data demonstrate that the effects on protein synthesis are subtle and result in modification in the pattern of gene expression rather than a general defect in protein synthesis.

In this paper we also demonstrate that incubation under the two nonpermissive conditions (47°C, or 30 to 35°C plus erythromycin) has profound and different effects on spore morphogenesis. At 47°C, no development occurs. At 30 to 35°C plus erythromycin, morphogenesis appears to be normal until forespores are formed. These forespores accumulate a cortex-like layer but form no coat.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* 168 was used as the parent sporulation strain. In the modified Schaeffer medium routinely employed for liquid growth (13) and on Trypticase-blood agar base agar medium (Difco Laboratories), this strain sporulates with reasonable efficiency at 25 to 47°C.

Ery^r Spo(Ts) strains E-2 and E-12 were independently isolated from wild-type 168 and characterized as previously described (24). Strain E-802 was derived by transformation of the parent to Ery^r by using DNA derived from strain E-2 (24).

Cells were grown in modified Schaeffer medium as previously described (24). Where indicated, erythromycin was added at 2.5 µg/ml of culture. Cultures were incubated at either 30 or 35°C, both permissive temperatures, and at 47°C, the restrictive temperature. Cultures were grown in side-arm flasks in a water bath shaker, and growth was followed with a Klett-Summerson colorimeter with a no. 66 filter. The appearance of phase-bright (refractile) spores was monitored by phase-contrast microscopy. "Heat-resistant" counts were determined, after the culture samples were heated for 15 to 20 min at 80°C, by plating dilutions of cultures on nutrient agar (Difco) plates. Viable cell counts were determined by plating dilutions of cultures without heat treatment.

Temperature and erythromycin shift experiments. Portions of cultures, growing at 30 or 35°C and in the absence of erythromycin, were transferred to prewarmed flasks in a 47°C water bath at various times during sporulation. Shifts into erythromycin were performed by transferring portions of a culture to flasks, at 30 to 35°C, containing sufficient drug to bring the concentration to 2.5 µg/ml. Samples of control and shifted cultures were processed for electron microscopy or pulse-labeled with [³H]phenylalanine or [³⁵S]methionine as described below.

Determination of rate of total protein synthesis. Samples (0.5 ml) of cultures growing at 35 or 47°C were transferred to tubes, at 35 or 47°C, containing erythromycin (final concentration, 2.5 µg/ml), chloramphenicol (final concentration, 50 µg/ml) plus erythromycin (2.5 µg/ml), or no drug (control). After 20 min, 2.5 µCi of L-[³⁵S]methionine (10 Ci/mmol) was added. Incorporation was stopped after 3 min by the addition of 5 ml of cold 5% trichloroacetic acid containing 2 mM unlabeled L-methionine. After 1 h in an ice bath, cells and precipitated protein were collected on GF/A filters (2.3-cm diameter; Whatman), which were washed with a solution containing 10 ml of cold 5% trichloroacetic acid and 10 ml of 95% ethanol and then dried. The filters were counted as previously described (24). A similar procedure (24) was used to determine the rate of L-[¹⁴C]phenylalanine incorporation.

Labeling of culture samples with L-[³⁵S]methionine for gel electrophoresis. Samples (8 ml) of cultures, growing or sporulating at 35 or 47°C or at 35°C in the presence of erythromycin (2.5 µg/ml), were labeled for 15 min with 200 µCi of L-[³⁵S]methionine (1,020 Ci/mmol). Cells were harvested and processed as described by Linn and Losick (15), except that cells were broken in a Bronwill model 2876 cell disruptor with 6 ml of 120-µm glass beads and 1 ml of lysis buffer (14) (pH 7.5) containing 0.5 mM phenylmethylsulfonyl fluoride and 2 mM EDTA. After the beads were allowed to settle and the supernatant was centrifuged at 10,000 × *g* for 20 min to remove cell walls, the resulting supernatant was centrifuged at 100,000 × *g* for 90 min. That supernatant (soluble protein) was precipitated with saturating concentra-

tions of $(\text{NH}_4)_2\text{SO}_4$. The precipitate was processed for electrophoresis as described by Linn and Losick (15). The initial $100,000 \times g$ pellet was solubilized by incubation for 30 min at 37°C in 0.05 M dithiothreitol-1% sodium dodecyl sulfate (SDS) in 0.5 M Na_2CO_3 buffer (pH 10). The mixture was again centrifuged at $100,000 \times g$, and the supernatant (SDS-soluble protein) was prepared for gel electrophoresis as before. The specific activity of all fractions was determined by measuring protein content (16) and counting samples in Aquasol 2 (New England Nuclear Corp.).

PAGE. Slab gel electrophoresis in the presence of SDS (SDS-PAGE) was performed as previously described (9), with one modification. Gels were formed as 5 to 20% linear gradients of acrylamide in slabs (13 cm by 15 cm by 3 mm or 30 cm by 15 cm by 0.75 mm). After electrophoresis, gels were stained with Coomassie brilliant blue and destained in 10% acetic acid as previously described (11). Fluorography was performed as described by Bonner and Lasky (2). Gels were exposed to Kodak RP/Royal X-Omat film for 7 to 10 days at -80°C .

Electron microscopy. Cells were fixed and sectioned for electron microscopy as previously described (8).

RESULTS

Sporulation under nonpermissive conditions: phase observations. Cultures of the parent (strain 168) and *Ery^r* mutants E-2, E-802, and E-12 had identical rates of turbidity increase during exponential growth and also during the postexponential sporulation period at 47°C . The parent sporulated with 80% efficiency at this temperature, but the *Ery^r* strains failed to sporulate (24) (Fig. 1, Table 1).

At 30 or 35°C , strains 168, E-2, E-12, and E-802 again grew with very similar kinetics (Table 1). The mutants also grew at 30, 35, or 47°C in the presence of 2.5 mg of erythromycin per ml at rates which were similar or only slightly retarded (Table 1); the parent fails to grow at 0.03 mg of erythromycin per ml (24). The parent and mutants sporulated with similar kinetics and efficiency at 30 or 35°C in the absence of erythromycin, but the mutants failed to sporulate at any temperature in the presence of the drug (Fig. 1, Table 1).

Shift of a 30°C culture of strain E-2 at T_{11} (see legend of Fig. 1) into the presence of erythromycin limited production of stage V forespores to 5% (Table 2) and phase-bright spore production to about 25% (Fig. 1). Observable phase brightness occurs before the true refractility that is characteristic of stage V forespores. Shift of the same culture at T_{11} to 47°C without the drug limited phase-bright spore production to about half of maximum (Fig. 1). T_{11} corresponds to 90% of the sporulation period at 30°C and, as these data demonstrate, marks the end of the period of sensitivity to a shift to 47°C (24).

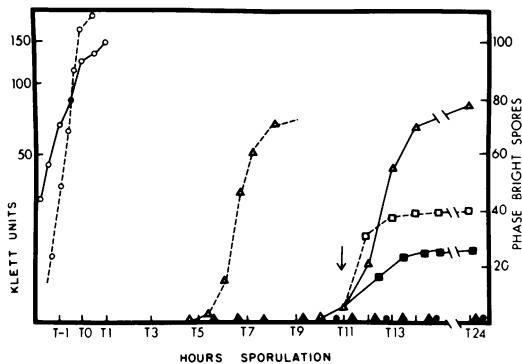


FIG. 1. Growth and sporulation. Time (T) in hours during sporulation is indicated by T_1 , T_2 , etc. T_0 is defined as the time at which exponential turbidity increase ceases. Cultures growing in modified Schaeffer medium were assayed for turbidity (Klett units) and for appearance of phase-bright spores. Symbols (turbidity): —○—, 30°C cultures; - -○- - -, 47°C cultures. Similar growth rates were obtained for strains 168, E-2, E-12, and E-802 and for *Ery^r* strains growing in the presence of erythromycin, and only one curve is presented for each temperature. Symbols (phase-bright spores): - -△- - -, produced by strain 168 at 47°C ; —▲—, produced by *Ery^r* strains at 47°C ; —△—, produced by a culture of strain E-2 growing at 30°C ; produced by a sample of this culture shifted to 47°C at T_{11} (—□—) or shifted at T_{11} (arrow) into the presence of 2.5 mg of erythromycin (at 30°C) (—■—); —●—, produced by a separate culture of strain E-2 growing at 30°C in the continuous presence of 2.5 mg of erythromycin per ml. A culture of strain E-12 growing at 30°C gave very similar data (not shown).

Electron microscopic observation: continuous incubation under nonpermissive conditions. Strains E-2 and E-12, growing continuously at 47°C not only failed to produce phase-bright spores, but they also failed to develop beyond the "axial filament" stage of morphological development (Fig. 2A and B). Condensation of the nucleoid at the cell axis occurs at T_1 during sporulation of wild-type *B. subtilis* at 37°C and is called sporulation stage 1 (21). This condensation is characteristic of stationary-phase cells and is not peculiar to sporulation. These cells were fixed at T_7 , but no spore septum was produced even after prolonged incubation at 47°C . At T_7 , 47°C or at T_{20} , 30°C , cells of strain 168 contained mature, refractile spores with a complete cortex and coats (not shown). At T_{20} , 30°C , cells of the *Ery^r* mutants also contained mature spores. However, at T_{20} after growth at 30°C in the continuous presence of erythromycin, cells of the *Ery^r* mutants did not contain phase-bright spores. Instead, they contained completely engulfed forespores surrounded by a heavily stained cell wall primordium and a fuzzy, atypical cortical region (Fig.

TABLE 1. *Growth and sporulation of parent (168) and Ery^r Spo(Ts) strains E-2 and E-802*

Strain	Temp (°C)	Doubling time (min)	Sporulation period (h) ^a	Maximal percent heat resistance ^b
168	30	41	11.5	85-90
	35	32	7	85-90
	47	24	6.5	80-85
E-2	30	45	12.5	85-90
	35	34	7.5	80-85
	47	27		1
E-802	30	42	12.5	85-90
	35	33	7.5	80-85
	47	26		1
E-802 + erythromycin (2.5 mg/ml)	30	50		2
	35	36		2
	47	28		0

^a The beginning of the sporulation period, T_0 , is defined as the time at which an abrupt break in the exponential rate of turbidity increase occurs (Fig. 1). The end of the sporulation period is arbitrarily defined as the time at which half-maximal accumulation of phase-bright spores has occurred (Fig. 1). The difference (in hours) is the sporulation period.

^b Heat resistance developed after phase brightness.

TABLE 2. *Distribution of morphological stages, estimated by electron microscopy, of strain E-2 cells grown at 30°C to $T_{11.5}$ and then shifted for 4.5 h into erythromycin or to 47°C*

Sample	Treatment after shift	No. counted	% At stage: ^a		
			III	IV	V
At Shift, T_{11}		89	88	12	0
Control, T_{16}	30°C	132	10	14	75
Shift, T_{16}	47°C	145	11	28	61
Shift, T_{16}	30°C + erythromycin	130	63	32	5

^a Stage III, nascent engulfed forespore; stage IV, cell wall primordium and some cortex present, and partial coat assembly visible; stage V, spores with mature coats and refractile core.

2C and D). No coat structures were visible, and no other structure, such as an exosporium-like layer (22, 24) or an outer-forespore membrane, could be seen surrounding this cortical region. Prolonged incubation did not lead to further morphological development.

Shift experiments. At T_{11} , 30°C, the vast majority of cells of the parent and both mutants

contained fully engulfed forespores (Table 2, Fig. 3A). These forespores were surrounded by a heavily stained cell wall primordium, but a cortex and coats were not visible. At T_{16} , cells of strain E-2, maintained at 30°C without erythromycin (control), contained almost mature phase-bright spores surrounded by a cell wall primordium, lightly stained cortex, and multi-layered coats (Table 2, Fig. 3B). The coats comprised both an inner lamellar layer and a densely stained and less clearly organized outer layer, surrounded by a more lightly stained layer that may be exosporium. Cells of the parent and of strain E-12 contained similar, almost mature spores at T_{16} , 30°C (not shown).

When shifted into the presence of erythromycin at T_{11} , 30°C and incubated at 30°C, cells of strain E-12, sampled at T_{16} (Fig. 4A), contained aborted forespores, essentially identical in appearance to those produced in the continuous presence of the drug (Fig. 2C), few being truly refractile (Table 2), although some phase brightening occurred (Fig. 1). They differed from the forespores present at the time of shift (Fig. 3A) in having a broad, atypical cortical area surrounding the cell wall primordium. Cells of strain E-2 (not shown) contained identical aborted forespores under the same conditions.

When shifted at T_{11} from 30 to 47°C, most of the parent cells produced free, mature refractile spores by T_{16} (not shown). Cells of Ery^r mutants E-2 and E-12, shifted from 30 to 47°C at the same time, contained aberrant spores when sampled at T_{16} (Table 2, Fig. 4B and C). Many variations were seen, but most contained almost mature spores of fairly normal appearances, being surrounded by a cell wall primordium, a poorly formed and patchily stained cortex, and coat layers, including both inner lamellar and outer layers. However, many cells contained evidence of visibly abnormal coat development. Coat sometimes surrounded the spore in duplicated layers (Fig. 4C) and was frequently assembled in completely inappropriate regions of the sporangium (Fig. 4B).

Rate of protein synthesis in sporulating cells. Under standard culture conditions, the incorporation of added L-[³⁵S]methionine by cells of strain 168 or E-802 sporulating at 35°C was very efficient, a maximal rate of incorporation was rapidly established (data not shown), and 3-min pulses gave a consistent pattern with respect to time during sporulation that probably reflected the true relative rates of protein synthesis (Fig. 5). The pattern of L-[¹⁴C]phenylalanine incorporation was also reproducible and was quite similar to the methionine uptake pattern (data not shown). Rates of incorporation of

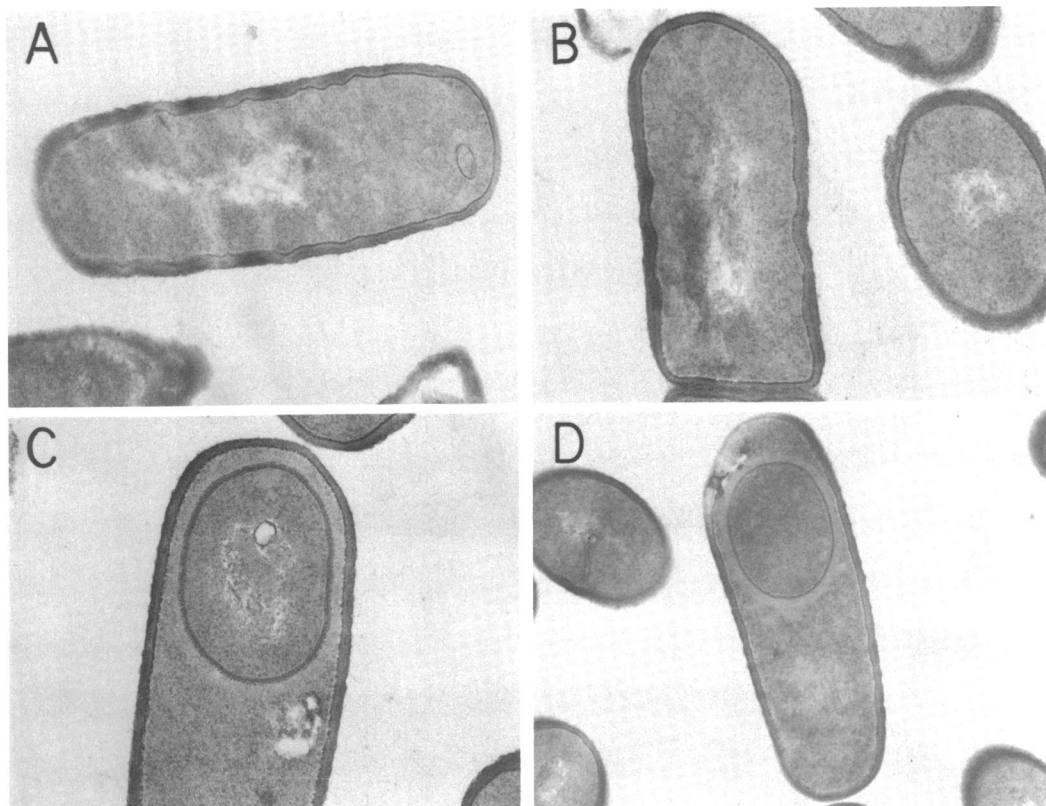


FIG. 2. Thin-section electron micrographs of strain E-12 at T_7 , grown continuously at 47°C (A), strain E-2 at T_7 , 47°C (B), strain E-12 at T_{20} , grown continuously in the presence of $2.5\ \mu\text{g}$ of erythromycin per ml at 30°C (C), and strain E-2 at T_{20} , 30°C plus erythromycin (D).

both phenylalanine and methionine were maximal at T_2 , declined rapidly from T_2 to T_5 , and then declined more slowly (Fig. 5). Even when the data were normalized to constant turbidity (Klett reading), the maximum incorporation rate still occurred at T_2 (not shown). In the continuous presence of erythromycin, the rate of incorporation of both labeled compounds in strain E-802 cells was little affected until T_4 to T_5 (Fig. 5), although incorporation of methionine into protein was almost abolished by T_6 (Fig. 5).

The turbidity data (Fig. 5) demonstrated that strains 168 and E-802 grew at the same rate at 35°C and that the continuous presence of $2.5\ \mu\text{g}$ of erythromycin per ml only slightly retarded the rate of growth of strain E-802. Postexponential turbidity patterns were also similar: the increase in turbidity for strain 168 from T_6 to T_8 paralleled the development of phase-bright spores (not shown) and was reflected by a slightly delayed but similar turbidity increase for strain E-802. In the E-802 culture grown in the continuous presence of erythromycin, this

increase did not occur, and refractile spores were not formed.

When erythromycin was added to E-802 cells at T_5 , 35°C and the rate of methionine incorporation was measured as a function of time (Fig. 6), about 40% of the incorporation seen in the absence of the drug was rapidly inhibited, whereas the residual 60% was resistant. The rate of methionine incorporation was determined, as a function of time in sporulation, after a 20-min exposure to erythromycin (Fig. 5). Again, no inhibition was seen until T_5 , after which the proportion of rapidly inhibitable incorporation increased (Fig. 5, insert) until by T_8 it was essentially total.

It appears that two periods of protein synthesis may be distinguishable in sporulating cells of both strains 168 and E-802 at 35°C . In the first (T_0 to T_3), protein synthesis is very active and is resistant to erythromycin in strain E-802, as is vegetative growth. In the second (T_4 to T_8), protein synthesis is less active and is erythromycin sensitive in strain E-802.

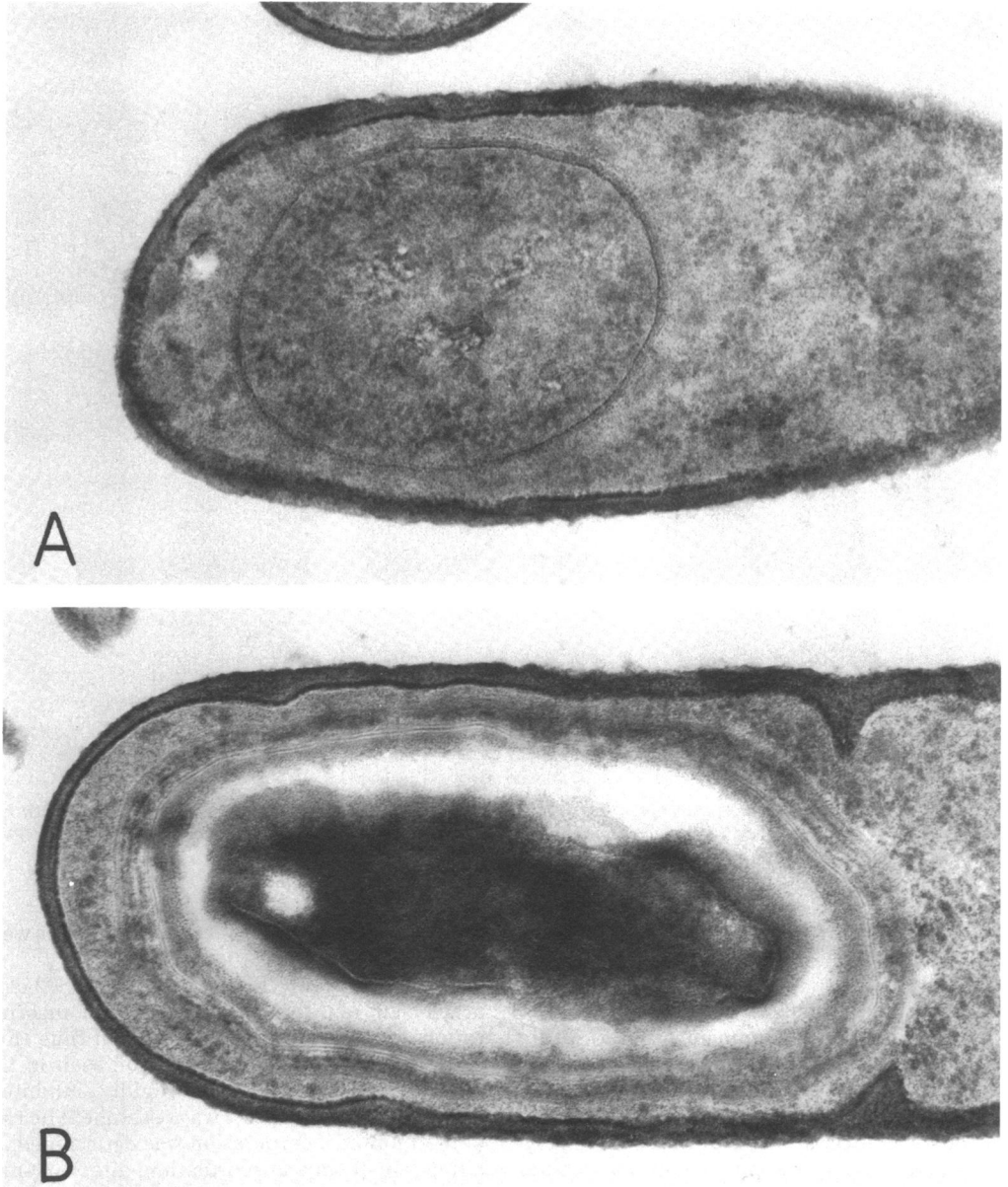


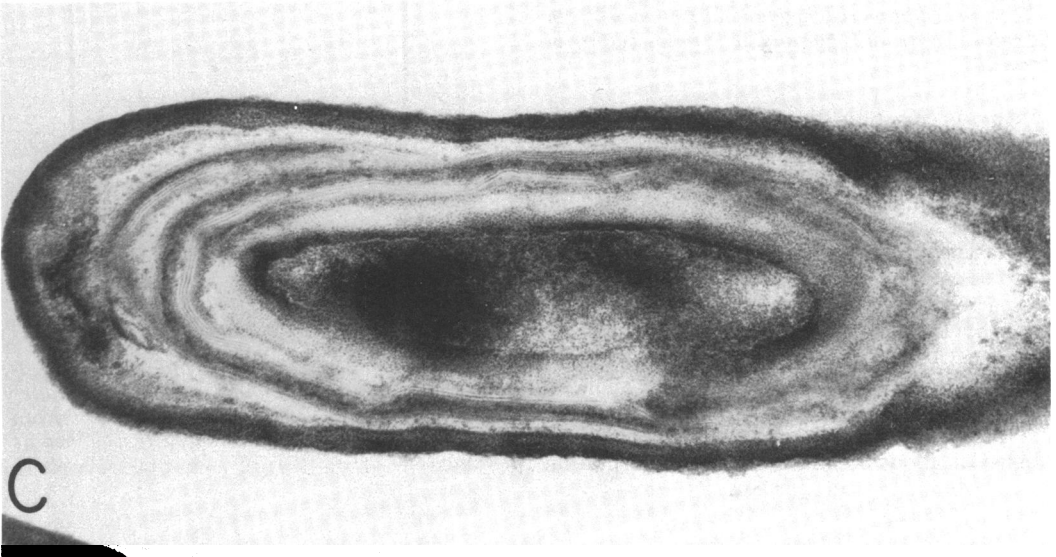
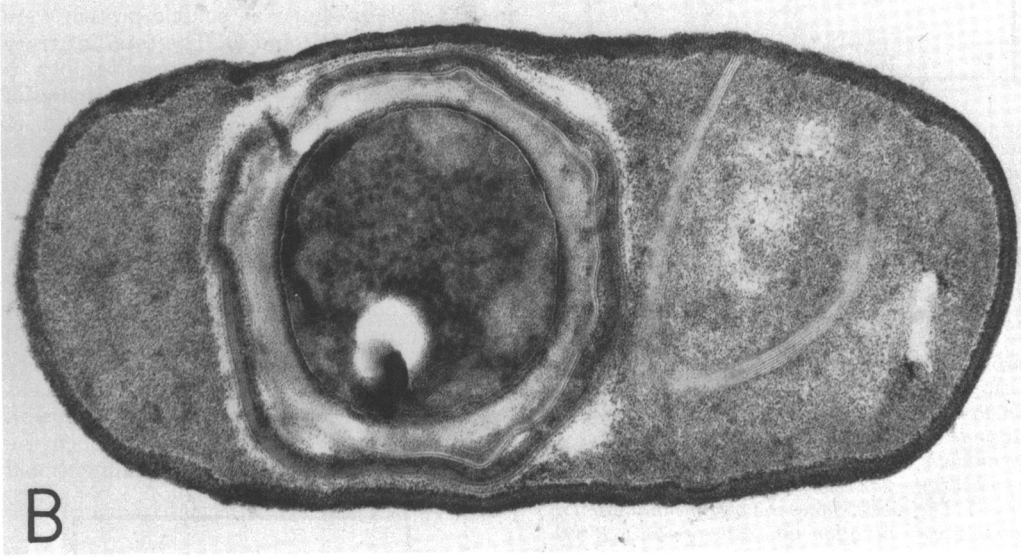
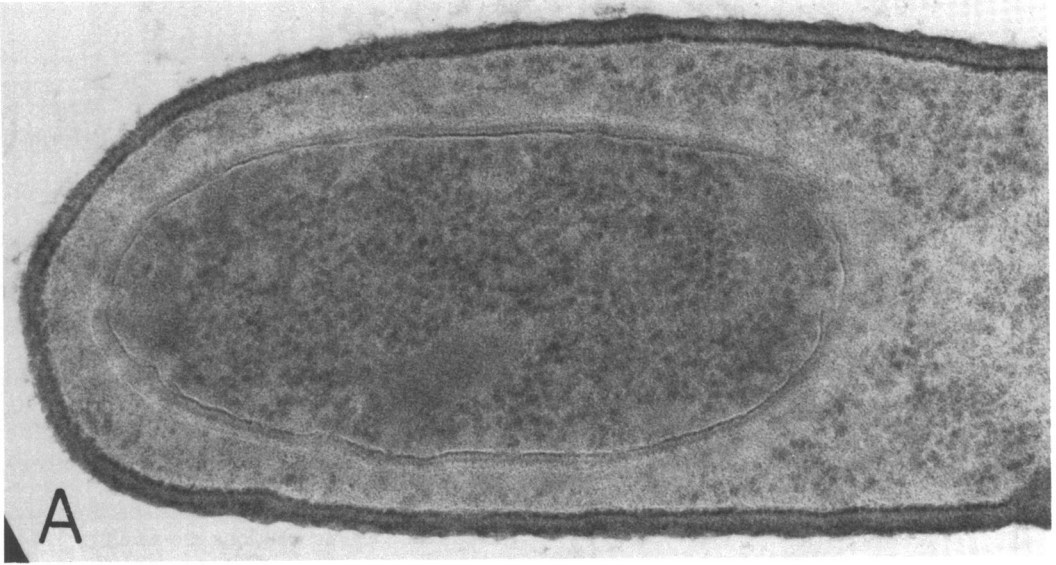
FIG. 3. Thin-section electron micrographs of strain E-2 during sporulation at 30°C at T_{11} (A) and T_{16} (B). The sporangium surrounding the almost mature spore shown in (B) contains an aborted septum near the right-hand end of the spore.

At 20 min after the addition of 50 μg of chloramphenicol per ml to sporulating cultures of strain 168 or E-802, incorporation of methionine was reduced to less than 5% (Fig. 5). The residual incorporation, at any stage of sporulation in cultures of strain E-802 in the presence of eryth-

romycin (continuously present or added 20 min previously), was also eliminated by the addition of chloramphenicol and so probably represents protein synthesis, as shown by autoradiography (see below).

Characterization of patterns of protein

FIG. 4. Thin-section electron micrographs of strain E-12, sporulating at 30°C and shifted at T_{11} (A) into the presence of 2.5 μg of erythromycin per ml and (B) to 47°C. Strain E-2, sporulating at 30°C, was also shifted at T_{11} to 47°C (C). All cells were harvested and fixed at T_{16} .



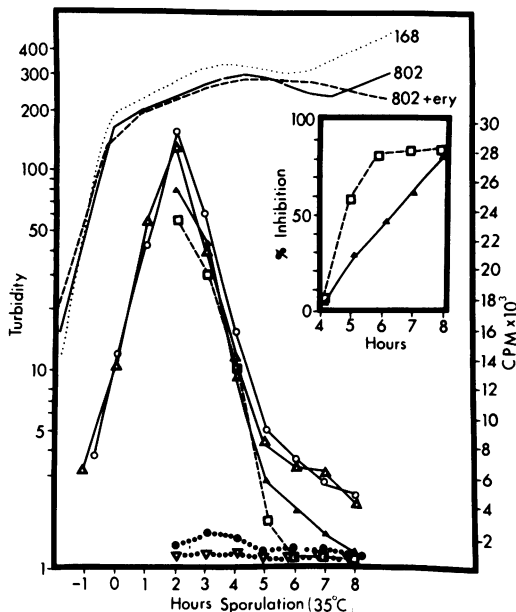


FIG. 5. Rate of incorporation of L - $[^{35}\text{S}]$ methionine during sporulation. Cultures were grown at 35°C . Turbidity, in Klett units (upper curves), is shown for strain 168 (.....), strain E-802 (—), and strain E-802 growing in the continuous presence of $2.5\ \mu\text{g}$ of erythromycin per ml (----). At the indicated intervals, samples of these cultures were withdrawn and labeled with L - $[^{35}\text{S}]$ methionine for 3 min (—○—, strain 168; —△—, strain E-802; ---□---, strain E-802 plus erythromycin). The effect of prior incubation for 20 min with $2.5\ \mu\text{g}$ of erythromycin per ml on incorporation in strains 168 (....●....) and E-802 (—▲—) was also determined. If chloramphenicol was present alone or with erythromycin during this preincubation, both strains gave similar residual incorporation (....▽....). The insert shows the percent inhibition of methionine incorporation into cells of strain E-802, at the indicated time, by erythromycin present continuously (---□---) or added 20 min before label (—▲—). Sporulation reached half maximum at $T_{7.5}$ in the culture of strain E-802 and at T_7 in the culture of strain 168.

synthesis by one-dimensional PAGE. Although the prolonged maintenance of postexponential viability of Ery^r cells at 47°C (24) suggests that normal proteins continue to be produced, measurement of rates of total protein synthesis says nothing about the quality of the synthetic products. We analyzed, by SDS-PAGE the patterns of proteins synthesized in cells labeled by 15 min of incubation with L - $[^{35}\text{S}]$ methionine. Cells of parental and Ery^r strains were labeled during vegetative growth and sporulation at 35 and 47°C and at 35°C in the continuous presence of erythromycin. Samples were labeled at about 30 and 80% of the sporulation

period at either temperature. At T_2 , cells formed sporulation septa. At T_5 (47°C) or T_8 (35°C), cells finish forespore engulfment and begin cortex and coat synthesis (21). Samples of 35°C cultures of both strains were also shifted at these times to 47°C or into the presence of erythromycin or erythromycin plus chloramphenicol. Label was added after 10 min. Labeled cells were broken and fractionated, as described above, into soluble proteins and proteins solubilized from the particulate fraction by SDS at pH 10, the conditions previously used to solubilize membrane and spore coat proteins (9). Breakage and subsequent recovery of protein fractions were consistently about 90%. The specific activities of the various fractions are given in Table 3. For the preparations from sporulating cells, samples containing equal quantities of ammonium sulfate-precipitated soluble protein were fractionated (Fig. 7 and 8). The total intensity of each column in these autoradiographs is roughly proportional to the specific activities of the applied samples (Table 3). Equal quantities of SDS-solubilized proteins were also fractionated (data not shown).

Soluble proteins synthesized during growth and sporulation in strain 168. Fractionation of soluble proteins from methionine-labeled vegetative cells of strain 168 showed at least 50 strong bands and 20 fainter bands. The vegetative patterns seen at 47°C (Fig. 7) and 35°C (Fig. 8) were remarkably similar. Very similar patterns were formed by vegetative cells of E-802 growing at 35 or 47°C (not shown). Although these patterns do not approach the complexity of patterns seen by two-dimensional

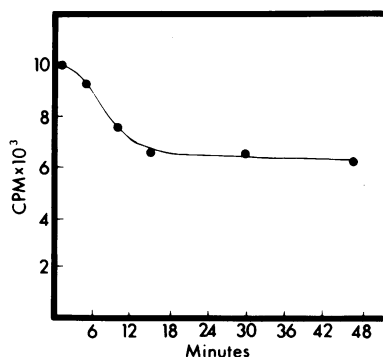


FIG. 6. Kinetics of inhibition of protein synthesis by erythromycin in E-802 cells at T_5 . Erythromycin was added to a final concentration of $2.5\ \mu\text{g}/\text{ml}$ to a strain of E-802 at T_5 , 35°C . At the indicated intervals, samples (0.5 ml) were withdrawn and immediately incubated with L - $[^{35}\text{S}]$ methionine for 3 min. Trichloroacetic acid-precipitable radioactivity was then determined.

TABLE 3. Specific activities of *L*-[³⁵S]Methionine-labeled soluble protein samples fractionated by SDS-PAGE^a

Labeling conditions	Time	Strain 168 cells		Ery ^r Spo(T ₅) cells	
		cpm/ μg	% Of control	cpm/ μg	% Of control
47°C (continuous)	Vegetative	9,200		9,090	
	T ₂	3,930		3,560	
	T ₅	2,110		1,820	
35°C + erythromycin (continuous)	T ₂			3,550	
	T ₆			190	
35°C (control) Shift to erythromycin Shift to 47°C	T ₂	2,750	100	2,740	100
	T ₂	290	10	2,270	83
35°C (control) Shift to erythromycin Shift to 47°C	T ₂	3,740	136	3,300	120
	T ₆	2,060	100	1,660	100
35°C (control) Shift to erythromycin Shift to 47°C	T ₆	150	7	780	47
	T ₆	3,970	193	4,190	252

^a See Fig. 7 and 8. Soluble proteins (3 μg) from vegetative cells and 18 μg of the proteins from all other cells were applied to the gel slots.

techniques (14), they are reproducible and characteristic of the stage of development. Consistent modifications occur in this pattern when the proteins from 168 cells, sporulating at 47°C, are examined at T₂ and T₅ (Fig. 7). The patterns seen at T₂ and T₆ in cells sporulating at 35°C (Fig. 8) are very similar to the 47°C patterns, as expected from the slightly longer sporulation period at 35°C.

Soluble proteins synthesized by E-802 cells at 35 and 47°C. At 35°C (permissive conditions), the patterns of proteins labeled at T₂ and T₆ in strain E-802 cells were essentially identical to those of proteins labeled in strain 168 cells at T₂ and T₆ (Fig. 8).

The protein patterns from T₂ and T₅ cells of this mutant grown at 47°C differed from the parental patterns (Fig. 7). Differences are indicated in Fig. 7 according to the scheme shown in Table 4. It is assumed that changes seen only in the sporulating (strain 168) cells may be sporulation specific. The classes indicated are those easily interpreted, and assignment is sometimes subjective, depending on changes in band intensities which may not reproduce accurately. Other classes, due for example to coincidence in migration of bands in different primary classes, such as b and c(c/b), a and C(a/C), etc., would give other predictable patterns. Inexplicable by this simple scheme are bands appearing only in strain E-802 cells, such as the bands indicated

by an X. Some of the band patterns (R) are consistent with a retardation of development in E-802 such that events normally occurring by T₂ only occur by T₅.

As expected from earlier data (24) and the data shown in Table 3, the total methionine incorporated by the Ery^r cells at T₂ and T₅, 47°C, as estimated from the total band intensities (Fig. 7), was similar to that incorporated by the strain 168 cells. Although some changes occurring in the sporulating 168 cells also occurred

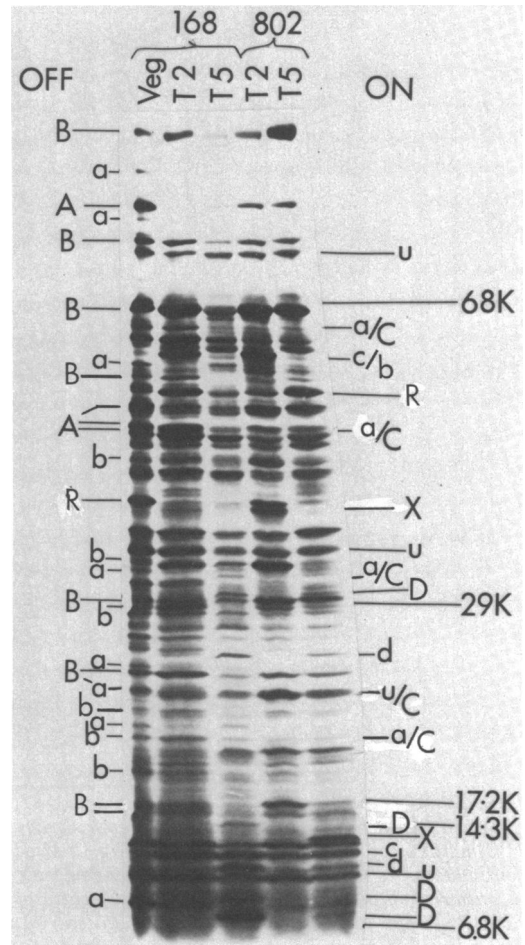


FIG. 7. Autoradiograph of soluble proteins from cells pulse-labeled during sporulation at 47°C with *L*-[³⁵S]methionine and fractionated on 30-cm SDS gels with a 5 to 20% acrylamide gradient. Molecular weight markers were: 68K, bovine serum albumin; 29K, carbonic anhydrase; 17.2 K, equine myoglobin; 14.3 K, lysozyme; and 5.8 K, bovine insulin. Symbols on the left indicate the disappearance of bands, and symbols on the right indicate appearance of bands during sporulation, according to the scheme given in Table 4.

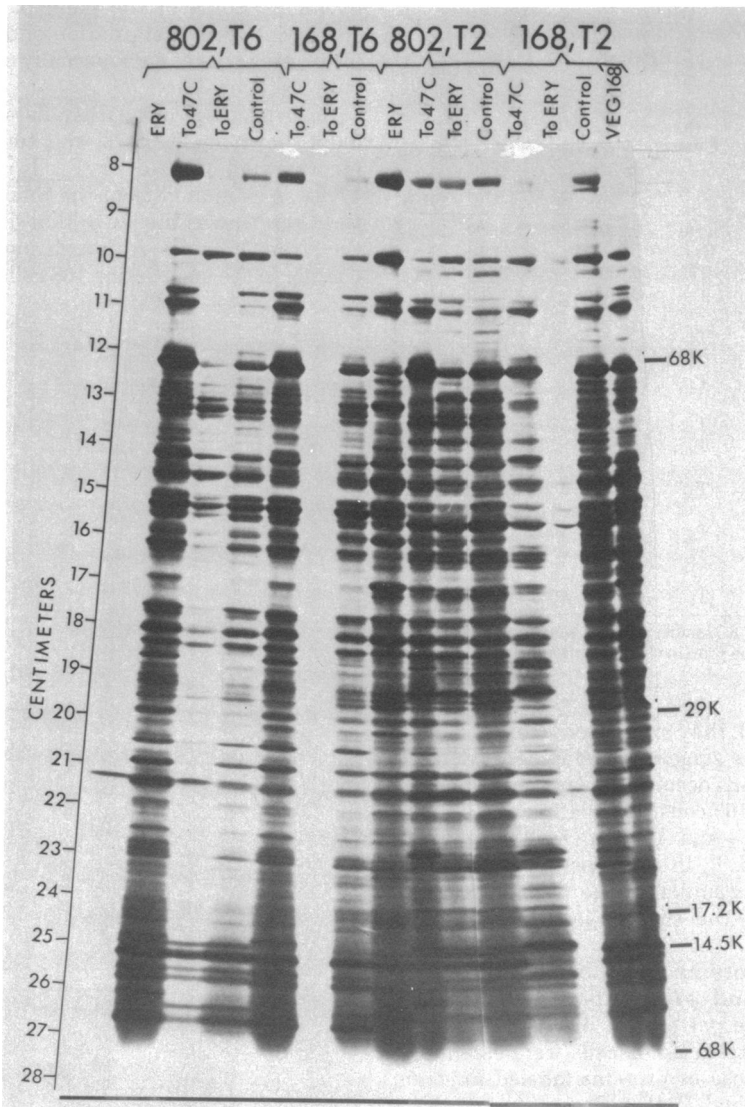


FIG. 8. Autoradiograph of soluble proteins from cells pulse-labeled during sporulation at 35°C with L - $[^{35}\text{S}]$ methionine and fractionated as in Fig. 7. Markers are as in Fig. 7. Migration distances are marked in centimeters. As in Fig. 7, gel slots were loaded with constant amounts of recovered protein. "Control" refers to patterns obtained from unshifted (35°C) cultures growing in the absence of erythromycin. "To47C" and "ToERY" refer to cultures shifted, at the indicated time (T_2 or T_6) to 47°C or into erythromycin at 35°C. ERY refers to cultures grown at 35°C in the continuous presence of erythromycin.

in the Ery^r cells, the band pattern in the latter remained more like that given by vegetative cells. Other bands, in both parent and Ery^r strains, remained apparently unchanged from the vegetative pattern. By these crude physical criteria, the Ery^r cells synthesized normal quantities of many normal proteins at 47°C.

SDS-soluble proteins synthesized at 47°C. The particulate fractions from breakage of sporulating cells consisted of wall and mem-

brane fragments at T_2 and of these fragments plus forespore integument fragments at T_5 . Fractionation of the SDS extracts of these fractions by SDS-PAGE followed by autoradiography (not shown) gave essentially the same information as fractionation of the proteins solubilized during breakage (Fig. 7). Although a higher background obscured minor bands, each extract gave a complex pattern that was highly reproducible, and distinct changes occurred during

TABLE 4. Changes in labeled protein band patterns during sporulation at 47°C^a

Class	Visibility of band						Change		
	Strain 168 Cells			Ery ^r E-802 Cells			Type	By:	Probably sporulation specific? ^b
	Vegetative	T ₂	T ₅	Vegetative	T ₂	T ₅			
U	+	+	+	+	+	+	None		
a	+	-	-	+	-	-	Off	T ₂	No
A	+	-	-	+	+	+	Off	T ₂	Yes
b	+	+	-	+	+	-	Off	T ₅	No
B	+	+	-	+	+	+	Off	T ₅	Yes
c	-	+	+	-	+	+	On	T ₂	No
C	-	+	+	-	-	-	On	T ₂	Yes
d	-	-	+	-	-	+	On	T ₅	No
D	-	-	+	-	-	-	On	T ₅	Yes
X	-	-	-	-	+ or -	+	On	Only in E-802	
R (on)	-	+	+/-	-	-	+	Retarded in E-802?		
R (off)	+	-	-	+	+	-			

^a See Fig. 7.^b Since only a few unique spore components have been identified, it is not known how many of the polypeptide bands appearing during sporulation are specific to this process. It seems likely that, since no morphological signs of sporulation occur in strain E-802 at 47°C, any change seen in both E-802 and 168 cells after T₂ may not be sporulation specific, whereas a change seen only in 168 cells may be sporulation specific.

the sporulation sequence in 168 cells. The same sequence of samples from E-802 cultures gave very similar patterns and levels of total incorporation. However, several potential sporulation-specific changes were seen only in the wild-type pattern. Major bands at about 9,000 and 16,000 daltons appeared at T₅ in 168 cells (Table 4, class D). Other major bands at about 60,000 and 75,000 daltons appeared at T₂ in 168 cells but not in E-802 cells (class C) and bands at 55,000 and 65,000 disappeared at T₅ in 168 cells but not in E-802 cells (class B).

Effect of erythromycin on patterns of soluble proteins synthesized at 35°C. Addition of erythromycin to strain 168 cells at T₂ or T₆, 35°C abolished incorporation of methionine added 10 min later (Table 3) except into a few discrete bands (Fig. 8). Addition of erythromycin to E-802 cells at T₂, 35°C caused some inhibition of methionine incorporation (Table 3) and had little effect on the pattern of proteins synthesized 10 min later (Fig. 8). In contrast, addition of erythromycin to E-802 cells at T₆, 35°C caused about 50% inhibition of total incorporation 20 min later (Fig. 5), which is reflected in a similar reduction in total band intensity (Table 3, Fig. 8). The effect on incorporation into major bands appeared to be highly selective. A series of six

low-molecular-weight bands were particularly resistant.

In the continuous presence of erythromycin, incorporation in vegetative cells of strain E-802 was little affected (data not shown), and the pattern seen at T₂ was very similar to that seen in 168 or E-802 at T₂ at 35°C in the absence of erythromycin (Fig. 8). However, a few bands were less prominent (12.1, 12.6, 13.5, and 15.5 cm), and others were more prominent (12.9, 15.8, and 16.8 cm). By T₆, incorporation was totally inhibited (Table 2, Fig. 8), except for a band at 21.3 cm and a few other fainter species (Fig. 8). A band at 21.3 cm also continued to be labeled in 168 T₂ cells exposed to erythromycin (Fig. 8).

Effect of erythromycin on SDS-soluble proteins. The patterns of SDS-soluble proteins produced in strain 168 cells at 35°C were as complex as those shown in Fig. 8 and showed no obvious correspondence to these soluble protein patterns. E-802 cells at 35°C produced patterns of SDS-soluble proteins that were very similar, and both strains showed reproducible changes from the vegetative pattern at T₂ and T₆ (data not shown). A shift into erythromycin abolished incorporation into 168 cells at T₂ or T₆ and had little effect on E-802 cells at T₂. The effect of erythromycin on E-802 cells at T₆ was less

marked than that seen on soluble proteins (Fig. 8). In the continuous presence of erythromycin, incorporation in E-802 cells was abolished by T_6 , but at T_2 the effects were minor.

Effects of brief shifts from 35 to 47°C. At 10 min after the shift of strain 168 or E-802 cultures from 35 to 47°C at T_2 and T_6 , marked changes in methionine-labeled soluble protein bands occurred (Fig. 8). Particularly noticeable were the strong bands appearing at 10.8, 12.2, 18.6, 22.6, and 24.8 cm. These changes seem to be characteristic of the shift and independent of the strain or sporulation stage. Labeling of the 8-cm band was stimulated at T_6 but not at T_2 . No selective effects on the Ery^r mutant were discernible, other than the appearance of a band at 26 cm at both T_2 and T_6 .

A shift to 47°C caused effects on SDS-soluble proteins which, like those seen for soluble proteins, seemed to be mostly independent of the strain or time. Major species appeared at 11.2, 13.3, 18.7, and 21.7 cm.

DISCUSSION

Spo(T_s) phenotype. The persistence of major components of the protein synthesis pattern of vegetative cells during sporulation (Fig. 7 and 8) is consistent with much previously published data (14), and this level of gene expression seems to be unaffected in the Ery^r mutants at 47°C. SDS-PAGE patterns suggest that normal amounts of many apparently normal proteins are being made in the cells at 47°C, and since no sporulation-specific morphogenesis occurs, there seems to be a selective defect in spore-specific gene expression.

The ribosomal modification in these strains has apparently disturbed one or more sporulation-specific ribosomal functions. Most directly, this function could be to discriminate between different messenger populations. This should soon be testable *in vitro* with the development of assays for translation of spore-specific messengers. (D. J. Tipper and G. H. Chambliss, unpublished data). If any specific ribosomal-membrane interactions occur during the synthesis and translocation of excreted proteins, then ribosomal mutations could affect the efficiency of such a process. Special interactions with forespore membranes would need to be invoked to explain a sporulation-specific effect. Third, the mechanisms of attenuation and stringency are models for indirect effects of ribosomal functions on gene expression at the level of transcription. The evidence correlating hyperphosphorylated nucleotide synthesis and sporulation (18-20) is inconclusive, and even if proven, the role of these substances and the participation of ribo-

somes in their production remains to be elucidated. This pathway remains a possible target for sporulation-specific mutation.

Ery^r phenotype. The Ery^r strains investigated in these physiological studies (E-2, E-802, and E-12) behave identically, substantiating the hypothesis (24) that all independent Ery^r strains arise by the same mutational event. These Ery^r strains are essentially indistinguishable from the parent strain (strain 168) during vegetative growth. Sensitivity of sporulation in these strains to high temperature or to erythromycin at permissive temperature extends from approximately 40 to 90% of the sporulation period, and viability is maintained for some time under either nonpermissive condition (24). This superficial similarity in the effects of these conditions on the mutants is belied by the data presented in this paper. Whereas high temperature precludes all morphological development and does not affect the rate of total protein synthesis, erythromycin affects only the late stages of morphogenesis, probably due to a severe inhibition of late protein synthesis. There is, therefore, no reason to suppose that the mechanisms of the effects of high temperature and erythromycin are related, even though they arise from the same modification in ribosome structure. The effects of shifting 35°C cultures of Ery^r strains into erythromycin late in sporulation mimic the effects of prolonged exposure to the drug because a major fraction of total late protein synthesis is rapidly inhibited. Development is aborted at this point.

The increased sensitivity of protein synthesis to erythromycin observed at T_4 to T_5 , 35°C in Ery^r cells coincides with the completion of forespore engulfment and could be a consequence of increased cellular permeability or of ribosomal modification. Because this altered sensitivity is peculiar to erythromycin among several related macrolide antibiotics tested (24; Tipper, unpublished data), a ribosomal change is suggested. There is no similar increase in sensitivity to erythromycin during sporulation in strain 168 cells (24). However, T_4 to T_5 also marks the termination of the period of rapid postexponential protein synthesis in 168 cells at 35°C (Fig. 5) and may correspond to the turnoff of several vegetative functions and the initiation of a period in which a greater proportion of total protein synthesis is sporulation specific. This is suggested by the SDS-PAGE patterns of proteins synthesized in 168 cells at T_5 , 47°C (Fig. 7) or T_6 , 35°C (Fig. 8). Thus, a change in ribosomal properties at this time may be consistent with the strategy of spore development. The proportion of rapidly inhibitable synthesis in Ery^r cells

increases slowly from T₄ to T₈ (Fig. 5), possibly as a result of asynchrony. The sensitivity of protein synthesis to prolonged exposure to erythromycin increases sharply between T₄ and T₅ and is probably a better indicator of synchrony. Mother cell proteins synthesis presumably predominates at later times, as forespore cytoplasm becomes refractile, and may account for the rapidly inhibitable fraction because of its greater accessibility. Differential sensitivity of compartmentalized ribosome populations might also explain the differential sensitivity of the labeling of specific protein bands to short-term exposure to erythromycin (Fig. 8). Residual incorporation may be into forespore proteins. The persistence of label incorporation into a few bands in cells continuously exposed to erythromycin is not easily explained.

The atypical cortical layer surrounding the aborted spores of erythromycin-inhibited Ery^r cells (Fig. 4C and D) seems to surround, rather than being surrounded by, an outer forespore membrane and so may not be cortical peptidoglycan. It may be incoherently assembled sporecoat made from precursors synthesized before T₄ (1, 17). This will be investigated chemically.

ACKNOWLEDGMENTS

We thank W. Charles Johnson for many helpful discussions during the course of this project and Michael Sadick for expert technical assistance.

This work was supported by Public Health Service grant AI 10806 from the National Institute of Allergy and Infectious Diseases.

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