ELIMINATION OF A GENETIC DETERMINANT FOR SPORULATION OF *BACILLUS SUBTILIS* WITH ACRIFLAVIN

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With evidence for at least three genetic loci (Spizizen, 1961), bacterial sporulation can be visualized as an intricate metabolic process involving diverse genetic determinants. We wish to communicate some experimental data which suggest the involvement of a cytoplasmic factor as one or more of these genetic determinants. A concept postulating the existence of genetic elements having the properties of episomes for sporulation of *Bacillus* has already been made (Jacob, Schaeffer and Wollman, 1960). A recent characteristic of the episomic state is its elimination in the autonomous state through acridine treatment. Acridines interact specifically with DNA (Lerman, 1963) and interfere with the self replication of intracellular structures containing nucleic acids. Such treatment allows a distinction between nuclear and extra nuclear genetic structures (Jacob and Wollman, 1961). Acridines can eliminate the sex factor in *E. coli* F^+ types while they are ineffective on Hfr types (Hirota, 1960) showing that they interfere with the autonomous episomic state but do not eliminate these same determinants in the integrated state.

Methods

Spizizen Minimal Medium (SMM) modified by the addition of 0.02% peptone and adjusted to pH 7.6 was used in the present

experiments. Neutral acriflavin was dissolved in distilled H_2O at 500 μg/ml and autoclaved. Each Klett flask containing modified SMM was inoculated with 0.4 ml of a 9 hour culture of B. subtilis strain Marburg grown in 1/2 strength Brain Heart Infusion broth at 37° C. The inoculated flasks consisted of approximately 10^6 viable cells per ml and were grown with shaking at an incubation temperature of 45° C. Acriflavin was added to flasks during various parts of the cells' growth curve to a final concentration of 6 μg/ml. At 24 hours or at shortly later times allowing for good growth in flasks containing acriflavin, cells were diluted and streaked on potato dextrose agar fortified with $MnSO_4$ and incubated at 37° C for 5 days. Spore forming colonies are opaque and dark brown in color. Asporogenic mutant colonies are transparent and white in color while "abortive" colonies appear midway between the wild type and mutant since sporulation goes to completion in only a very small proportion of these latter type bacteria.

Results

Acriflavin treated and control cells yielded the results recorded in Table 1. Colonies were identified by their appearance as described in the methods and verified through observation under the phase contrast microscope. The presented data represent total observed colonies compiled from several experiments. One experiment representing a specific point of the early log phase of growth yielded an average of 33 per cent asporogenic mutants. Samples of these mutants have maintained their stability through fourteen transfers and are being checked for transformability with wild type DNA. Since early growth yielded the most significant results, these periods became the main area of concentration in the present, incipient stages of this study. These findings suggest the presence

TABLE 1

The Response of B. subtilis to Acriflavin at Various Stages of Growth

Growth in flasks 24 hr after acriflavin addition	Stage of growth at the time of addition of acriflavin*	Total No. of colonies examined	No. of "abortive" colonies	No. of asporogenic mutant colonies	% "abortive" colonies	% asporogenic mutant colonies
Good growth	Early lag	868	868	0	100	0
	Late lag	763	583	0	76.3	0
	Early log	2,189	487	171	22.2	7.8
	Mid log	319	0	2	0	0.6
	Late log	300	0	3	0	1.0
	Stationary	242	0	0	0	0
	Untreated control	1,996	0	1	0	0.05
No noticeable growth	Early lag	1,505	2	0	0.13	0
	Late lag	343	0	0	0	0

* Acriflavin final concentration, 6 μg/ml.

of an autonomous genetic determinant for sporulation during early stages of growth which becomes integrated somewhere in the log phase of growth. Table 1 also indicates that if acriflavin eliminates any cytoplasmic determinant's function, we must assume that growth, possibly essential for chromosomal detachment or cytoplasmic replication of the determinant, is necessary for the effect. As a result of permeability to the toxic mutagen at early growth periods, much difficulty was encountered in inducing further growth after acriflavin addition. In other cases abundant lysis was observed; yet, only where this growth occurred in lag and early log phases did a good number of resulting cells appear as asporogenic and "abortive" forms.

Discussion

The phenomenon may be due to a mutagenic action of acriflavin giving an increased frequency of mutation varying in its effect due to permeability differences at various stages of growth. Highly stable, proflavin-induced, rII mutants have been reported for T4 bacteriophage (Brenner, Benzer and Barnett, 1958). A more attractive alternative is the episome hypothesis. During the lag phase a genetic determinant detaches from the chromosome and replicates. At this point acriflavin interference with episomic replication or through episomic agglutination (Hirota, 1960) will tend to yield the high percentage of "abortive" spore formers found. True asporogenic mutants may not be observed due to the prevalence of additional complex integrated and chromosomal sporulation determinants, (Schaeffer and Ionesco, 1960). Early log phase can be visualized as the state of initial chromosomal replication and the phase of episomic integration where this genetic determinant can be considered in part unstably attached to the host chromosome and in part autonomous (Watanabe and Okada, 1964). Combined acriflavin interference with episomic integration and with chromosomal

replication producing errors of insertions or deletions of nucleotides (Lerman, 1963) belonging to non episomic genetic sporulation determinants may in combination be responsible for the effect noted in early log (Table 1). The mutants observed at later growth phases may simply be a result of error in chromosomal replication. Owing to the absence of conjugation in Bacillus, we must at this time solely rely on the evidence that a particle in the autonomous state may be eliminated through acridine treatment. Similar evidence for an episome in a non conjugating system has been reported for penicillinase production (Harmon and Baldwin, 1964).

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