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Properties of Purified Sporlets Produced by *spoII* Mutants of *Bacillus subtilis*

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A number of abortively disporic *spoII* mutants of *Bacillus subtilis* released their forespore compartments (termed stage II sporlets) after mother cell lysis during sporulation in nutrient exhaustion or resuspension media. Stage II sporlets were viable and contained levels of ATP and a number of enzymes similar to those in cells 2 to 3 h after sporulation. However, stage II sporlets carried out essentially no macromolecular synthesis, a result suggesting that they were in a quiescent state. The nucleoid of these quiescent stage II sporlets was significantly condensed relative to that in the original vegetative cells, as was previously found to take place 1 to 2 h after initiation of sporulation (B. Setlow, N. Magill, P. Febbroriello, L. Nakhimousky, D. E. Koppel, and P. Setlow, J. Bacteriol. 173:6270–6278, 1991). Stage II sporlets may be a useful model system for analysis of forespore properties early in stage II of sporulation.

Members of the gram-positive genera *Bacillus* and *Clostridium* undergo a developmental process called sporulation, in which a single cell differentiates into a sporangium encompassing both a larger mother cell and a smaller forespore that develops into the dormant endospore. Two early events in sporulation are the condensation of the forespore nucleoid (20) and the synthesis of the asymmetric septum that divides the sporangium into the mother cell and forespore compartments (14). The role of forespore nucleoid condensation in sporulation is unclear, but this process could play a role in differential gene expression in the mother cell and forespore (20, 22).

Bacillus subtilis spoII mutants are arrested just after asymmetric septum formation, although forespore nucleoid condensation does occur (14, 20). A number of these *spoII* mutants, including *spoIIAC* mutants, exhibit a phenotype called abortively disporic (14, 15), in which asymmetric septa are laid down at both ends of the sporangium, creating two forespore compartments in a single sporulating cell. In previous work, we found that sporulating cells of these abortively disporic *spoII* mutants contained two condensed nucleoids, one in each of the forespore compartments at the poles of the cells, with little if any DNA in the intervening mother cell (20). When incubation of these sporulating cells was continued, an increase in the number of cells exhibiting this disporic phenotype was observed (data not shown); this increase was followed by lysis of the mother cell compartment and release of the free ellipsoid to coccoid forespore compartments (termed stage II sporlets), which contained condensed nucleoids (Fig. 1a and b). Since these small cells were stained with the DNA stain 4,6-diamidino-2-phenylindole (DAPI) (Fig. 1b) (and were viable; see below), they were true cells, not minicells. The asymmetric sporulation septum normally contains very little peptidoglycan (6, 14). Although peptidoglycan synthesis is required for the synthesis of the asymmetric septum (2, 7), much of this peptidoglycan is degraded, allowing forespore engulfment to proceed (14). However, for the released stage II sporlets to be osmotically stable (as they are), one would predict that at some period in their formation they would become surrounded by a thick, more cell wall-like peptidoglycan layer. Analysis of *spoIIAC* mutant cells 3 to 4 h (t_3 to t_4) after initiation of sporulation by electron microscopy revealed a number of abortively disporic forms, with only a small amount of peptidoglycan in the asymmetric septa (Fig. 1c, arrow; data not shown). However, the stage II sporlets were completely surrounded by a thick peptidoglycan layer, in some cases with one end further encompassed by what was presumably the cell wall from the original mother cell (Fig. 1d, arrows). While the overall process leading to stage II sporlet production is not clear, the absence of DNA from the mother cell compartment (20) may contribute to lysis of the mother cell. Other workers have noted the release of a forespore compartment during the sporulation of asporogenous mutants, with the released forespores being termed pygmy cells (28), pseudoforespores (29), or sporlets (14); we use the term stage II sporlets throughout this work. For analysis of the production of stage II sporlets by asporogenous mutants, cells were grown in 2×SG medium (4) at 37°C with high aeration, and samples were taken at 12, 24, 36, and 48 h. Cells were washed, fixed with glutaraldehyde, stained with DAPI, and observed through epifluorescence optics as described previously (20). This analysis of stage II sporlet production in known abortively disporic *spoII* mutants showed that four (*spoIIAA69*, *spoIIAC1*, *spoIIE61*, and *spoIIE64*) produced stage II sporlets but that one (*spoIIG55*) did not. Other *spoII* mutants tested (*spoIIB131*, *spoIIC298*, and *spoIID66*) did not produce significant amounts of stage II sporlets, although a small amount (<5% of total cells) was seen in a *spoIIJ* mutant (*spoIIJ*::Tn*917*Ω*HU19*) after 48 h of incubation. The one *spoIII* mutant tested, *spoIIIGΔ1*, did not produce any sporlets.

Stage II sporlets could be potentially useful for studying chromosome condensation and forespore properties early in stage II of sporulation. Consequently, we optimized stage II sporlet production and purification to allow their characterization. Initially, we found that the use of resuspension medium (25) to induce sporulation resulted in a much higher percentage of stage II sporlets than was obtained when sporulation was induced by nutrient exhaustion in 2×SG medium (4). The *spoIIAC1* and *spoIIE61* strains routinely produced 90 to 95% stage II sporlets at t_{24} in resuspension medium. After being harvested at t_{24} by centrifugation (8,000 × *g*, 10 min), the stage II sporlets were purified to <2% contamination by two or three low-speed centrifugations (480 × *g*, 5 min) to remove sporulating cells and then by a

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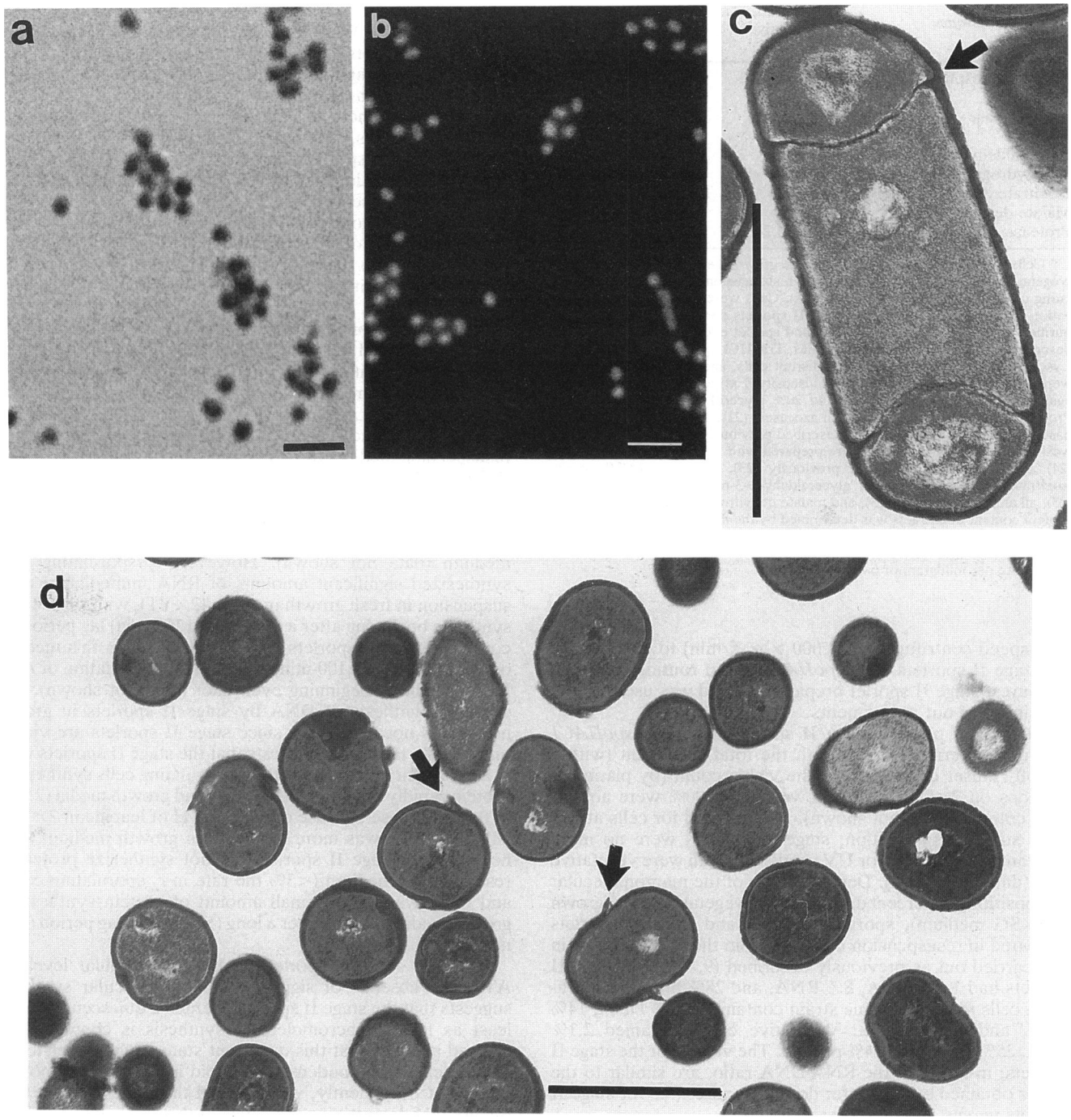

FIG. 1. Photomicrographs of sporulating cells and stage II sporlets from strain *spoIIAC1*. (a) Light micrograph of t_{24} sporlets from resuspension medium. Cells were fixed with glutaraldehyde (2.5%) as described previously (20), stained with crystal violet (0.02%), and viewed through bright-field objectives. Bar, 2.9 μm. (b) Epifluorescence micrograph of t_{24} sporlets from resuspension medium. Cells were fixed with glutaraldehyde as noted above, stained with the DNA stain DAPI, and viewed through epifluorescence optics as described previously (20). Bar, 2.9 μm. (c and d) Electron micrographs of an abortively disporic cell (c) and t_{24} sporlets from resuspension medium (d). Samples were fixed with 2.5% glutaraldehyde in cacodylate buffer (3, 19). Samples were then treated with OsO_4, stained with 0.5% uranyl acetate, dehydrated, embedded, sectioned, and viewed as previously described (19). The sporlets are surrounded by a complete cell wall, and the site of attachment to the mother cell can be seen on some (arrows in d). Bars, 1 μm.

TABLE 1. Specific activities of various enzymes in cells and stage II sporlets[a]

Enzyme	Sp act (nmol/min/mg of protein)[b] in:		
	Vegetative cells	t_2 sporulating cells	Stage II sporlets
Alkaline phosphatase	<3	<4	<6
Aspartase	43	<1	28
Asparaginase	37	11	24
Enolase	106	51	41
Glyceraldehyde-3-phosphate dehydrogenase	3.7	7.6	23
Isocitrate dehydrogenase	157	57	45
Malate dehydrogenase	3,460	210	450
Protease	0.24	6.7	4.6

[a] Cells of strain PS1175 (*spoIIAC1*) were grown in either 2×SG medium (4) (vegetative cells) or Sterlini-Mandelstam resuspension medium (25) (t_2 sporulating cells and purified stage II sporlets). Cells were harvested by centrifugation (8,000 × *g*, 10 min), and stage II sporlets were harvested at t_{24} and purified as described in the text. Cell and sporlet extracts were prepared as described previously (23) with 50 mM Tris-HCl (pH 7.4)–5 mM $CaCl_2$. Lysozyme (0.5 mg/ml) was used to disrupt cells, and the resulting extracts were sonicated briefly to reduce their viscosity. Extracts were dialyzed at 4°C against 50 mM Tris-Cl (pH 7.4) and 20% glycerol and stored at −80°C. Protease activity was measured with azocasein (21). Aspartase and asparaginase activities were measured as described previously (26). Enolase activity was measured in extracts that were prepared with 1 mM β-mercaptoethanol (24) and assayed as described previously (23), as were NADP-specific isocitrate dehydrogenase (23), glyceraldehyde-3-phosphate dehydrogenase (23), alkaline phosphatase (13), and malate dehydrogenase (8) activities. The protein content of extracts was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard.

[b] Protease specific activities are given as micrograms of azocasein degraded per minute per milligram of protein.

high-speed centrifugation (8,000 × *g*, 5 min) to concentrate the stage II sporlets. The *spoIIAC1* strain routinely yielded the purest stage II sporlet preparations and was used for the remainder of our experiments.

Analysis of purified stage II sporlets from the *spoIIAC1* strain by determination of both the total cell count (with a Petroff-Hauser chamber) and the viable count (by plating of dilutions on 2×SG plates) showed that >90% were able to form colonies (data not shown). As expected for cells at this early stage of sporulation, stage II sporlets were no more resistant to heat (60°C) or UV irradiation than were vegetative cells (data not shown). Determination of the macromolecular composition (as percent dry weight) of vegetative cells (grown in 2×SG medium), sporulating cells, and stage II sporlets (prepared in resuspension medium) from the *spoIIAC1* strain was carried out as previously described (9, 18, 23). Stage II sporlets had 1.5% DNA, 8% RNA, and 28% protein. Sporulating cells (t_2) of the same strain contained 1.3% DNA, 14% RNA, and 28% protein. Vegetative cells contained 2.1% DNA, 25% RNA, and 24% protein. The values for the stage II sporlets, in terms of the RNA/DNA ratio, are similar to the values obtained by others for dormant spores (5.3 for stage II sporlets versus 5.7 for spores [23]).

Analysis of enzyme levels showed that protease specific activities in stage II sporlets and t_2 sporulating cells were similar, although they were higher than those in vegetative cells; however, the *spoIIAC1* mutant made no alkaline phosphatase, as expected (14) (Table 1). The specific activities of aspartase and asparaginase were significantly higher in stage II sporlets than in t_2 sporulating cells, a result indicating that most of these enzymes are produced in the forespore compartment of sporulating cells, as suggested previously (26). Enzymes of intermediary metabolism showed specific activities in stage II sporlets similar to or higher than those in t_2 sporulating cells; these values were generally similar to those found previously with *B. megaterium* t_2 sporulating cells or forespores isolated at t_4 of sporulation (23). While the enzyme complement of stage II sporlets may represent the enzyme composition of t_2 forespores before engulfment (indeed, as noted above, it is similar to that in t_4 forespores), there certainly could be changes in enzyme levels between t_2 forespores and purified stage II sporlets, because of the prolonged incubation needed to allow sporlet release.

Analysis of the level of ATP in t_2 sporulating cells and stage II sporlets of the *spoIIAC1* strain by methods described previously (23) showed that both the cells and the sporlets had similar amounts of ATP per unit of wet weight (data not shown) and thus similar intracellular ATP concentrations. Previous work with *B. megaterium* had shown that the ATP concentration in $\sim t_3$ forespores was similar to that in t_3 sporulating cells or vegetative cells but that by $\sim t_5$ essentially no ATP remained in the forespores (23). To further examine the metabolic state of stage II sporlets, we measured their synthesis of macromolecules in comparison with that of t_2 sporulating cells. As described previously (19), radiolabelled precursors were added to cultures resuspended in either spent resuspension medium or fresh growth medium (2×YT [10] for DNA or RNA synthesis or 2×SG for protein synthesis), and the incorporation of label into acid-insoluble material was measured. Stage II sporlets and t_2 sporulating cells exhibited no significant synthesis of DNA or RNA in resuspension medium (data not shown). However, t_2 sporulating cells synthesized significant amounts of RNA immediately upon suspension in fresh growth medium (2×YT), with rapid DNA synthesis beginning after a long (75- to 100-min) lag period. In contrast, stage II sporlets synthesized RNA in this medium only after a long (≥100-min) lag period with initiation of rapid DNA synthesis beginning even later (data not shown). The eventual synthesis of DNA by stage II sporlets in growth medium is not surprising, since stage II sporlets are viable, but the long lag period suggests that the stage II sporlets were in a quiescent metabolic state. t_2 sporulating cells synthesized protein rapidly in both resuspension and growth media (2×SG in the latter case because of a high level of leucine in 2×YT), although there was more synthesis in growth medium (data not shown). Stage II sporlets did not synthesize protein in resuspension medium (<5% the rate in t_2 sporulating cells), and there was only a small amount of protein synthesis in growth medium, again after a long (>100-min) lag period (data not shown).

Although stage II sporlets had normal cellular levels of ATP, the absence of significant macromolecular synthesis suggests that the stage II sporlets were in a quiescent state, at least as far as macromolecular synthesis is concerned. It seemed possible that this quiescent state might be related to the presence of a condensed nucleoid in the stage II sporlets (20, 22). Consequently, we analyzed the stage II sporlets in the hope of determining the cause of this nucleoid condensation. One possibility is changes in DNA supercoiling, changes that do take place during sporulation (12, 20). However, such changes are due to α/β-type, small, acid-soluble spore proteins that are only synthesized ~2 h after forespore nucleoid condensation. Indeed, these proteins were absent from stage II forespores, and plasmid supercoiling in stage II forespores was identical to that in t_2 sporulating cells (data not shown). Another possible cause of forespore nucleoid condensation is a change in the level of a DNA-binding protein—possibly the synthesis of a major new sporulation-specific protein. Indeed,

a number of DNA-binding proteins have been identified for *B. subtilis*, and the levels of some have been reported to change during sporulation (1, 5, 11, 16). Consequently, Southwestern (DNA-protein) blot analysis as described by Wagar and Stephens (27) was carried out with either total *B. subtilis* DNA (digested with *Eco*RI, *Hin*dIII, or *Pst*I, with subsequent mixing of the digests) or plasmid pUC19 as a probe. This analysis revealed that the amounts of nonspecific DNA-binding proteins decreased markedly early in the sporulation of the wild-type (*spo*$^+$), *spoIIAC1*, and *spoIIE64* strains (data not shown). Although high levels of nonspecific DNA-binding proteins were not found in spores or stage II sporlets by this Southwestern blot analysis, HU protein (also termed HBsu) was detected in vegetative cells, spores, and stage II sporlets by Western blot (immunoblot) analysis (11, 17) with anti-HU antibody. An analysis of the amounts of HU in vegetative cells, spores, and stage II sporlets relative to DNA contents showed that HU levels were similar in vegetative cells, spores, and stage II sporlets (data not shown).

In a second type of analysis of DNA-binding proteins, cells ruptured under dry conditions were extracted with 2 N HCl (17) and soluble proteins were analyzed by polyacrylamide gel electrophoresis either at a low pH or with sodium dodecyl sulfate (17). As noted above, the levels of most acid-soluble proteins, many of which might be DNA-binding proteins, decreased greatly early in sporulation of the wild-type and *spoIIE64* strains, although one new minor protein appeared at $\sim t_0$ to t_2 (data not shown). HU protein was also detected, at similar levels in extracts of these vegetative cells and stage II sporlets (data not shown). It is not clear whether the disappearance of putative DNA-binding proteins early in sporulation is related to forespore nucleoid condensation, but this decrease was observed with both wild-type and *spoII* mutant strains. However, it is certainly possible that major DNA-binding proteins were not detected by the assay procedures used. In any event, at present, the specific cause(s) of both forespore nucleoid condensation and the metabolic quiescence of stage II sporlets remains unclear.

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REFERENCES

1. **Brehm, S. P., F. Le Hegarat, and J. A. Hoch.** 1974. Developmental modulation of deoxyribonucleic acid-binding proteins of *Bacillus subtilis* during sporulation stages. J. Bacteriol. **120:** 1443–1450.
2. **Dancer, B. N.** 1979. Requirement for peptidoglycan synthesis during sporulation of *Bacillus subtilis*. J. Bacteriol. **140:**786–797.
3. **Francesconi, S. C., T. J. MacAlister, B. Setlow, and P. Setlow.** 1985. Immunoelectron microscopic localization of small, acid-soluble proteins in sporulating cells of *Bacillus subtilis*. J. Bacteriol. **170:**5963–5967.
4. **Goldrick, S., and P. Setlow.** 1983. Expression of a *Bacillus megaterium* sporulation-specific gene during sporulation of *Bacillus subtilis*. J. Bacteriol. **155:**1459–1462.
5. **Hirschbein, L., N. Guillen, and F. Le Hegarat.** 1977. Isolation and characterization of nucleoids of *Bacillus subtilis* during growth and sporulation, p. 373–390. *In* A. N. Barker, J. Wolf, D. J. Ellar, G. J. Dring, and G. W. Gould (ed.), Spore research. Academic Press, Inc., New York.
6. **Illing, N., and J. Errington.** 1991. Genetic regulation of morphogenesis in *Bacillus subtilis*: roles of σ^E and σ^F in forespore engulfment. J. Bacteriol. **173:**3159–3169.
7. **Jonas, R. M., S. C. Holt, and W. G. Haldenwang.** 1990. Effects of antibiotics on synthesis and persistence of σ^E in sporulating *Bacillus subtilis*. J. Bacteriol. **172:**4616–4623.
8. **Kitto, G. B., and R. G. Lewis.** 1967. Purification and properties of tuna supernatant and mitochondrial malate dehydrogenase. Biochim. Biophys. Acta **139:**1–15.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
10. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
11. **Micka, B., N. Groch, U. Heinemann, and M. A. Marahiel.** 1991. Molecular cloning, nucleotide sequence, and characterization of the *Bacillus subtilis* gene encoding the DNA-binding protein HBsu. J. Bacteriol. **173:**3191–3198.
12. **Nicholson, W. L., and P. Setlow.** 1990. Dramatic increase in negative superhelicity of plasmid DNA in the forespore compartment of sporulating cells of *Bacillus subtilis*. J. Bacteriol. **172:**7–14.
13. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination, and outgrowth, p. 391–450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons, Inc., New York.
14. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40:**908–962.
15. **Remsen, C. C., and D. G. Lundgren.** 1965. Multiple septation in variants of *Bacillus cereus*. J. Bacteriol. **90:**1426–1431.
16. **Salti, V., F. Le Hegarat, and L. Hirschbein.** 1985. Isolation and characterization of small heat-stable acid-soluble DNA-binding proteins from *Bacillus subtilis* nucleoids. J. Gen. Microbiol. **131:**581–590.
17. **Sanchez-Salas, J.-L., M. L. Santiago-Lara, B. Setlow, M. D. Sussman, and P. Setlow.** 1992. Properties of *Bacillus megaterium* and *Bacillus subtilis* mutants which lack the protease that degrades small, acid-soluble proteins during spore germination. J. Bacteriol. **174:**807–814.
18. **Schneider, W. C.** 1957. Determination of nucleic acids in tissues by pentose analyses. Methods Enzymol. **3:**680–684.
19. **Setlow, B., A. R. Hand, and P. Setlow.** 1991. Synthesis of a *Bacillus subtilis* small, acid-soluble spore protein in *Escherichia coli* causes cell DNA to assume some characteristics of spore DNA. J. Bacteriol. **173:**1642–1653.
20. **Setlow, B., N. Magill, P. Febbroriello, L. Nakhimovsky, D. E. Koppel, and P. Setlow.** 1991. Condensation of the forespore nucleoid early in sporulation of *Bacillus* species. J. Bacteriol. **173:**6270–6278.
21. **Setlow, P.** 1979. Protease and peptidase activities in growing and sporulating cells and dormant spores of *Bacillus megaterium*. J. Bacteriol. **122:**642–649.
22. **Setlow, P.** 1991. Changes in forespore chromosome structure during sporulation in *Bacillus* species. Semin. Dev. Biol. **2:**55–62.
23. **Singh, R. P., B. Setlow, and P. Setlow.** 1977. Levels of small molecules and enzymes in the mother cell compartment and the forespore of sporulating *Bacillus megaterium*. J. Bacteriol. **130:**1130–1138.
24. **Spring, T. G., and F. Wold.** 1971. The purification and characterization of *Escherichia coli* enolase. J. Biol. Chem. **246:**6797–6802.
25. **Sterlini, J. M., and J. Mandelstam.** 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem. J. **113:**29–37.
26. **Sun, D., and P. Setlow.** 1991. Cloning, nucleotide sequence, and expression of the *Bacillus subtilis ans* operon, which codes for L-asparaginase and L-aspartase. J. Bacteriol. **173:**3831–3845.
27. **Wagar, E. A., and R. S. Stephens.** 1988. Developmental-form-specific DNA-binding proteins in *Chlamydia* spp. Infect. Immun. **56:**1678–1684.
28. **Waites, W. M., D. Kay, I. W. Dawes, D. A. Wood, S. C. Warren, and J. Mandelstam.** 1970. Sporulation in *Bacillus subtilis*, correlation of biochemical events with morphological changes in asporogenous mutants. Biochem. J. **118:**667–676.
29. **Young, I. E.** 1964. Characteristics of an abortively disporic variant of *Bacillus cereus*. J. Bacteriol. **88:**242–254.