JOURNAL OF BACTERIOLOGY, May 1979, p. 314-319
0021-9193/79/05-0314/06$02.00/0

Vol. 138, No. 2

Specific Inhibition of Outgrowth of *Bacillus subtilis* Spores by Novobiocin

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Received for publication 17 February 1979

Spores of a *Bacillus subtilis* mutant temperature sensitive in deoxyribonucleic acid (DNA) replication proceeded through outgrowth at the nonpermissive temperature to the same extent as the wild-type parent spores. In contrast, the DNA synthesis inhibitor novobiocin completely prevented spore outgrowth while displaying a marginal effect on logarithmic growth during one generation time. Inhibition of outgrowth by novobiocin occurred in the absence of DNA replication, as demonstrated in an experiment with spores of the temperature-sensitive DNA synthesis mutant at the restrictive temperature. Novobiocin inhibited the initial rate of ribonucleic acid synthesis to the same extent in germinated spores and in exponentially growing cells. A novobiocin-resistant mutant underwent normal outgrowth in the presence of novobiocin. Therefore, novobiocin inhibition was independent of its effect on chromosome replication per se.

Ginsberg and Keynan (15) confirmed earlier observations (6, 24, 26, 28, 33) pointing to the independence of *Bacillus subtilis* spore outgrowth from DNA synthesis. They demonstrated that outgrowth of *B. subtilis* 168 proceeds normally under restrictive conditions in mutants temperature sensitive in DNA synthesis. Two inhibitors of DNA synthesis, nalidixic acid and 6-(*p*-hydroxyphenylazo)uracil, impaired outgrowth. However, the inhibition of outgrowth and not that of DNA synthesis is reversed by glucose (15).

In probing the generality of these observations, we were surprised to observe that the antibiotic novobiocin, also a DNA synthesis inhibitor, completely impaired outgrowth even under glucose-enriched conditions. This drug interacts with and inhibits the enzyme DNA gyrase (*Eco* DNA topoisomerase II) responsible for inserting negative superhelical twists into the *Escherichia coli* chromosome (9, 13). This paper demonstrates that novobiocin at a concentration that completely inhibited DNA synthesis interfered with outgrowth but interfered substantially less with exponential growth.

MATERIALS AND METHODS

Bacteria and phages. The genotypes of bacterial and phage strains used are given in Table 1.

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Growth of bacteria. LB agar plates contained (in grams per liter): tryptone (Difco), 10; yeast extract (Difco), 5; NaCl, 5; and agar, 15. Soft agar for phage overlay contained (in grams per liter): nutrient broth (Difco), 8; NaCl, 5; and agar, 7. Vegetative growth, sporulation, outgrowth, and preparation of spores were as described elsewhere (26) with the exception that the final step of purification on a discontinuous Renografin gradient was eliminated. The purified spore stocks contained between 82 and 97% of the spores in the phase-bright state. Spore stocks were checked for the expected genetic markers and heat resistance by plating spores after heating for 10 min at 80°C. Spores were not heat activated before transfer into the broth. Changes in optical density were followed in a Klett-Summerson photoelectric colorimetric equipped with red filter no. 66.

Nucleic acid synthesis. Synthesis of DNA and RNA were measured by incorporation of [^{3}H]thymidine and [^{14}C]uracil, respectively (final concentration in the culture, 5 μCi/ml), into trichloroacetic acid-insoluble material (26) in culture samples containing at least 10^8 exponentially growing cells. For outgrowth, 5 μCi of [^{3}H]thymidine or 0.625 μCi of [^{14}C]uracil per ml was used. DNA was also estimated by the colorimetric method of Burton (2).

Genetic transformation. Competent cell cultures of *B. subtilis* 168 *trpC2* were prepared by the method of Bott and Wilson (1). Purified donor DNA (10 μg) (23) from strain CO169 was mixed with 1 ml of competent cells for 30 min at 37°C. Cells were then collected by centrifugation, washed with 5 ml of Penassay broth (PAB), and resuspended in 10 ml of the same medium. Two generations of growth were then permitted to obtain marker segregation. Cells were concentrated by centrifugation and spread on LB plates containing 5 μg of novobiocin per ml.

TABLE 1. *Strains used*

Strain	Genotype	Source
B. subtilis 168	*trpC2*	Our stock
B. subtilis ANS-1	Spontaneous *trp*$^+$ revertant of 168	Our stock
B. subtilis BD54	*leuA8 metB5 ile-1*	D. Dubnau
B. subtilis BD43 *dnaC*	*metB5 ile-1 dnaC30*	D. Karamata (19)
B. subtilis CO169	*trpC2 thyA thyB novA1*	N. Sueoka (16)
B. subtilis 168 *novA*	*trpC2 novA1*	Our stock
B. subtilis BC50	*ade-16 leuA8 metB5 nic*	J. Copeland
B. licheniformis ATCC 8480		American Type Culture Collection
Phage AR-9		A. A. Prozorov via D. Dubnau

Transduction. Transduction was used to ascertain the genetic location of the *nov* marker transferred into strain 168. AR-9 phage was titrated by infection of 0.1 ml of a *Bacillus licheniformis* culture in PAB (at a cell density of 60 to 80 Klett units). After 10 min at 37°C, the suspension was spread onto M agar plates (34) with a 2.5-ml soft agar overlay. Distinct plaques appeared after about 20 h of incubation at 37°C.

Transducing lysates were obtained by infecting 0.1 ml of the donor strain culture containing the *nov* marker (5×10^8 cells per ml) with phage at a multiplicity of infection of 1 and allowing 10 min of incubation before transfer of the mixture to an M plate by the soft overlay method. After 20 h at 27°C, the plate was flooded with 5 ml of phage buffer (8), and the top layer of the plate was removed with a sterile spatula. The plate was further rinsed with 5 ml of phage buffer. The collected suspension was centrifuged, and the agar particles were discarded. The resultant supernatant fraction was filtered through a membrane filter (Millipore Corp.; type HA, 0.45-μm pore diameter). Titers of phage stocks varied from 5×10^9 to 5×10^{11} plaque-forming units per ml.

Transduction was performed by inoculating a culture of the recipient strain BC50 in PAB at 20 Klett units, followed by 5 h of incubation at 37°C with rotary agitation. At this time cultures displayed cells of high motility and were then infected with the transducing phage at a multiplicity of infection of 1. This was followed by gentle agitation for 15 min. Cells were collected by centrifugation, washed with mineral salts medium (26), and plated on selective medium.

Chemicals. Novobiocin and nalidixic acid were purchased from Sigma Chemical Co. Rifampin was a product of Schwarz/Mann. [CH_3-^{3}H]thymidine (6.7 Ci/mmol) and [2-^{14}C]uracil (57 mCi/mmol) were obtained from New England Nuclear Corp.

RESULTS

Effect of novobiocin on spore outgrowth. The presence of 4.6 μg of novobiocin per ml had no effect on the germination of *B. subtilis* spores but completely inhibited their outgrowth (Fig. 1A). The same concentration of the antibiotic, when introduced at a midpoint during exponential cell multiplication, displayed a marginal effect on the increase of turbidity during one generation time (about 20 min) (Fig. 1B). The first cycle of DNA replication in the outgrowing cell system was completely suppressed by novobiocin (Fig. 1C). Its addition to a vegetative cell culture brought an immediate inhibition of DNA synthesis (Fig. 1D). Similar experiments revealed that 1.7 μg of novobiocin per ml had no effect on spore outgrowth (data not shown).

Outgrowth is not dependent on DNA replication. Ginsberg and Keynan (15) demonstrated that the outgrowth process occurred almost normally in mutants conditional for DNA replication under nonpermissive conditions. Spores of a thymidine-dependent mutant were also capable of outgrowth in the absence of thymidine (15). Both of these experiments were performed in defined media. The same results were obtained in rich medium. Germination kinetics and the time of cell emergence were the same for a mutant temperature sensitive for DNA synthesis and its isogenic parent (Fig. 2A and B). Novobiocin prevented outgrowth in both cultures. Thus, novobiocin sensitivity was not dependent upon DNA replication.

Time of escape from inhibition of outgrowth by novobiocin. Addition of the antibiotic 25 min after the initiation of germination still prevented subsequent increase in cell mass (Fig. 3). Germination was well completed by 20 min. Loss of sensitivity of the system to the addition of novobiocin began to occur at 60 min, approximately coincident with the time of initiation of DNA replication (Fig. 1C), implying that replication released the germinated spore from novobiocin inhibition.

RNA synthesis in the presence of novobiocin. Addition of the antibiotic at 25 min of outgrowth resulted in a 63% decrease in accumulation of labeled RNA within the next 35 min (Fig. 4A). Exposure of exponentially growing cells to novobiocin reduced RNA synthesis by essentially the same degree (Fig. 4B). Rifampin (8 μg/ml) immediately inhibited RNA synthesis in both cultures when introduced at the same time as novobiocin (data not shown).

Novobiocin resistance mutation removed the novobiocin effect during outgrowth.

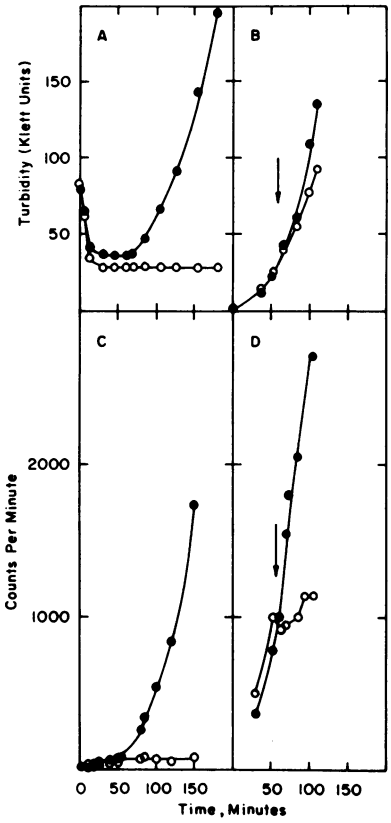

FIG. 1. *Effect of novobiocin on cell mass and DNA synthesis of outgrowing cells and exponentially growing cells of B. subtilis strain ANS-1 at 37°C. Spores (A and C) were suspended in PAB containing [^{3}H]thymidine with (○) and without (●) 4.6 μg of novobiocin per ml. Exponentially growing cells are shown in (B) and (D). At 55 min (indicated by the arrows) one culture (○) received novobiocin; the other culture (●) served as a control.*

The *novA1* marker from strain CO169 was transduced into *B. subtilis* 168. The antibiotic resistance mutation in the latter strain was mapped and shown to display 15% cotransduction (51/305) to the *purA* mutation. This value compares well with other reported *novA/purA* cotransduction frequencies (14 to 18%) (16). Spores of strain 168 *novA1*, when exposed to novobiocin from the beginning of the induction of germination, synthesized DNA normally and displayed normal outgrowth kinetics (Fig. 5B). Outgrowth and DNA synthesis of the parental isogenic strain were completely sensitive to the drug (Fig. 5A).

Partial outgrowth occurred in the presence of nalidixic acid. Nalidixic acid allowed partial outgrowth in the absence of detectable DNA synthesis in strain 168, as well as in its corresponding *nov* mutant (Fig. 5A and B).

Sporulation occurred normally in the presence of novobiocin. A number of independent studies have determined that chromosome replication is completed within 2 h after logarithmic growth ceases (10, 21) and that chromosome termination is a prerequisite for the completion of sporulation (10, 21, 22). Novobiocin added after the last round of DNA replication had no effect on the final sporulation level observed either in the parental strain or in its novobiocin-resistant derivative (data not shown).

DISCUSSION

Early outgrowth of bacterial spores includes a period during which RNA and protein syntheses occur in the absence of gene replication. Furthermore, outgrowth can proceed during artificial inhibition of the first cycle of DNA replication. The lack of dependence of outgrowth on concomitant DNA synthesis led to the conclusion that inhibition of outgrowth by novobiocin cannot be due to inhibition of DNA synthesis per se.

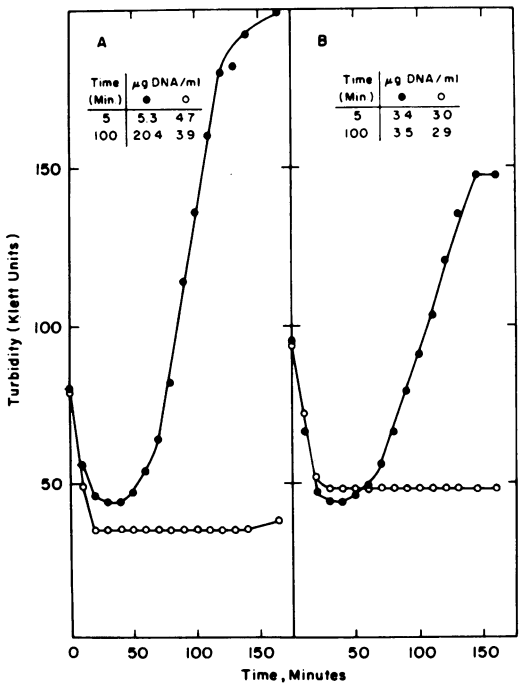

FIG. 2. *Effect of novobiocin on outgrowth of a mutant temperature sensitive in initiation of DNA replication and of the parental strain. Spores of BD54 (A) and BD43 dnaC (B) were suspended in PAB with (○) and without (●) 4.6 μg of novobiocin per ml, and outgrowth was followed at 48°C. The table inserts present DNA content as determined on the cultures by the Burton colorimetric method (2).*

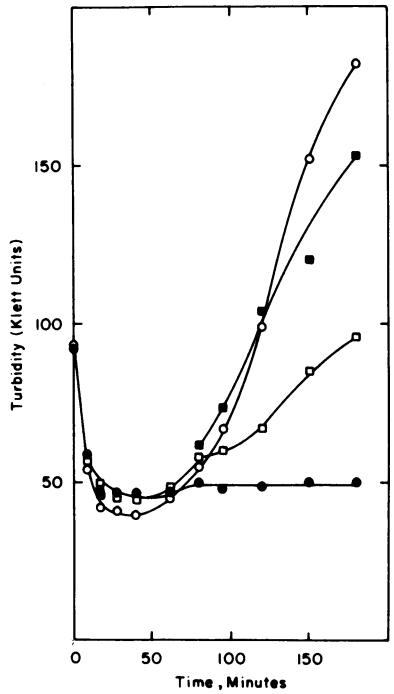

FIG. 3. *Escape from novobiocin inhibition of outgrowth. Strain ANS-1 spores were suspended in PAB and incubated at 37°C. No novobiocin added* (○). *Novobiocin (4.6 μg/ml) was added at 25 min* (●), *60 min* (□), *and 110 min* (■).

Many studies on the control of outgrowth have revealed that the genome is not transcribed randomly and that enzymes appear in an ordered manner during progression of the spore toward a vegetative cell (for a review, see reference 20). There is some evidence that spore outgrowth may represent a unique developmental stage with functions specific to it. Conditional outgrowth mutants have been described by Galizzi et al. (12) to possess normal vegetative growth at the nonpermissive condition in rich medium. These mutations are distributed throughout the chromosome (12). Dawes and Halvorson (7) isolated a collection of temperature-sensitive mutants, blocked at different stages during outgrowth and defective in vegetative growth at the restrictive temperature. These mutants were obtained by nitrosoguanidine and could suffer from multiple mutations.

Analyses of nonribosomal RNA synthesized during outgrowth demonstrated that certain RNA transcripts in *Bacillus cereus* are absent or are present in lower concentrations in vegetative cells (30). More recent hybridization competition experiments show that early in the outgrowth period there are RNA species transcribed from the heavy DNA strand which are unique and undetectable during logarithmic growth (R. Rudner, personal communication).

A probable candidate for the site of action of novobiocin in our studies is DNA gyrase, which catalyzes the ATP-dependent introduction of negative superhelical twists into the chromosome (9, 13). This enzyme has been implicated in replication (for a review, see reference 5), recombination (25), and repair (17) of viral and bacterial DNA. The purified enzyme from *E. coli* is inhibited by nalidixic acid (18) and novobiocin (14) and has been shown to be composed of two subunits, one the product of the gene

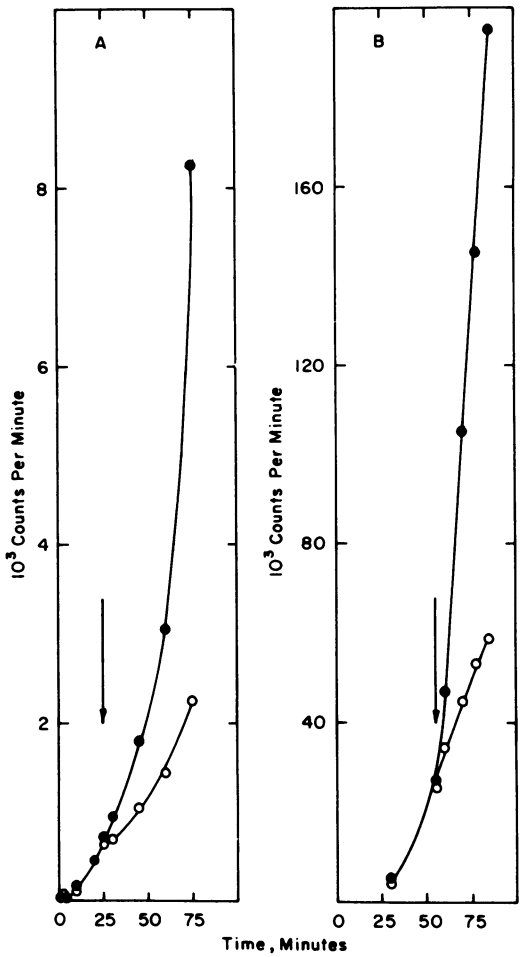

FIG. 4. *Novobiocin effect on RNA synthesis during spore outgrowth and exponential growth of strain ANS-1. Two parallel cultures of spores and vegetative cells were started in PAB containing [^{14}C]uracil at 37°C. Arrows indicate the time of addition of 4.6 μg of novobiocin per ml to one of the cultures* (○); *the other received no novobiocin* (●). *The optical density profile for (A) is shown in Fig. 3, and that for (B) is shown in Fig. 1B.*

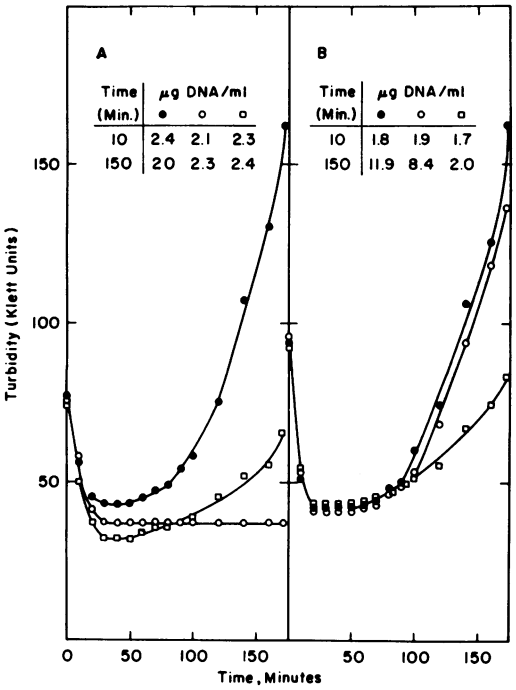

FIG. 5. *Novobiocin and nalidixic acid effect on outgrowth of strains 168 and 168 novA. Spores of strain 168 (A) and strain 168 novA (B) were suspended in PAB supplemented with tryptophan, with* (○) *and without* (●) *4.8 μg of novobiocin per ml or with* (□) *10 μg of nalidixic acid per ml, and incubated at 37°C.*

controlling sensitivity to the first drug and the other the product of an unlinked gene controlling sensitivity to novobiocin (18). Relevant to our experiments are the reports that reveal the role of DNA gyrase in the process of transcription in *E. coli* (11, 27, 29, 32). Recently, Smith et al. (31) demonstrated that nalidixic acid, novobiocin, and coumermycin impair phage promotor-dependent read-through transcription of the *trp* operon in φ80p*trp* but not RNA transcription initiated from the promotor internal to the *trp* operon itself.

Whereas the addition of novobiocin to an exponentially growing culture of *B. subtilis* had only a slight effect on the increase in cell mass (Fig. 1B), it had a greater effect on the initial rate of RNA synthesis (Fig. 4B). In contrast, when novobiocin was added to outgrowing spores, it completely inhibited the increase in turbidity (Fig. 1A), whereas its effect on RNA synthesis (Fig. 4A) was similar to that observed with exponentially growing cells. The effect of novobiocin on the increase in cell mass suggests that outgrowth involves novobiocin-sensitive reactions that are absent in vegetatively growing cells.

Unexplained was the observation that partial outgrowth occurred in the presence of nalidixic acid and in the absence of detectable DNA synthesis (Fig. 5). Nalidixic acid is known to induce new proteins in *E. coli* (4). If the mode of action of nalidixic acid was similar in *B. subtilis*, the synthesis of some proteins in the presence of the antibiotic may be sufficient to support partial outgrowth.

We have not been successful in detecting DNA gyrase in vitro in *B. subtilis* with the purpose of confirming that the 168 *novA* strain contains a mutation in the structural gene of one of the subunits of the gyrase. Nevertheless, *novA* is located very close to a mutation controlling nalidixic acid resistance in *B. subtilis* (3), which encourages the speculation that in this organism the genes coding for the two subunits of the DNA gyrase are closely linked. Alternatively, the *novA* mutation might result from a permeability property preventing the entrance of the antibiotic into the cell. The latter is not an improbable occurrence given the reported existence of other *nov* markers not linked to the *ade-16* mutation (16).

Our observations suggest a role of the enzyme DNA gyrase in gene expression during cell emergence before the first round of DNA replication. This does not seem to be the case during sporulation.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant PCM 76-11693 and Public Health Service grant GM18904 from the National Institute of General Medical Sciences.

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