Axial Filament Formation in *Bacillus subtilis*: Induction of Nucleoids of Increasing Length after Addition of Chloramphenicol to Exponential-Phase Cultures Approaching Stationary Phase

JAMES E. BYLUND, MICHAEL A. HAINES, PATRICK J. PIGGOT, AND MICHAEL L. HIGGINS*

Department of Microbiology and Immunology, Temple University
School of Medicine, Philadelphia, Pennsylvania 19140

Received 21 September 1992/Accepted 14 January 1993

When chloramphenicol was added to a culture of *Bacillus subtilis* in early exponential growth, microscopic observation of cells stained by 4',6-diamidino-2-phenylindole showed nucleoids that had changed in appearance from irregular spheres and dumbbells to large, brightly stained spheres and ovals. In contrast, the addition of chloramphenicol to cultures in mid- and late exponential growth showed cells with elongated nucleoids whose frequency and length increased as the culture approached stationary phase. The kinetics of nucleoid elongation after the addition of chloramphenicol to exponential-phase cultures was complex. Immediately after treatment, the rate of nucleoid elongation was very rapid. The nucleoid then elongated steadily for about 4 min, after which the rate of elongation decreased considerably. Nucleoids of cells treated with 6-(*p*-hydroxyphenylazo)-uracil (an inhibitor of DNA synthesis) exhibited the immediate rapid elongation upon chloramphenicol treatment but not the subsequent changes. These observations suggest that axial filament formation during stationary phase (stage I of sporulation) in the absence of chloramphenicol results from changes in nucleoid structure that are initiated earlier, during exponential growth.

Many investigators have sought to describe the arrangement of the nucleoplasm in the procaryotic cell. The subject has remained elusive, partly because the nucleoid undergoes rearrangement during the most gentle manipulations (e.g., rapid filtration and temperature changes, etc. [8]). Thus, after decades of effort, only a few noncontroversial statements can be made: the nucleoid of the exponential-phase cell is localized primarily in the center of the cell but does extend to the periphery of the cytoplasm (2, 7, 8, 12, 13); in stationary-phase cells, the nucleoid becomes confined to the center, and this results in a decrease in the density of this portion of the cell (3, 6, 21).

Recently there has been a resurgence of interest in nucleoid structure. In part, this has resulted from the development of cryofixation techniques for electron microscopy (13), the availability of DNA-specific fluorescent stains such as 4',6-diamidino-2-phenylindole (DAPI) (11, 17, 19, 25), and a rich supply of mutants whose phenotypes include characteristics that could be, or are, related to chromosome structure and function. In this light we have developed a method which uses image analysis to characterize the nucleoid in terms of size, shape, and distribution in a cell as the cell proceeds from exponential- to stationary-phase growth. The distinct internal organization of *Bacillus subtilis* as it undergoes symmetric and asymmetric division offers a model system for exploring nucleoid structure and function during exponential growth and post-exponential-phase differentiation.

MATERIALS AND METHODS

Growth conditions. *B. subtilis* BR151 *trpC2 lys-3 metB5* (source, F. E. Young) was used for this study. Bacteria were grown in modified Schaeffer's sporulation medium (26). Cultures were grown aerobically (medium-to-flask volume ratio, 1:10) with agitation (120 rpm) at 37°C. Cell growth was

measured by monitoring turbidity at 600 nm (20). Media were inoculated with 1/100 volume of cultures of exponentially growing cells (optical density at 600 nm, between 0.4 and 0.8). The mass doubling time of cells grown in modified Schaeffer's sporulation medium was 35 ± 5 min. The initiation of stationary-phase conditions was defined as a deviation from linearity of culture turbidity measurements plotted as a function of time on a semilogarithmic scale. By this definition, stationary-phase conditions occurred at an adjusted optical density at 600 nm of 1.6 ± 0.3 . The system was reproducible enough to consider samples taken at optical densities at 600 nm of approximately 0.2, 0.4, and 0.8 to be three, two, and one mass doublings, respectively, before the onset of stationary phase or to represent samples taken at early, mid-, and late exponential growth. When it was required, bacteria were treated with chloramphenicol (Calbiochem-Behring Diagnostics, La Jolla, Calif.) (50 µg/ml) and/or 6-(*p*-hydroxyphenylazo)-uracil (HPUra) (a gift of B. Langley, Imperial Chemical Industries) (15 µg/ml) for 15 min (unless otherwise stated) at 37°C with agitation (120 rpm).

Fixation. To prevent autolysis and to minimize the rearrangement of cellular components, we chose to base our work on fixed material. Control experiments indicated that fixed cells gave the same DAPI staining profile as unfixed cells. However, with time, unfixed cells gave variable DAPI staining profiles. Cells were fixed by the simultaneous addition of formaldehyde and glutaraldehyde to final concentrations of 0.37 and 0.001%, respectively. Formaldehyde at a final concentration of 0.37% was selected because it was the lowest concentration which did not result in an overnight decrease in the optical density of cells. Even though the optical density of formalin-fixed cultures was stable for at least 48 h, some cell-to-cell variation in the intensity of DAPI staining was observed. Critical experiments studying this problem showed that 0.001% glutaraldehyde in combination with 0.37% formaldehyde eliminated any visible variation in DAPI staining intensity. Higher concentrations of glutaral-

* Corresponding author.

dehydrate gave no further advantage in decreasing the variation in staining but, instead, imposed the disadvantage of producing a concentration-dependent increase in the clumping of cells.

Microscopy and staining procedure. All microscopy was accomplished with a Zeiss Photomicroscope II (Carl Zeiss, Oberkochen, Germany). Nucleoid morphology was observed by a combination of fluorescence and transmitted phase-contrast microscopy after staining with DAPI (Boehringer Mannheim, Indianapolis, Ind.). The DAPI staining procedure is a modification of the method of Hiraga et al. (11). Culture samples (1 ml) were fixed in 0.37% formaldehyde and 0.001% glutaraldehyde (wt/vol, final concentration) for 30 min at room temperature, harvested by centrifugation, washed twice with water, and resuspended in 50 μ l of water. Ten microliters of the suspension was applied to a coverslip treated with 0.1% polylysine. The coverslip was then inverted and placed sequentially onto two successive 100- μ l lots of first 50, then 70, and finally 95% ethanol (30 s for each treatment). Finally, the coverslip was placed onto methanol for 1 min, washed by rapid submersion in water, then placed onto a drop of DAPI solution (0.25 μ g/ml) for 20 min in the dark. Excess liquid was removed by blotting the coverslip on a paper towel. The coverslip was then placed on a microscope slide, sealed with paraffin wax, and observed.

Image analysis. Images of DAPI-stained material were recorded on Kodak Tri-X 35-mm film (Eastman Kodak Co., Rochester, N.Y.). Nucleoid lengths were measured in photographs with DUMAS image analysis equipment (Drexel University, Philadelphia, Pa.). The edges of the nucleoids were determined visually with an interactive graphic tablet. Differences in DAPI staining intensity led to some variation in mean nucleoid lengths between sets of experiments. However, within a set of experiments this difference in staining intensity was not a factor. At least 150 nucleoids were measured for each cell population studied.

RESULTS

DAPI is a fluorescent stain which has a high affinity for DNA. We have used this compound to study the morphology of the nucleoids of *B. subtilis* during early, mid-, and late exponential growth, before and after treatment with chloramphenicol. In our experiments we defined early-exponential-phase cultures as cells that are at least three mass doublings from stationary-phase conditions (see Materials and Methods for details). Cells in mid- and late exponential growth were defined as being two and one mass doublings, respectively, from stationary-phase conditions.

Consistent with results seen by many laboratories, our controls showed that the nucleoid morphology of *B. subtilis* cells changes from irregular spheres and dumbbells to axial filaments during early stationary phase (1, 15) (Fig. 1). The mean nucleoid length in cells taken from post-exponential-phase cultures is 2.24 μ m (median = 2.08 μ m).

When nucleoids from cells in early, mid-, and late exponential growth were compared, they seemed to be very similar in shape, size, and distribution (Fig. 2a, 2b, and 2c, respectively). Upon analysis, the frequency distributions of the lengths of the nucleoids seen in each population (i.e., from the early-, mid-, and late-exponential-phase cultures shown in Fig. 2) were almost superimposable (Fig. 3A).

Having established the similarity of nucleoid morphology during exponential growth, we questioned the configuration of these nucleoids when cells were exposed to chloramphenicol. As shown in Fig. 2d, treatment of early-exponential-

FIG. 1. (A) Light micrograph of DAPI-stained cells taken from post-exponential-phase culture (time = 1 h). Bar = 5 μ m. (B) Frequency distribution of length measurements of DAPI-staining figures (nucleoids) observed in micrographs of cells taken from post-exponential-phase cultures (time = 1 h).

phase cells resulted in brightly stained oval or spherical bodies; however, as cultures approached stationary phase, chloramphenicol treatment resulted in an increasing fraction of the population forming elongated nucleoids (Fig. 2e and 2f). Distribution of nucleoid lengths in these cells showed a progressive increase in size as the culture neared the end of exponential growth (Fig. 3B). The mean nucleoid length in late-exponential-phase cells treated with chloramphenicol was 1.48 μ m (median = 1.42 μ m). This was compared with the untreated control cells, which had a mean nucleoid length of 0.93 μ m (median = 0.82 μ m), or treated early-exponential-phase cells, which had a mean nucleoid length of 1.04 μ m (median = 1.09 μ m).

Kinetics of nucleoid elongation. Figure 4 shows the distribution of nucleoid lengths after various periods of chloramphenicol treatment. In these experiments, the drug was added to late-exponential-phase cultures, and the cells were fixed at the times shown. The first treated sample (Fig. 4) underwent about 20 s of treatment (i.e., the shortest time in which the sample could be fixed after the addition of chloramphenicol and mixing). The kinetics of nucleoid elongation is complex. In the first seconds of treatment, mean nucleoid length increased to 70% of the maximum length observed (from 0.77 to 0.93 μ m). Mean nucleoid length increased substantially during the next 4 min of treatment (to 1.20 μ m), with a further small increase (to 1.31 μ m) noted during the remainder of the 15-min observation period. Treatment beyond 15 min showed little change in mean nucleoid length (1.36 μ m for a 60-min treatment). It is possible that these represent three distinct periods of elongation. Another possibility is that a portion of the population undergoes rapid elongation in the first few seconds. Thereafter, increasingly smaller portions of the population undergo nucleoid elongation. Such behavior might be linked to chromosome replication, since cells treated with chloramphenicol can continue to synthesize DNA. It might be that nucleoids cannot fully elongate until chromosome replication is complete. To test this possibility, HPUra, an inhibitor of DNA synthesis (4), was added to late-exponential-phase cells in the absence and presence of chloramphenicol.

The effect of HPUra on nucleoid length. When HPUra was added to exponential-phase cultures at a final concentration

FIG. 2. Light micrographs of DAPI-stained cells taken from early-, mid-, and late-exponential-phase cultures before (panels a, b, and c, respectively) and after (panels d, e, and f, respectively) the addition of chloramphenicol for 15 min. The bar in panel c represents 5 μm and applies to all micrographs.

of 15 $\mu\text{g/ml}$, [^3H]thymidine incorporation into cold-acid-precipitable counts was reduced within 5 min to less than 5% of that of the untreated controls (results not shown). The addition of HPUra to cells without chloramphenicol resulted in a small decrease (ca. 18%) in the nucleoid distributions (mean = 0.54 μm) in comparison to those of the untreated controls (mean = 0.71 μm) (Fig. 5). When HPUra was added with chloramphenicol to late-exponential-phase cultures (Fig. 5), the distributions (mean = 0.82 μm) increased above those of untreated cultures. The increase was much smaller than that of cells treated only with chloramphenicol (mean = 1.20 μm). This supports the idea that the nucleoid elongation process is dependent on residual DNA synthesis.

DISCUSSION

The addition of chloramphenicol to a variety of bacteria in exponential growth has been shown to result in the confinement of the nucleoid into a spherical or ovoid body (a partial list includes *B. subtilis* [9, 22], *Staphylococcus aureus* [10], *Streptococcus faecalis* [6, 7], and *Escherichia coli* [2, 12–14, 16, 25, 27]). An exception, reported by Van Iterson, is that elongated nucleoids appear in germinated heat-shocked

FIG. 3. Measurements of nucleoid length from exponential-phase cells before and after a 15-min treatment with chloramphenicol. (A) Frequency distributions of measurements of DAPI-staining figures (nucleoids) observed in micrographs of cells taken from early (squares)-, mid (crosses)-, and late (circles)-exponential-phase cultures. (B) The three distributions of nucleoid length in panel A were combined (triangles) to allow comparison with the distributions of nucleoid length obtained after cultures had been treated with chloramphenicol. Results observed with early (squares)-, mid (crosses)-, and late (circles)-exponential-phase cell nucleoids are shown.

spores of *B. subtilis* after treatment with chloramphenicol for periods greater than 110 min (23). It is clear that the elongation of the nucleoid noted here in the latter stages of exponential growth is an unusual observation in the microbial world. That elongation occurs with greater frequency as cells approach the end of exponential growth raises the question of whether the phenomenon is related to the axial filament formation designated stage I of spore formation (1, 15). This question can be addressed by proposing a hypothetical scheme of how nucleoid elongation could occur upon chloramphenicol treatment as a culture approaches stationary phase and how this process could be related to the normal course of endospore development.

We suggest that as cells approach stationary phase, the nucleoid changes in some unknown physical manner such that, upon the inhibition of protein synthesis (but with continued DNA synthesis), it has an increasing probability of forming an elongated structure. This structural change could involve an alteration in the number or placement of chromosome interactions with the envelope, in the number or type of DNA-binding proteins, or in the concentration of supercoils. We have no insight into which mechanism might

FIG. 4. Effects of chloramphenicol, with time, on the distribution of nucleoid length in late-exponential-phase cells. Cells were removed and fixed for analysis 10 min before (squares), upon (crosses), 2 min after (circles), 4 min after (triangles), and 15 min after (diamonds) treatment.

be relevant, but Setlow et al. (19) have shown that supercoiling decreases between late exponential phase and early stationary phase (i.e., the period when axial filament formation would be expected).

The elongated nucleoids induced by the addition of chloramphenicol to late-exponential-phase cells are about one-half to two-thirds the length of axial filaments formed (in the absence of chloramphenicol) during sporulation. We suggest that during sporulation, two (or more) nucleoids of a cell elongate (in part because of a reduction of protein synthesis in the cell [6]) and fuse to form an axial filament. This would be consistent with the fusion of nucleoids in *E. coli* as reported by Schaechter and Laing (18). In fact, other data from this study (to be published separately) support a model in which chloramphenicol-induced oval and elongated nucle-

oids also result from the fusion of at least two normal nucleoids. We suggest that, as in the nucleoid occlusion model of Woldringh et al. (24), by traversing the length of the cell the axial filament inhibits septum formation. When one chromosomal copy is confined to the mass necessary for spore formation, the resulting asymmetrical gap between nucleoids then becomes the site of the spore septum. The cause of asymmetric chromosome confinement remains unknown. The counteridea that axial filament formation is not a stage of sporulation cannot at present be excluded. It is based on the observation that all *spo0* mutants examined form elongated nucleoids (1, 15). In addition, the process is not specific to presporulating cells, because the formation of elongated nucleoids, in late-exponential-phase cells treated with chloramphenicol, was observed in a medium that does not support sporulation (Luria broth) (5). The isolation of mutants defective in axial filament formation would best resolve this problem. However, it is possible that disruption of the mechanism for axial filament formation involves essential genes. If this is the case, it may prove difficult to identify and isolate conditional mutants which fail to form axial filaments. If the potential for nucleoid elongation is regulated in relation to some external signal and if elongation is an adaptation in preparation for sporulation, it may be necessary to redefine when the process of sporulation is initiated.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI10971 from the National Institute of Allergy and Infectious Diseases and by grant DMB-8912323 from the National Science Foundation.

REFERENCES

- Balassa, G. 1971. The genetic control of spore formation in *Bacilli*. *Curr. Top. Microbiol. Immunol.* **56**:101-192.
- Bohrmann, B., W. Villiger, R. Johansen, and E. Kellenberger. 1991. Coralline shape of the bacterial nucleoid after cryofixation. *J. Bacteriol.* **173**:3149-3158.
- Bourbeau, P., D. Dicker, M. L. Higgins, and L. Daneo-Moore. 1989. Effect of cell cycle stages on the central density of *Enterococcus faecium* ATCC 9790. *J. Bacteriol.* **171**:1982-1986.
- Brown, N. 1971. Inhibition of bacterial DNA replication by 6-(*p*-hydroxyphenylazo)-uracil: differential effector repair and semi-conservative synthesis in *Bacillus subtilis*. *J. Mol. Biol.* **59**:1-16.
- Bylund, J. E. Unpublished data.
- Daneo-Moore, L., D. Dicker, and M. L. Higgins. 1980. Structure of the nucleoid in cells of *Streptococcus faecalis*. *J. Bacteriol.* **141**:928-937.
- Daneo-Moore, L., and M. L. Higgins. 1972. Morphokinetic reaction of *Streptococcus faecalis* (ATCC 9790) cells to the specific inhibition of macromolecular synthesis: nucleoid condensation on the inhibition of protein synthesis. *J. Bacteriol.* **109**:1210-1220.
- Edelstein, E., L. Parks, H.-C. Tsien, L. Daneo-Moore, and M. L. Higgins. 1981. Nucleoid structure in freeze fractures of *Streptococcus faecalis*: effects of filtration and chilling. *J. Bacteriol.* **146**:798-803.
- Frehel, C., and A. Ryter. 1971. Etude de la liaison du noyau a la membrane chez *B. subtilis* apres traitement au chloramphenicol. *J. Microsc. (Paris)* **12**:59-72.
- Giesbrecht, P., and H. Ruska. 1968. Über Veränderungen der Feinstrukturen von Bakterien unter der Einwirkung von Chloramphenicol. *Klin. Wochenschr.* **46**:575-582.
- Hiraga, S., H. Niki, T. Ogura, C. Ichinose, H. Mori, B. Ezaki, and A. Jaffé. 1989. Chromosome partitioning in *Escherichia coli*: novel mutants producing anucleate cells. *J. Bacteriol.* **171**:1496-1505.

FIG. 5. Effects of HPUra (30-min treatment) on the distribution of nucleoids in late-exponential-phase cultures with and without subsequent chloramphenicol (15-min) treatment. Symbols represent cell nucleoids that were HPUra treated (circles), untreated (squares), chloramphenicol treated (crosses), and HPUra and chloramphenicol treated (triangles).

12. Hobot, J. A., M.-A. Bjornsti, and E. Kellenberger. 1987. Use of on-section immunolabeling and cryosubstitution for studies of bacterial DNA distribution. *J. Bacteriol.* **169**:2055–2062.
13. Kellenberger, E. 1991. Functional consequences of improved structural information, on bacterial nucleoids. *Res. Microbiol.* **142**:229–238.
14. Kellenberger, E., A. Ryter, and J. Sechaud. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *J. Biophys. Biochem. Cytol.* **4**:671–678.
15. Mandelstam, J., D. Kay, and D. Hranueli. 1975. Biochemistry and morphology of stage I in sporulation of *Bacillus subtilis* cells, p. 181–186. In P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), *Spores VI*. American Society for Microbiology, Washington, D.C.
16. Morgan, C., H. S. Rosenkranz, H. S. Carr, and H. M. Rose. 1967. Electron microscopy of chloramphenicol-treated *Escherichia coli*. *J. Bacteriol.* **93**:1987–2002.
17. Ryter, A., P. Schaeffer, and H. Ionesco. 1966. Classification cytologique, par leur stade de blocage, des mutants de sporulation de *Bacillus subtilis* Marburg. *Ann. Inst. Pasteur* **110**:305–315.
18. Schaechter, M., and V. O. Laing. 1961. Direct observation of fusion of bacterial nuclei. *J. Bacteriol.* **81**:667–668.
19. Setlow, B., N. Magill, P. Febroriello, 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.
20. Shockman, G. D. 1962. Amino acids, p. 567–673. In F. Kavanagh (ed.), *Analytical microbiology*. Academic Press, Inc., New York.
21. Valkenburg, J. A. C., and C. L. Woldringh. 1984. Phase separation between nucleoid and cytoplasm in *Escherichia coli* as defined by immersive refractometry. *J. Bacteriol.* **160**:1151–1157.
22. van Iterson, W., and J. A. Aten. 1976. Nuclear and cell division in *Bacillus subtilis*. *Antonie Leeuwenhoek* **42**:365–386.
23. Van Iterson, W., P. A. M. Michels, F. Vyth-Dreese, and J. A. Aten. 1975. Nuclear and cell division in *Bacillus subtilis*: dormant nucleoids in stationary-phase cells and their activation. *J. Bacteriol.* **121**:1189–1199.
24. Woldringh, C. L., E. Mulder, P. G. Huls, and N. Vischer. 1991. Toporegulation of bacterial division according to the nucleoid occlusion model. *Res. Microbiol.* **142**:309–320.
25. Woldringh, C. L., and N. Nanninga. 1976. Organization of the nucleoplasm in *Escherichia coli* visualized by phase-contrast light microscopy, freeze fracturing, and thin sectioning. *J. Bacteriol.* **127**:1455–1464.
26. Wu, J.-J., M. G. Howard, and P. J. Piggot. 1989. Regulation of transcription of the *Bacillus subtilis* *spoIIA* locus. *J. Bacteriol.* **171**:692–698.
27. Zusman, D. R., A. Carbonell, and J. Y. Haga. 1973. Nucleoid condensation and cell division in *Escherichia coli* MX74T2 *ts52* after inhibition of protein synthesis. *J. Bacteriol.* **115**:1167–1178.