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Biochemical Genetics of Bacterial Sporulation

I. Unidirectional Pleiotropic Interactions among Genes Controlling Sporulation in *Bacillus subtilis*

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Summary. Pleiotropic interactions among genes controlling the formation of bacterial spores and of sporulation-associated products are studied. In order to obtain sporulation mutants, spores have been germinated in the presence of chloramphenicol and then treated with nitrosoguanidine. In the most favorable conditions 25% of sporulation mutants have been found among the 40% surviving bacteria. This number is at least four times higher than the number of auxotrophic mutants, therefore a rough estimate of the number of genes involved in sporulation is 800.

Rapid plate-tests have been developed for the oxidation of tetrazolium salts, the formation of various proteolytic enzymes and the production of antibiotics. Although the exact biochemical nature of the products is not yet known, the results suggest that distinct factors, probably various enzymes (including several proteases) are detected by these tests. All of them are associated with spore formation and absent from a large number of sporulation mutants. Using these tests, the phenotypes of 500 randomly selected sporulation mutants were determined. No important differences were found between asporogenous and oligosporogenous mutants. The number of mutants deficient for several sporulation-associated characters is large, pleiotropic interactions following a defined pattern are observed. Statistical analysis indicates the existence of a unidirectional pleiotropic system. All the results agree with the hypothesis of sequential gene activation. Consequently, the sporulation-associated characters can be ordered into a linear sequence, presumably reflecting the consecutive steps in spore formation. The order obtained is the following: gelatinase, proteases acting on casein and on denatured albumin, oxidation of tetrazolium No 7, digestion of protamine, production of antibiotics (against a *Staphylococcus* and a *Bacillus*), hydrolysis of hemoglobin, oxidation of tetrazolium No 2, digestion of native albumin, synthesis of elastase. Another category of mutants, blocked in a late step of sporulation and apparently derepressed for the formation of elastase, is also described.

In conclusion, arguments are put forward in favor of sequential gene activation. Sporulation genes, related by unidirectional pleiotropic interactions, form a sporulon. Generalization of this concept to other differentiating systems (a differon), its predictions and possible experimental confirmation are considered.

Introduction

Spore formation in Bacilli involves a sequence of morphological and biochemical changes (MURRELL, 1967). The object of this paper is to describe some of the events associated with spore formation and to analyze the pleiotropic interactions among sporulation genes. This was accomplished in three steps. First, a large

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collection of sporulation mutants[1] was constituted. In this respect, the induction of sporulation mutants by nitrosoguanidine was studied in some detail. Next, rapid agar-plate tests have been developed for several characters associated with spore formation. The nature and possible physiological significance of these characters will be briefly discussed. Finally, using the mutant collection and the available plate-tests, the pleiotropic effects of sporulation genes have been demonstrated and their pattern analyzed. This lead to the conclusion that most or all the sporulation genes are parts of a genetic control system which manifests itself by unidirectional pleiotropic interactions.

Sporulation mutants of *B. subtilis* have been obtained in several laboratories spontaneously (SCHAEFFER, 1967), after UV irradiation (SCHAEFFER, 1967), heat treatment (NORTHROP and SLEPECKY, 1967), or with acridines (BOTT and DAVIDOFF-ABELSON, 1966; ROGOLSKY and SLEPECKY, 1964). Available evidence (SCHAEFFER, 1967; SCHAEFFER, IONESCO, RYTER and BALASSA, 1965; SPIZIZEN, 1965; TAKAHASHI, 1965), obtained by transformation and transduction, indicates the existence of at least twenty sporulation genes. In the present investigation I used the powerful chemical mutagen N-methyl-N′-nitro-N-nitrosoguanidine (NTG) (ADELBERG, MANDEL and CHEN, 1965) to obtain a large collection of sporulation mutants. The high frequency of such mutants facilitated quantitative studies and permitted a critical test of the criterium used to recognize sporulation mutants (white colonies on nutrient broth).

To gain a better understanding of the role of these genes in spore formation, further genetic studies should be preceeded by a phenotypic examination of the various sporulation mutants. It is known from electron microscopic examination that spore formation could be blocked in mutants at any of the six known morphological steps (RYTER, SCHAEFFER and IONESCO, 1966). Electron microscopy, however, is not a sufficiently rapid method for scanning a large number of mutants. Fortunately several biochemical properties have been found associated with sporulation. Among these are the production of antibiotics (BALASSA, IONESCO and SCHAEFFER, 1963; BERNLOHR and NOVELLI, 1963; SPIZIZEN, 1965) and of proteolytic enzymes (BERNLOHR, 1964; BALASSA, 1964a; MICHEL, 1967; SCHAEFFER, 1968; SPIZIZEN, 1965), all suitable for rapid examination by standard microbiological techniques. Although such techniques are currently used in several laboratories for the classification of sporulation mutants, frequently the results

1. Nomenclature and abbreviations: *sporulation mutants* (Sp^M) include all the mutants with altered capacity to sporulate, including both true asporogenous (Sp^-) and oligosporogenous ($Sp^\pm$) mutants.

The following abbreviations will be used for various plate-tests: Ab(St) and Ab(BH)-test for antibiotic activity on *Staphylococcus* and *Bacillus H* indicators; TZ2 and TZ7-oxidation of the tetrazolium salts No 2 and No 7; Gel, Cas, Alb, Protam, Hgl, AlN and El-tests for proteolytic activities using gelatin, casein, denatured albumin, protamine, native hemoglobin, native albumin or elastin as substrate. Pr is used as a generic term for the proteases. The superscripts +, —, $\pm$ indicate the phenotype of sporulation mutants on the designated tests, M includes both — and $\pm$ phenotypes. The superscript D is used for an apparently derepressed proteolytic activity.

The genes in which the mutations occur are not identified in this study, thus all the preceeding symbols indicate the *phenotype* of the sporulation mutants. Thus Sp^M Gel^+ $Cas^\pm$ is a single sporulation mutant with normal activity on a gelatin plate and reduced activity on a casein plate.

are not comparable or contradictory (BALASSA, IONESCO and SCHAEFFER, 1963; SPIZIZEN, 1965; MICHEL, 1967; SCHAEFFER, 1968). Therefore a systematic investigation of the appropriate microbiological methods with a large number of randomly selected sporulation mutants appeared necessary. Since the multiplicity of proteolytic activities is a likely source of difficulties (MICHEL, 1967; SCHAEFFER, 1968), an attempt was made to differentiate proteolytic activities with the help of various proteins as substrates. As a result, tests have been developed for the characterization, on agar plates, of two antibiotic activities and at least six apparently distinct proteolytic activities. All these activities are definitely associated with sporulation. When *Bacillus subtilis* undergoes *en masse* sporulation, the oxygen consumption of the bacteria and the specific activity of the particle-bound NADH-oxidase increase several times (SZULMAJSTER, 1964). Since these changes occur normally two or three hours after the onset of sporulation and fail to occur in early sporulation mutants (SZULMAJSTER, 1964), they are likely to be related to an intermediate step in spore formation. Such observations suggested to investigate several tetrazolium salts as possible indicators of respiratory activities. Two of these compounds have been used with success.

In an attempt to characterize the phenotypes of various sporulation mutants, several hundreds of them have been submitted to tests for the production of antibiotics, the appearance of proteolytic activities and the oxidation of tetrazolium salts. The results indicate that several biochemical characters are associated with spore formation in *B. subtilis*. They are altered or missing in many sporulation mutants. A very large number of phenotypes, many of them with multiple deficiencies, were found among bacteria carrying single mutations in a sporulation gene. In addition, in several cases it was proved by transformation that mutations in any one of several genes could lead to the same phenotypic defect (see also SCHAEFFER, IONESCO, RYTER and BALASSA, 1963; SPIZIZEN, 1965; SCHAEFFER, 1968). Such results indicate that sporulation genes have pleiotropic effects on several phenotypic traits.

Similar conclusions have been reached by previous studies (BALASSA, IONESCO and SCHAEFFER, 1963; SCHAEFFER, 1968) which showed that the production of at least one of the antibiotics and one of the proteases are associated with early events of spore formation, occurring before the bacteria reach the morphological step I.

The explanation offered, implicity or explicity, for such pleiotropic interactions is that sporulation consists of a linear sequence of successive events, possibly under sequential genetic control (HALVORSON, 1965; MURRELL, 1967; SCHAEFFER, 1968). The formation of several sporulation-associated products is, following this hypothesis, related to different points of the sporulation sequence; if a mutated gene is involved in sporulation before such a point, the mutant will be negative for the substance in question; if the gene is involved later, the mutant will be positive.

This hypothesis of sequential gene activation leads to two predictions: 1) the distribution of multiple biochemical deficiencies among the mutants should not be at random; various characters could be ordered as "early" or "late"; 2) each sporulation gene (or, at least, many of them) serves as a regulatory gene controlling the activity of "later" genes. The main purpose of the present paper

is to analyze the observations made with several hundred randomly selected sporulation mutants in terms of this hypothesis and to present further evidence for the role of regulatory genes in sporulation. Phenotypic analysis of the mutants leads to the ordering of the sporulation-associated characters and to the establishment of unidirectional pleiotropic interactions among the sporulation genes.

Materials and Methods

I. Bacteria and Chemicals

Strains. The following two strains are derived from the Marburg strain of *Bacillus subtilis:* 1) SA3, met$^-$thr$^-$leu$^-$ (Yoshikawa and Sueoka, 1963), from Dr. N. Sueoka; 2) SB5, try$^-$his$^-$ur$^-$ (Ganesan and Lederberg, 1964), from Dr. E. Lederberg. A his$^+$ revertant, SB5—2, of the latter has been used in most experiments.

Media. The solid sporulation medium "DN", modified from Schaeffer (Schaeffer, Ionesco, Ryter and Balassa, 1965), has the following composition: Difco Nutrient Broth 4 g, KCl 0.5 g, glutamic acid 50 mg/l, glucose 0.01%, $MgSO_4$ $5 \cdot 10^{-5}$ M, $CaCl_2$ $5 \cdot 10^{-3}$ M, $FeCl_2$ $5 \cdot 10^{-6}$ M, Bactoagar 2.2%; pH adjusted to 7.4. The liquid medium is Schaeffer's nutrient broth (D-medium). For germination, the same medium is used with 0.5% of glucose.

The soft agar, used in overlays, contains 0.65% of Bactoagar in DN medium or in distilled water.

The basic synthetic medium contains: potassium phosphate buffer at pH 6.2, 0.1 M; $(NH_4)_2SO_4$, 0.2%; sodium citrate, 0.2% and bivalent metals as in Schaeffer's medium. The agar concentration of the solid medium is 1.5%. The medium is supplemented with glucose (0.5%) and the required amino acids (20 μg/ml each) for the minimal medium or with glucose (0.5%) and casamino acids (0.2%) for the "GCA" medium. (The latter medium is similar to Spizizen's synthetic medium (Spizizen, 1958)).

Dilutions of bacteria are carried out in a buffer consisting of the basic medium without $(NH_4)_2SO_4$ but supplemented with glucose (0.5%).

Cultures. The incubation temperature is, when not specified otherwise, 37° C. Liquid cultures are incubated in a Metabolyte gyratory shaker (New Brunswick Co., New Brunswick, N.J.) at 200 tours/mn.

Spore stocks were prepared by incubating bacteria for 24 hours at 45° C. The spores were washed in distilled water and heated for 1 hr at 60° C and for 10 min at 80° C. The germination medium was inoculated with 10^7—10^8 spores/ml.

Chemicals. Solutions of NTG (N-methyl-N'-nitro-N-nitrosoguanidine, Aldrich) were freshly prepared in 0.1 M acetate buffer at pH 5.0 and used without sterilization.

EMS (ethyl methyl sulfonate) and chloramphenicol were obtained respectively from Eastman Organic Chemicals and from Parke, Davis and Co.

Replication. An aluminum block with 25 blunt aluminum needles was used.

II. Tetrazolium Tests

Sporulation mutants were transferred with a replicator block to DN nutrient agar plates and unless otherwise indicated, incubated at 37° for 4 days. The tetrazolium salts listed in Table 5 have been obtained from Nutritional Biochemicals Co. (Cleveland, Ohio). Stock solutions at a concentration of 2 mg/ml were kept in the dark at 4° C. 2.5 ml of the stock solution was added to 100 ml of soft agar at 50°, and 10 ml of the soft agar was poured to cover the colonies of each plate. The development of coloration was observed after 30 min at room temperature or after overnight incubation at 30°.

III. Tests for Enzyme Activities

Several proteins known as substrates for specific proteolytic enzymes were selected for their commercial availability. Insoluble and denatured proteins were mixed with soft agar and used in the overlay. Soluble proteins were incorporated directly into the soft agar and enzyme activity was observed after "developing" the plates by flooding with 10 ml of a precipitating solution ("Hg-sol"): $HgCl_2$ 4% in HCl 10%.

Since it was expected *a priori* that only a small proportion of the sporulation mutants will be different from wild type in certain tests, 50 randomly chosen sporulation mutants have been replicated in preliminary experiments for the development of the methods. After standardization, the tests have been repeatedly performed on more than 500 mutants. Halos were observed on a colony-counter, with direct or lateral illumination. As a rule, a mutant was considered positive if a normal size halo appeared at the same time as with wild type colonies, and negative if there was no halo even with prolonged incubation. Small halos or delayed activity were scored $\pm$. Since the "kitchen-work" involved in the preparation of each plate is important for reproducible observations, each test will be described separately.

Gelatin (Gel). A stock solution of 5% gelatin (Nutritional Biochemicals Co., Cleveland, Ohio) is heated to 80°C and diluted 5 times in soft agar. After overnight incubation the gelatin is precipitated with "Hg-sol".

Denatured Albumin (Alb). 1.5 ml of a stock solution of sterile-filtered 10% albumin (bovine, fraction V, Calbiochem, Los Angeles) is mixed with 100 ml of melted soft agar containing 10^{-3} M $MgSO_4$ and autoclaved for 5 min at 120°C. Before pouring the overlay, the precipitate is dispersed by repeated pipetting. Big halos develop after 20—30 hours of incubation.

Casein (Cas). This test is best performed by mixing casein with the bulk DN agar. DN agar is prepared as described, and 5 g/l of casein (Difco) is mixed with the agar before autoclaving. On the resulting turbid plates the proteolytic activity becomes manifest after 1—1.5 days as a transparent halo of lysis around the colonies, surrounded by a dense white ring of precipitated casein.

Protamine (Protam). A 10% solution of protamine sulfate (Calbiochem, Los Angeles) is autoclaved and diluted 20 times in melted soft-agar. After 3 days of incubation, the plates are precipitated with "Hg-sol". (The halos are less clear than in other tests, therefore the results are less reproducible and $\pm$ colonies often not recognized.)

Hemoglobin (Hgl). A 10% solution of hemoglobin (Difco) is centrifuged twice to discard the insoluble residue, sterilized by filtration and diluted five times in soft agar at 50°C. Lysis is observed after 3 or 4 days, and becomes more apparent when the plates are flooded with "Hg-sol".

Native Albumin (AlN). One liter of DN agar at 50°C is supplemented with 60 ml sterile-filtered 10% undenatured albumin. The plates are transparent. After 5 days of incubation the albumin is precipitated by "Hg-sol". The halos seem to be restricted to the immediate vicinity of the colonies and are best observed after removing the colonies with a tooth-pick. (Reading of this test requires some practice, and the identification of $\pm$ colonies remains uncertain.)

Elastin (El). Partially purified elastin from calf embryo ligaments or from 9 year old bovine ligament nuchae was obtained from Dr. L. SANDBERG (Biology Division, Caltech). A 5% suspension of the elastin (reduced to fine powder) is autoclaved and diluted ten times in soft agar. The overlayed plates are checked daily for dissolution of the elastin granules. Positive colonies are surrounded with a halo after 5—7 days; with negative mutants, the insoluble elastin granules remain visible around and under the colonies after an incubation of 7—10 days.

Amylase (Amy) was detected on plates overlayed with soluble amydon (Malinckrodt, 0.25%, autoclaved) and developed after overnight incubation by adding 5 ml of a 0.02% alcoholic iodine solution.

IV. Antibiotic Production

The indicator strains for antibiotic assay were *Staphylococcus aureus* ATCC 1057, from the American Type Culture Collection, and *Bacillus H* from Dr. J. SPIZIZEN (Scripps Institute for Biological Research, La Jolla, Calif.). Activity against *Staphylococcus (Ab(St))* was tested as described previously (BALASSA, IONESCO and SCHAEFFER, 1963), by overlaying a plate with 10 ml of DN soft agar, containing a 10^3—10^5 times dilution of an overnight *Staphylococcus* culture, and then replicating onto it the *B. subtilis* mutants. Plates prepared with a 100 times dilution of *Bacillus H* were used to detect activity against this strain *(Ab(BH))*. Antibiosis halos developed after overnight incubation. (Since the detection of activity in both cases depends strongly on the relative growth rate of the producer and

indicator bacteria and on the density and physiological state of the indicator culture, results are much less reproducible than with enzyme tests. Each test was therefore repeated at least four times. Inoculation of the plates with drops of liquid culture of the mutants gives more clear-cut and reproducible results, and the phenotypes of certain mutants have been confirmed in this way.)

V. Transformation

This was performed by the method of Anagnostopoulos and Spizizen (1961), using crude phenol-treated DNA preparations. Sp^+ transformants have been selected indirectly by a two-step method: uracil-or tryptophan-independent transformants have been first selected on a glucose-casamino acid medium (GCA) and then replicated to ascertain their properties concerning sporulation and exoenzyme formation.

VI. Numerical Analysis

Correlations between various properties of sporulation mutants were established with the help of a computer, programmed in Fortran IV.

Results

I. Production of Sporulation Mutants

1. High Mutation Rate Induced by NTG

In *Escherichia coli*, the highest mutagenic and the lowest lethal effect are yielded by NTG if DNA synthesis is blocked (Adelberg, Mandel and Chen, 1965). Since the onset of DNA synthesis can be completely prevented after germination if chloramphenicol is present (Balassa and Contesse, 1965), the following conditions of NTG treatment have been developed for highly efficient production of sporulation mutants: 10^7—10^8 spores/ml were incubated in the germination medium containing 100 μg/ml chloramphenicol. 90—99% of the spores germinated after 1 hr. NTG was then added to the cultures and mutagenesis terminated by dilution in cold buffer. Finally the bacteria were plated on DN agar. Since colonies of sporulation mutants remain white, in contrast to the brown colonies of the wild type (Schaeffer, Ionesco, Ryter and Balassa, 1965), the number of white colonies was determined after three days of incubation at 30 or 37° C As shown in Table 1, extremely high frequencies of sporulation mutants can be obtained reproducibly in these conditions with little lethal effect.

Table 2 shows the kinetics of mutagenesis in one experiment. The number of mutants increases with the time of treatment up to 25—30% of the surviving cells. This saturation value is attained when more than 10% of the cells survive. (After more prolonged treatment the surviving bacteria are mostly composed of ungerminated spores.)

Variations of the optimal NTG concentration and of the treatment time may be attributed to the instability of the mutagen. Similar results were obtained if the germinated spores were centrifuged and resuspended in a pH 6.0 buffer before adding NTG. (Lower pH kills the cells.) A higher temperature of incubation, 46° C, does not change the results significantly.

Although the best results have been obtained with germinating spores, treatment of exponentially growing or of sporulating cells with NTG also leads to sporulation mutants. Such conditions however, as well as another chemical mutagen, ethyl methyl sulfonate, were less efficient. Finally, it was noticed

Table 1. *Induction of sporulation mutants by NTG*

Expt.	NTG (μg/ml)	Time of treatment	Survival of cells (%)	Sporulation mutants at 37° C (%)	Sporulation mutants at 30° C (%)
A	50	1 hr	30	20	
	500	1 hr	10	20	
B	40	1 hr	38	25	18
	40	1 hr at 46° C	12	27	20
C	50	10 min	12	27	
D[a]	300	30 min	2	13	

Germination medium, containing 100 μg/ml chloramphenicol and inoculated with 10^7—10^8 spores/ml was incubated at 37 °C for 1 hr, then NTG was added to the cultures. After the time indicated the cultures were diluted and plated on DN-agar. The plates were incubated at 30 or 37° C. White colonies were counted as sporulation mutants. In all cases the figures are based on several hundred colonies.

Strains used: Exp. A-SB5; Exp. B and C-SA3; Exp. D-SB5-2.

[a] In the experiment D, bacteria in stationary phase have been submitted to the mutagen one hour after growth stopped.

Table 2. *Kinetics of NTG-induced mutagenesis*

Time of treatment (min)	Survival (%)	Sporulation mutants (37° C) (%)	Time of treatment (min)	Survival (%)	Sporulation mutants (37° C) (%)
0	100	< 0.1	10	12	27
0[a]	100	5[a]	20	2.4	25
5	40	16	40	1	5[b]

Germinated spores were treated with 50 μg/ml of NTG. Experimental conditions as in Table 1 (Experiment C).

[a] Dilution immediately *after* addition of NTG.

[b] No correction was made for the ungerminated spores.

that germinated spores, treated with NTG, never give segmented colonies; white-and-brown-segmented colonies were sometimes observed when growing or sporulating bacteria were treated with NTG.

2. General Characterization of the Sporulation Mutants

The great majority of the mutants develop colonies of normal size, both on DN-agar and on synthetic media. Some of them have a tendency for autolysis after growth is completed. They all reach the same optical density in D-medium and raise the pH of the DN-agar after growth to 8.0 as does the wild type. A striking characteristic of the mutants is the variety in the morphology of the colony types, with respect to their rough or smooth surface, rhizoid form, etc. With few exceptions, the mutants conserved all the biochemical markers of the original strain. No abnormal reversion rates or segregation pattern have been observed; most mutants are stable. Several mutants form small colonies, appear to be slowly growing and have been discarded.

Two kinds of sporulation mutants are known: asporogenous (Sp^-) mutants, unable to form any spores, and oligosporogenous ($Sp^{\pm}$) mutants, with a genetically determined sporulation frequency, from 10% to 10^{-7} or less (SCHAEFFER, IONESCO, RYTER and BALASSA, 1965). A crude indication of the sporulation frequency is the color of the colony: mutants with high sporulation frequencies are often of a more or less pronounced yellow color. About half of the mutants described here produce yellow colonies.

True asporogenous mutants can be recognized by the chloroform-replica technique (BALASSA, IONESCO and SCHAEFFER, 1963): 3-day old DN-agar plates are exposed for 1 hr to chloroform vapors, then replicated with a velvet: Sp^- mutants are killed, while spores of $Sp^{\pm}$ mutants are transfered to the replica. Using this technique only 10—15% of the sporulation mutants appear Sp^- (Table 3). Measurement of the number of spores found in liquid cultures after

Table 3. *Frequency of Sp^- mutants*

Expt.	Number of Sp^M colonies	Sp^- mutants	
B	376	61	16%
C	213	32	15%
D	266	29	11%

The number of true asporogenous mutants among Sp^M mutants was measured by the chloroform replica technique.

48 hr of incubation in D-medium at 30° C shows a broad spectrum of sporulation frequencies (from 0 to 10^8 spores/ml).

The number of white colonies among NTG-treated bacteria is considerably lower if the plates are incubated at 30° C instead of 37° C (Table 1). When incubated at 30, 37 or 42° C, many mutants exhibit temperature-sensitive coloration. With such mutants, the number of spores in liquid cultures might be 10 to 10^5 times higher at 30° C than at 37° C.

3. Validity of the Selection of Sporulation Mutants

The procedure of counting and isolating sporulation mutants was based on the color of the colonies on DN-medium. Since Schaeffer's original observation (see SCHAEFFER, IONESCO, RYTER and BALASSA, 1965) this method, although used in several laboratories, has not been submitted to a critical test. I investigated therefore on one hand its efficiency and reproducibility and, on the other hand, the assumption that *all* sporulation mutants form white colonies on DN-agar.

The coloration of the colonies is reproducible and the number of white colonies measured either on the original plates or after isolation of random colonies is the same. (Rare "albino" mutants, white but sporulating normally, have been described (SCHAEFFER, IONESCO, RYTER and BALASSA, 1965) but were not encountered here.)

To look for asporogenous mutants with normal pigmentation, more than 300 brown colonies have been isolated in an experiment where the frequency of white sporulation mutants was over 20%. The chloroform-replica technique

failed to detect any Sp⁻ mutant among these clones. If pigment-forming asporogenous mutants exist, they are thus at least 20 times rarer than white sporulation mutants.

Comparison of the DN-agar with other media which have been sometimes used to detect sporulation mutants (BOTT and DAVIDOFF-ABELSON, 1966; SPIZIZEN, 1965) also indicates that Schaeffer's medium permits the isolation of the majority if not all the sporulation mutants (BALASSA, unpublished).

4. Auxotrophic Mutations

In order to compare the frequency of sporulation mutants with the frequency of auxotrophic mutants, the number of colonies which have acquired a new nutritional requirement, was measured. Due to the high mutagenic power of NTG, this could be done without selection: the NTG-treated cells were first plated on DN-agar, then individual colonies were reisolated and replicated on a synthetic medium containing either casamino acids or only the nutrients required by the original strain. Mutants unable to grow on the medium (*AA*⁻ mutants) are auxotrophic, presumably for one of the 17 missing amino acids (the exact requirement was not determined). The results (some of them shown in Table 4) indicate that nutritional mutations can be obtained under the same

Table 4. *Frequency of auxotrophic mutants*

Expt.	Colonies isolated	Sp^+		Sp^M		AA^- Total
		Total	AA^-	Total	AA^-	
B	245	190	5 (2.5%)	55 (22%)	7 (12%)	12 (5%)
D	—	150	4 (2.5%)	150 (12%)	11 (7.5%)	(3%)[a]

Experimental conditions: see Table 1.

In experiment B randomly isolated colonies were replicated, as described in the text, to measure the number of auxotrophic mutants (AA^-) among both Sp^+ and Sp^M cells. In experiment D two sets of colonies have been first isolated, one Sp^+, the other Sp^M, and the number of auxotrophic mutants measured in each set. The frequency of Sp^M mutants was determined independently in this experiment.

[a] Calculated from the preceding figures.

conditions as sporulation mutations. The frequency of sporulation mutants is in both experiments four times higher than the frequency of auxotrophic mutants.

In all experiments 7—15% of the Sp^M mutants acquired simultaneously an amino acid requirement. This number of double mutants (Sp^M AA^-) is considerably higher than expected on the basis of independent mutations, suggesting that the population is heterogeneous with regard to sensitivity of the mutagen. (This population heterogeneity is one of the inconveniences of using germinated spores.) Because of this heterogeneity and since in several experiments the number of sporulation mutants is already at saturation value, our results are not accurate measurements of the ratio of the mutation rates of genes of sporulation and of

amino acid synthesis. In fact, all these factors tend to underestimate the mutation rate of sporulation genes. The conclusion is therefore that the mutation rate toward an alteration in sporogenesis is *at least* 4—5 times as high as the rate of mutations to amino acid requirement. This estimation permits to guess the total number of sporulation genes (see Discussion).

II. Oxidation of Tetrazolium Salts by Sporulation Mutants

Tetrazolium salts (TZ) have been successfully used in the isolation of respiratory mutants in yeast (OGUR, ST. JOHN and NAGAI, 1957), of sugar metabolism mutants in *Escherichia coli* (LEDERBERG, 1948), and in bacterial cytochemistry (VANDERWINKEL and MURRAY, 1962). Since no logical approach seems available at present in the choice of a particular indicator of oxidation-reduction in relation to the actual biochemical defect, I carried out a systematic screening of thirteen TZ derivatives. The following *a priori* considerations have been helpful in the evaluation of the results: 1. No difference is expected between wild type and mutant cells during vegetative growth, thus the screening should be done under conditions of spore formation (e.g. with old colonies). 2. To be useful, an indicator should be oxidized by the wild type and by several but not all the mutants (thus permitting the division of mutants into two classes). 3. If several steps are involved in the change of oxidative metabolism during sporulation, various indicators might allow a differential classification.

Wild type bacteria and fifty randomly chosen sporulation mutants have been incubated on DN nutrient agar for 1, 4, or 7 days at 37° C and then overlayed with TZ-containing soft agar. Thirteen different TZ salts have been tested on young and old plates (Table 5). Six TZ derivatives developed, even with young colonies, little or no color and have been discarded. The remaining seven have been oxidized by one day old colonies to strongly colored formazans. At this stage the wild type and all the mutants showed the same activity. When older colonies (4 to 7 days) were tested, it was found that several of the TZ compounds were oxidized only by a fraction of the mutants (Table 5). Since this was the desired result, two of these derivatives, TZ2 and TZ7 (together with TZ1 as a control) have been submitted to a more detailed investigation with several hundred sporulation mutants, with the following results.

None of the three salts permitted any distinction between young colonies of various mutants. Old colonies (up to 7 days) of the 500 sporulation mutants (with the possible exception of two) all oxidized TZ1. However, when TZ2 or TZ7 were used, differences appeared among the mutants after 4 days of incubation. Longer incubation up to 7 days did not change the results. 40% of the sporulation mutants examined (Table 6) were inactive with TZ2 and 20% show an intermediate phenotype (weak but significant coloration), therefore the total number of mutants with a reduced ability to oxidize TZ2 ($TZ2^M$) attained 60%. Among the mutants obtained in the experiment C only 30% appeared negative with TZ7. These mutants ($TZ7^M$) were all (with two exceptions) also $TZ2^M$. With the help of two TZ compounds, one could therefore divide sporulation mutants into three phenotypic classes: $TZ2^+ TZ7^+$, $TZ2^M TZ7^+$ and $TZ2^M TZ7^M$ (all the three being $TZ1^+$). (Sporulation mutants obtained in the experiment D

Table 5. *Coloration of tetrazolium salts by Bacillus subtilis*

Nb	Tetrazolium salt	Color	Rapidity of coloration[a]	Mutants detected[b]
1	Thiazolyl blue (MTT)	blue	+++	no
2	Triphenyl tetrazolium Cl (TTC)	red	+++	yes
4	Tetrazolium violet	violet	+++	±
7	P-Tolyl tetrazolium red	red	+++	yes
9	Iodonitro tetrazolium violet (INT)	violet	+++	±
11	Neo-tetrazolium (NT)	violet	++	±
12	M-nitro neotetrazolium chloride	violet	+	no
14	Tetrazolium blue (BT)	blue	++	yes
16	M-nitro blue tetrazolium chloride	blue	±	no
18	P-nitro blue tetrazolium chloride	blue	+	no
20	P-anisyl P-nitro blue tetrazolium chloride	blue	±	no
21	Piperonyl tetrazolium blue	blue	±	no
23	Veratryl tetrazolium blue	blue	±	no

[a] Strong color visible after: +++ 30 min; ++ two hours; + overnight incubation; ± only weak color after long incubation.

[b] Yes = several sporulation mutants are clearly distinguished by their coloration on 4 day old plates; ± = mutants distinguished only on 7-day old plates; no = no clear-cut distinction between various mutants.

Table 6. *Oxidation of tetrazolium salts by sporulation mutants*

Exp.	Dye	Number of sporulation mutants tested	Fraction of sporulation mutants with TZ^M phenotype (in %)		
			—	±	total (— and ±)
C	TZ2	211	43	17	60
	TZ7	235	12	17	29
D	TZ2	315	36	24	60
	TZ7	315	36	24	60

TZ^M phenotypes: — = no coloration with tetrazolium dye; ± = weak but significant coloration with tetrazolium dye; + = coloration similar to that with wild type bacteria.

had the same phenotype when tested with TZ2 or TZ7. This difference, attributed to a difference between the original strains, was not further investigated.)

Is the correlation between asporogeny and the lack of ability to reduce certain TZ compounds apparent or real? The large number of $TZ2^-$ mutants among asporogenous mutants makes a fortuitous coincidence rather unlikely. Moreover, oxidation of all three TZ compounds was clearly seen in 200 Sp^+ colonies, isolated from a culture submitted to the same mutagenic treatment (despite the interference of the brown color of the sporulating colonies with the color of the formazans). The complete absence of Sp^+ TZ^M colonies supports the notion of a definite relationship between sporulation and the ability of the bacteria to oxidize, in stationary phase, certain TZ compounds.

III. Exoenzymes and Antibiotics

1. Proteolytic Activities of Wild Type *B. subtilis*

Various native, denatured and insoluble proteins have been used in agar-plate tests for the determination of proteolytic activities. A zone of lysis was observed around *B. subtilis* colonies with all the seven protein substrates used in this investigation. The appearance of large halos suggests that this is due to the activity of exocellular enzymes. This activity does not appear at the same time on different substrates; the following order is observed:

Gel (12 hrs) — Alb and Cas (1 day) — Protam (3 days) — Hgl (4 days) — AlN (5 days) — El (7 days). (Observations made at 37° C, the times indicate the development of a normal size halo.)

Delayed observation of an enzyme activity could be due to late synthesis, slow diffusion or slow action of the enzyme. To eliminate the last two possibilities, the following alternative methods of observation have been used: 1. Instead of substrate-containing plates, the bacteria were first grown on DN nutrient agar, then killed with chloroform vapors or with streptomycin and overlayed with the substrate in soft agar. Halos of enzyme activity were detected in most cases after a few hours, suggesting that diffusion or hydrolysis itself are not the timing factors. 2. In a more quantitative but time-consuming method, small test tubes are filled with nutrient agar containing the substrate and inoculated with bacteria. The extent of lysis is followed daily. It was found for instance that with wild type bacteria Alb is digested at the rate of 12 mm/day.

2. Proteolytic Activities of the Sporulation Mutants

Five hundred sporulation mutants have been tested for proteolytic activity on the various substrates. All the tests have been repeated at least twice. Many mutants developed no activity in one or several tests (Pr^-), others had an intermediate ($Pr^{\pm}$) phenotype. The number of Pr^M mutants varied from one test to another (Table 7). By the frequency of sporulation mutants with altered proteolytic activity, the substrates can be ordered as follows: Gel — Alb, Cas and Protam — Hgl — AlN — El. (In this order, intermediate phenotypes have been combined with negative phenotypes, but considering only the latter, a similar order would arise.) Note the similarity of this order with the timing of enzyme appearance.

In a few cases, the phenotype of the mutants was confirmed in soft agar tubes. For instance, $Alb^{\pm}$ mutants produce 5 mm/day of lysis (instead of 12 mm); Alb^- mutants remain negative for 5 days. Several mutants with either "derepressed" (earlier than wild type) or delayed protease synthesis have also been found.

3. Multiplicity of Proteolytic Enzymes

The existence of mutants, positive on one substrate and negative on another, might suggest for example that the two activities are due to different enzymes or that one test is much more sensitive than the other. Nevertheless, considering two substrates X and Y, if both X^+Y^- and X^-Y^+ phenotypes are found among the mutants, this strongly suggests that the activities are due to different enzymes. (The other alternative: mutations in the structural gene, changing the specificity of a single enzyme, is very unlikely.) Table 8 shows the existence of such mutants:

for instance, among the 500 mutants examined, 8 were found with the phenotype $Alb^{-}Hgl^{+}$ and 40 with the complementary phenotype $Alb^{+}Hgl^{-}$. Applying the same reasoning to the other tests the data suggest the presence of at least six distinct proteolytic activities (see Discussion).

Table 7. *Biochemical defects of sporulation mutants*

Test	Exp.[a]	Nb of colonies examined[b]	Phenotype (%)			% of mutants (mean value)
			—	±	mutant[c]	
			Proteolytic enzymes			
Gelatin	C	235	0.85	10	11	11
	D	292	1.7	9	11	
Albumin (denatured)	C	235	15	8.7	24	21
	D	317	9.5	9.5	19	
Casein	C	233	0.85	19	20	20
	D	317	4	14	19	
Protamine	C	211	3.3	16	20	22
	D	309	6	18	24	
Hemoglobin	C	235	21	18	39	41
	D	314	15	27	42	
Albumin (native)	C	235	50	9.3	60	54
	D	316	33	15	49	
Elastin	C	230	43	41	84	60
	D	298	14	32	46	
			Antibiotics			
Bacillus H	C	228	17	27	44	32
	D	315	6.7	18	24	
Staphylococcus	C	211	20	38	58	45
	D	308	14	25	39	

[a] Mutagenesis experiments C and D.
[b] All the colonies have been isolated as sporulation mutants (Sp^{-} or $Sp^{\pm}$).
[c] Sum of the preceeding two columns.

Table 8. *Mutants indicating distinct proteolytic enzymes*

Negative phenotype	Positive phenotype					
	Gel^{+}	Alb^{+}	Cas^{+}	Hgl^{+}	AlN^{+}	El^{+}
Gel^{-}	X	1	1	0	0	
Alb^{-}	32	X	16	8	1	3
Cas^{-}	4	4	X			
Hgl^{-}	67	40		X	12	3
AlN^{-}	166	126		78	X	18
El^{-}		72		46	15	X

The figures represent the number of mutants positive for one proteolytic activity and negative for another. A total of 500 mutants (from experiments C and D) have been examined. Intermediate (±) phenotypes have been excluded. Empty cases: not determined.

4. Relationship Between Protease Production and Sporulation

The above experiments indicate that one or more of the proteolytic activities are absent from a large number of sporulation mutants. This is, of course, not a sufficient proof for a physiological relationship between spore formation and proteolysis. The following results demonstrate that such a relationship indeed exists. Simultaneously with the isolation of sporulation mutants, 250 Sp^+ bacteria, submitted to the same mutagenic treatment, were isolated. When replicated on the various protein test-plates, they were without exception positive. Assuming that sporulation and enzyme production are independent, the expected frequency of protease-negative (Pr^-) mutants among Sp^+ colonies could be calculated from the data of Table 7. (A correction factor of three was introduced because of the higher frequency of auxotrophic mutations among Sp^M, see Table 4.) On the assumption of random coincidence of independent mutations one would expect to find among the 250 Sp^+ colonies the following phenotypes: 9 Gel^M, 16 Alb^M, 16 Cas^M, 32 Hgl^M etc. None were found. Thus the total absence of the Sp^+Pr^M phenotypes and the high frequency of Pr^M among Sp^M argue against this hypothesis. These observations are further confirmed by the fact that the proportion of Pr^M increases very little with the frequency of sporulation mutants with increasing doses of the mutagen (Table 9, compare with the frequency of mutants negative for the sporulation-independent enzyme amylase).

Table 9. *Phenotype of sporulation mutants obtained with various doses of NTG*

	Sp^M isolated in experiments with mutation frequencies of		
	2.3—5%	11—15%	25—27%
	% of mutant phenotypes among the Sp^M		
Gel	5	10	13
Alb	16	17	28
Protam	21	19	22
Hgl	29	41	41
AlN	49	46	62
El	47	53	76
Ab (St)	31	47	58
Amy	1	7	12

Mutagenesis experiments have been grouped following the frequency of sporulation mutants obtained, and the phenotype of mutants, isolated in each group, has been characterized on various plates. The figures indicate the frequencies of mutant (— and $\pm$) phenotypes among sporulation mutants in each group.

No distinction was made in the preceeding data between asporogenous and oligosporogenous mutants. Two questions are of interest in this respect: is there any correlation between the presence or absence of proteolytic activities and the frequency of sporulation, and could some of the intermediate phenotypes be attributed to oligosporogenous mutants? Table 10 shows that the frequency of protease-negative mutants is roughly the same among true asporogenous mutants and oligosporogenous mutants. (Yellow colonies included sometimes less Pr^-

mutants; since they sporulate with frequencies as high as 10%, this number of sporulating bacteria might produce detectable quantities of the enzymes.) When the ratios of mutants, with negative or intermediate phenotype (—/±, Table 10) were compared among the three classes of sporulation mutants, it was found that the frequency of intermediate phenotypes increases with the sporulation frequency; completely negative mutants occur more often among true asporogenous mutants. At least some of the intermediate phenotypes could thus be explained by the presence of sporulating bacteria in the oligosporogenous colonies. Similar conclusions have been reached concerning the oxidation of tetrazolium salts.

5. Production of Antibiotics

It was shown in two laboratories that the Marburg strain of *B. subtilis* produces antibiotic-like substances against a *Staphylococcus* (Balassa, Ionesco and Schaeffer, 1963) and a *Bacillus* species (Spizizen, 1965); the production of these substances is related to sporulation. In order to question the identity of the two products, I tested their presence in all the sporulation mutants described here. 32 and 45% of these were altered in their ability to produce an antibiotic against *Bacillus H* and against *Staphylococcus*, respectively (Table 7). Eleven mutants were found with normal activity against the first indicator but inactive with the second; although no mutant of the opposite phenotype was observed, fifteen mutants developed normal antibiosis on *Staphylococcus* and only a weak activity on *Bacillus H*. The existence of these two classes suggests the non-identity of the two antibiotics.

Table 10. *Distribution of enzyme deficiencies among various sporulation mutants*

		Sporulation mutants		
		Asporogenous	Oligosporogenous	
			White	Yellow
Sporulation	% [a]	13	68	20
Alb	% [b]	13	70	16
	r [c]	3.5	1.4	0.3
Hgl	%	15	72	13
	r	0.8	1.0	0.6
AlN	%	14	70	17
	r	5.4	4.0	1.8
El	%	10	71	20
	r	1.3	1.0	0.5
Ab (St)	%	13	70	16
	r	1.0	0.5	0.6
TZ2	%	9	69	22
	r	4.8	2.4	1.3

Data combined from experiments C and D.

[a] Distribution of all the sporulation mutants in per cent.

[b] Distribution of the mutant (negative + intermediate) phenotypes for proteolytic activities among the three classes of sporulation mutants.

[c] r = ratio of negative to intermediate phenotypes (—/±).

None of the 150 Sp^+ colonies was defective in the production of either of the antibiotics. Oligosporogenous mutants showed intermediate phenotypes more frequently than asporogenous mutants (Table 10). These last observations confirm previous results (BALASSA, IONESCO and SCHAEFFER, 1963; SCHAEFFER, IONESCO, RYTER and BALASSA, 1965) establishing a strict relationship between spore formation and the production of these antibiotics.

6. Sporulation-Independent Exoenzymes

Beside proteases *B. subtilis* produces in stationary phase several hydrolytic enzymes (POLLOCK, 1962; SCHAEFFER, 1968), but no relationship between their production and sporulation was reported (except for lysozyme, SCHAEFFER, 1967) The presence of such enzymes in protease-less mutants could be used as a control to prove that these mutants reach stationary phase and are able to produce and excrete exoenzymes. All the mutants have been examined therefore for amylase activity. This activity appears after 12 hours of incubation (as gelatinase activity). Amy^- mutants were found among both Sp^+ and Sp^M colonies; Sp^M Amy^- strains are certainly double mutants since the two characters can be separated by transformation and since their frequency depends strongly on the mutagenesis frequency (Table 9). Lipase activity (BALASSA, unpublished) is also independent of spore formation and present in the majority of sporulation mutants.

IV. Pleiotropic Effects of Sporulation Genes

1. Mutants with Multiple Phenotypic Changes

Following the results described above, we have now microbiological tests available for eleven phenotypes associated with sporulation: the oxidation of two tetrazolium salts, the presence of two antibiotics and the production of various proteolytic activities, presumably corresponding to several distinct enzymes. More than 500 sporulation mutants have been tested and their phenotype in respect to these properties was noted. A superficial look at the results is sufficient to detect a great variety of phenotypes as illustrated with a few mutants in Table 11. As we saw, the absence of proteolytic activities is not due to random coincidence of distinct mutations. This suggests the existence of pleiotropic interactions between sporulation genes. Further analysis of the data

Table 11. *Phenotypes of randomly selected sporulation mutants*

Mutant	Sp	TZ2	Cas	Hgl	El	Gel	Alb	Ab(St)	AlN	Ab(BH)	Protam
CA 1	±	+	+	+	—	+	+	±	—	+	+
2	±	+	+	+	—	±	—	±	+	±	—
3	—	+	+	+	+	+	+	+	+	+	+
4	±	—	±	±	+	+	+	+	+	+	+
5	±	+	+	+	—	+	+	—	±	±	+
6	—	—	+	±	0	+	+	0	±	0	+
7	±	±	+	—	0	±	—	0	—	0	±
8	±	+	+	±	+	±	—	+	—	+	±
9	±	+	+	+	±	+	+	+	—	+	±
10	±	+	+	±	0	+	—	0	—	—	+

was therefore attempted in order to reveal correlations between the presence or absence of various sporulation-associated characters.

One conceivable reason for the existence of $Sp^{M}Pr^{M}$ type mutants is that each sporulation-associated substance (protease, etc.) is required for spore formation. In this case one would expect that mutants with multiple phenotype changes of the $Sp^{M}Gel^{M}Alb^{M}$ type would arise by random coincidence of distinct mutations (e.g. of $Sp^{M}Gel^{M}$ and $Sp^{M}Alb^{M}$). To show that this is not the case, the frequency of multiple changes (the number of mutants lacking two or more of the sporulation-associated products) was calculated. To simplify the problem, one could first consider all the tests two by two, and compile, for each pair, the distribution of the mutants with various phenotypes. A complete set of data is given for the two tests Alb and Ab(St) in Table 12. Assuming no pleiotropic effects, the expected number of $Alb^{M}Ab^{M}$ mutants, calculated on the basis of the frequency of each phenotype, is $\frac{Alb^{M} \times Ab^{M}}{T} = 42$ (see Table 12); the observed number is 67. Similar results are obtained with other pairs of characters: there is always an excess of the double mutant phenotypes. Considering two characters, this excess is in general not very pronounced. If, however, several characters are considered, the excess becomes spectacular: for instance, the number of mutants defective in the production of four proteolytic activities is 10—30 times higher than expected on the basis of random coincidence of various phenotypes (Table 13). Such a frequent occurrence of certain multiple deficiencies not only confirms the presence of pleiotropic interactions among sporulation genes but also suggests that they follow a definite pattern. This can be investigated by further analysis of the distribution of mutant phenotypes.

Table 12. *Distribution of mutants with various phenotypes in respect to Alb and Ab(St)*

		Ab(St)				
		—	±	*M*	+	Total
Alb	—	12	21	*33*	11	**44**
	±	17	17	*34*	13	**47**
	M	*29*	*38*	*67*	*24*	**91**
	+	56	118	*174*	252	**476**
Total		**85**	**156**	*241*	**276**	**T = 517**

M = mutant phenotypes, sum of the two preceeding columns or lines (— and ±). T = total number of colonies examined.

Table 13. *High frequency of multiply deficient mutants*

Phenotype	Number of mutants	Number of colonies examined	Expected number of mutants[a]
$Gel^{M}\ Alb^{M}Cas^{M}Hgl^{M}$	34	522	0.9
$Alb^{M}Cas^{M}Hgl^{M}AlN^{M}$	57	545	5.0

[a] Calculated by multiplying the total number of colonies by the frequency of each individual mutant phenotype in the population.

2. Asymmetry of the Pleiotropic Interactions

In the hypothesis of sequential gene activation, pleiotropic interactions depend on the order in which the various sporulation genes act during spore formation. A more stringent test of this hypothesis can be developed by the following reasoning: if the absence of an "early property" indicates a mutation in an "early gene", all the "late" properties should be absent in "early" mutants. On the other hand, mutants devoid of a "late" property might or might not possess early properties, insofar as the block is before or after the early genes in question. (In other terms, mutations have no retroactive effects.) As a consequence, the correlation between the various characters should be asymmetrical. To verify this prediction, data for various couples of characters were compiled as in Table 12, and the frequency of mutants with "asymmetrical" phenotypes (positive for a character X and negative for a second character Y or *vice versa*) was calculated. To measure the degree of asymmetry, the ratio of the two complementary asymmetrical phenotypes X^-Y^+ and X^+Y^- was calculated and

Table 14. *Coefficients of asymmetry*

	Gel	Alb	Protam	TZ7	Ab(BH)	Hgl	Ab(St)	AlN	TZ2
Cas	2.2 2.5								
Alb	3.6 2.5								
Protam		1.3 1.2							
TZ7	+ 1.7	1.9 1.3							
Ab(BH)	42 2.3	1.5 1.5							
Hgl		3.3 2.8	1.4 1.8	1.4 1.3	1.5 1.2				
Ab(St)	4.3 3.8	2.6 3.0	6.2 2.3	2.3 2.0	+ 4.4				
AlN	+ 7.62	18.4 8.2		4.3 3.0		2.9 1.8	1.4 1.1		
TZ2	3.3 3.5	2.1 2.3		1.6 1.5	1.7 2.1		1.1 1.4	1.2 1.2	
El				15.3 5.5		3.1 2.1		0.9 1.4	0.9 1.0

The coefficients are calculated as described in the text. For each case the first line is for the negative phenotypes (C^-), the second for the totality of mutant phenotypes (C^M). The data are based on the phenotypes of 500 mutants.

The tests have been ordered in a way to obtain coefficients larger than one. High values mean excess of the $A^M B^+$ phenotype over $A^+ B^M$, if A is the test indicated in the first column. For instance when comparing Hgl and Alb, the high coefficient of asymmetry indicates that the Hgl activity is involved in sporulation *later* than the Alb activity.

+ = large values with no precision because of a small divider.

corrected for the ratio of the two negative phenotypes X^- and Y^- in the total population. The resulting coefficient of asymmetry is thus:

$$C^- = \frac{X^- Y^+}{X^+ Y^-} \Big/ \frac{X^-}{Y^-}$$

(A similar coefficient C^M is calculated for each pair of tests to include all the mutant phenotypes X^M and Y^M.) Such a ratio indicates the excess of the asymmetrical phenotypes over the numbers expected on the basis of complete randomness. The calculation is formally similar to that of recombination data in a population with unequal gene inputs. The results of these calculations, summarized in Table 14, show that an asymmetry indeed exists; it may be very pronounced in certain cases, weaker in others. Moreover, following the increasing coefficients of asymmetry, it is possible to order all the tests into a linear order. This means that the negative phenotype of a given mutant in one test predicts with high probability that the mutant will be negative in all the following tests, without having any implication on the results of the previous tests. The order thus obtained is the following:

Gel — Cas, Alb — TZ7, Protam, Ab(BH) — Ab(St), Hgl — TZ2 — AlN, El

V. Mutants Apparently Derepressed for Enzyme Formation

The previous results suggest that certain sporulation genes play the role of regulatory genes by controlling the expression of other sporulation genes. One could therefore expect to find mutants having the properties of regulatory mutants for the production of sporulation-associated enzymes. Several mutants developing either earlier or larger halos of proteolytic activity than wild type colonies were found, they suggest a derepressed synthesis or overproduction of the enzyme. This effect was particularly clear in the case of elastase.

Elastase activity normally appears after 5—7 days of incubation at 37°C. A large number of mutants do not develop this activity even after 8 days (El^-). Both kinetic evidence and the results described above suggest that this enzyme is related to a late event in sporulation. Surprisingly however, several sporulation mutants develop very early big halos of proteolytic activity on elastin plates. Among 528 sporulation mutants examined, 141 (27%) developed elastase activity earlier than wild type colonies, some of them already after two days of incubation at 37°C, the exact time being a reproducible property of each mutant. This suggests strongly the early production of the enzyme at an abnormally high rate, therefore these mutants will be tentatively called "derepressed" (El^D).

No mutant with El^D phenotype was found among 250 Sp^+ colonies isolated after treatment with nitrosoguanidine, therefore the "derepressed" phenotype is associated with the altered capacity of the mutants to sporulate. Five independent El^D mutants were further investigated by transformation: the Sp^M El^D try^- ur^- strains have been treated with DNA of the wild type. Since direct selection procedures for Sp^+ transformants may involve undesirable complications (e.g. imperfect expression of the phenotype), first try^+ or ur^+ transformants have been selected. These transformants have been either reisolated and tested for sporulation, or replicated after treating the plates with chloroform vapors

to select Sp^+ colonies among the transformants. A total of 133 Sp^+ transformants have been isolated this way and tested on elastin plates. They all lost the "derepressed" phenotype and showed elastolytic activity only at the time when wild type colonies did (after 5—7 days). No recombinant phenotypes (Sp^+El^D) occurred. As a control, 200 ur^+ or try^+ but Sp^M transformants have been isolated; they conserved without exception the "derepressed" phenotype. It is clear therefore that a single mutation can simultaneously derepress elastase synthesis and block spore formation.

Since elastase is the last sporulation-associated proteolytic enzyme to appear and seems to be related to a rather late step of spore formation, it is likely that the sporulation block in El^D mutants is also a late one. Therefore El^D mutants are expected to be positive for most or all the other proteolytic activities and for antibiotic production. Table 15 shows that this is true for most of the mutants: the "earlier" proteases are not overproduced, and the frequency of mutants with reduced proteolytic activity on other substrates or with altered antibiotic production is much lower among El^D mutants than in the whole population.

A similar situation arises with the native albumin (AlN) test: 12% of the sporulation mutants appear to be derepressed: more than half of them are also El^D. Their behavior in other tests confirms their similarity to the El^D mutants (Table 15), and in transformation experiments involving Sp^M El^D AlN^D mutants no segregation was observed. It seems therefore that at least some of the El^D mutations have a pleiotropic effect on two phenotypes.

Table 15. *Phenotypes of derepressed mutants*

	Phenotype	Test						
		Gel	Alb	AB(BH)	Ab (St)	Hgl	AlN	El
El^D	—	0	4	3	11	7	12	—
	±	5	6	19	30	22	16	—
	% of mutants	4	7	16	29	20	20	—
AlN^D	—	0	1	1	3	0	—	2
	±	1	1	4	16	7	—	13
	% of mutants	1.6	3.2	8	30	11	—	25
Total population % of mutants (from Table 7)		11	21	32	45	41	54	60

The phenotype of 140 El^D mutants and 64 AlN^D mutants was examined. 36 mutants have the phenotpye El^D AlN^D.

Discussion

The ultimate goal of this paper is to present evidence concerning the pleiotropic interactions between sporulation genes. Since these data have been obtained with a new mutant collection, first the conditions of mutagenesis and the basis for the selection of sporulation mutants will be discussed. Phenotypic characterization of the mutants was made possible by several known or newly developed microbiological tests for sporulation-associated characters. The biochemical background of these tests and the possible reasons of functional relationship between

sporulation and the various phenotypes will be considered here. Classification of the mutants following such tests leads to the establishment of a pattern of unidirectional pleiotropic interactions among sporulation genes. The nature of such interactions, the finding of mutants of a "regulatory" type and the possible mechanisms of the underlying genetic control system will constitute the least part of this discussion.

I. Specific Sporulation Genes

1. Mutagenesis Induced by Nitrosoguanidine

NTG is the most potent mutagen known for *E. coli*. When applied to resting cells, it induces mutation in a large number of genes (probably in all the genes) with a relatively low lethality. However treatment of dividing cells is not satisfactory because of the low survival and the possibility of preferential mutagenesis at the growing point (ADELBERG, MANDEL and CHEN, 1965; CERDA-OLMEDO *et al.*, 1968). These considerations lead to the use of spores, germinated in the presence of chloramphenicol, for mutagenesis experiments. The high frequency of both auxotrophic and sporulation mutants obtained justified this approach. (It should however be emphasized that none of the experimental data suggest that the physiological conditions of the cells lead to selective induction of sporulation mutants.)

Additional advantages of these conditions of mutagenesis are the absence of spontaneous mutants among the untreated cells, the absence of multinucleate cells and of chains, and, consequently, the lack of segregation. Two inconveniences of the method should also be mentioned: the heterogeneity of the population with regard to mutagen sensitivity and the presence of ungerminated spores which renders quantitative data with low survival values unreliable.

2. Recognition of Sporulation Mutants

The use of the color formation by colonies on nutrient agar for the purpose of selecting sporulation mutants deserves a few comments. This criterion was used by SCHAEFFER *et al.* (1965a) and by others to detect non-sporulating *B. subtilis* mutants. It was shown in this paper that asporogenous mutants displaying brown coloration are rare or non-existent. (The existence of oligosporogenous mutants with brown colonies was not excluded.) It seems therefore likely that a mutant collection, based on this criterion, contains a random sample of all the possible sporulation mutations. Other laboratories used different conditions to recognize sporulation mutants. For instance, SPIZIZEN's (1965) mutations have been first isolated as large colonies on a synthetic medium. Exploratory experiments with the nitrosoguanidine-induced mutants indicate that many sporulation mutants would not be recognized as such on synthetic media (BALASSA, unpublished). It is therefore possible that mutant collections obtained by Spizizen's methods represent a non-random selection of sporulation mutants. This might explain some of the differences between results obtained by the Paris group (BALASSA, IONESCO and SCHAEFFER, 1963; SCHAEFFER, 1967; SCHAEFFER *et al.*, 1965a) and by SPIZIZEN (1965).

Collections have been made previously of sporulation mutants obtained spontaneously or induced by UV, heat, or acridine-orange (BOTT and DAVIDOFF-

Abelson, 1966; Northrop and Slepecky, 1967; Schaeffer *et al.*, 1965a; Schaeffer, 1967). As far as one can judge from published data, these mutants and the nitrosoguanidine-induced mutants reported here exhibit a similar spectrum of phenotypes. For instance, the frequency of oligosporogenous mutants and the proportion of mutants altered in antibiotic production seems to be the same in all these conditions. The same conditions are known to give rise also to auxotrophic mutations, although less frequently than to sporulation mutants.

3. "True" and "False" Sporulation Mutants

It was shown that the white or yellow colonies selected after NTG treatment all have altered abilities to form spores. This does not imply that the genes carrying the mutations are directly concerned with spore formation. It is a known fact that bacteria, deficient in an enzyme of intermediary metabolism, are often unable to sporulate, although their growth seems normal under most conditions. This was clearly demonstrated in the case of an aconitase-less mutant (Szulmajster and Hanson, 1965). Other mutants have been originally obtained as Sp^- and later found to lack enzymes of the Krebs-cycle, oxidative enzymes, etc. (Bott and Davidoff-Abelson, 1966; Freese and Fortnagel, 1967). In the latter cases it is not yet clear what is the primary function of the mutated genes; many of these enzymes are absent from vegetative cells and appear only during sporulation. These observations make it clearly necessary to distinguish the true sporulation mutants from more or less "hidden" mutations of the intermediary metabolic pathways. Unfortunately this is a difficult task since the first event in sporulation (the "commitment" or "induction") is not known. (The first morphologically recognizable events (formation of the axial filament and of the sporulation septa, Ryter, Schaeffer and Ionesco, 1966), are undoubtedly preceded by several biochemical steps, and (contrary to the established belief) the first steps in spore formation are not irreversible in *B. subtilis* (Balassa, unpublished)). The following observations indicate at least the absence of gross metabolic deficiencies among the majority of the mutants. All the mutants described here appear to grow normally both on complex and on synthetic media. Young colonies oxidize the various tetrazolium salts and old colonies oxidize the compound TZ1. In addition, all the mutants excrete enzymes unrelated to sporulation, e.g. amylase, and finally, fifty mutants examined all perform the pH changes (drop followed by a rise) characteristic for the wild type. Of course none of these points can eliminate definitely the possibility of "hidden" metabolic mutations. This question will be further discussed in connection with the sporulation-associated characters.

4. Estimates for the Number of Sporulation Genes

Genetic mapping (by transformation and transduction) leads to the localization of several sporulation genes on the chromosome; other mutants have been proved unlinked among themselves by transformation (Spizizen, 1965; Schaeffer *et al.*, 1965a; Takahashi, 1965; Rouyard, Ionesco and Schaeffer, 1967). Compilation of such data, together with the fact that independent mutations rarely fall into the same cluster, leads to a minimum estimate of at least 20

sporulation genes. The large variety of phenotypes in respect to the morphological block (RYTER, SCHAEFFER and IONESCO, 1966) and to various sporulation-associated characters also suggests the existence of at least several dozen distinct sporulation genes.

NTG treatment of spores germinated in presence of chloramphenicol gives rise to sporulation mutants with a high frequency: up to 25% mutants can be obtained under conditions of 40% cell survival. Since the mutation frequency is known to vary from gene to gene, a single (or a few) sporulation gene with particularly high mutation rate could be postulated to account for the high frequency of sporulation mutants. However this explanation seems unlikely in view of the large variety of distinct phenotypes observed among the mutants. Assuming on the other hand that the mutation rate of the sporulation genes is comparable to that of other genes, sporulation would require the participation of a large number of genes. In my experiments, the total number of sporulation mutants is at least 4 times higher than the number of mutants deficient in the synthesis of amino acids. If the number of genes concerned with the synthesis of amino acids is approximately 200, the number of sporulation genes could be estimated to 800.

II. The Nature of the Sporulation-Associated Phenotypes and their Relationship to Spore Formation

1. Oxidation of Tetrazolium Salts

The biochemical defect leading to TZ^- phenotype is not known. It is therefore not possible to ascertain the exact nature of the relationship between the observed effect and spore formation. A likely hypothesis is that the oxidation of TZ by cells in stationary phase reflects the presence of a new oxidative enzyme, normally synthesized during one of the steps leading to sporulation. $TZ2^-$mutants would be blocked, in this hypothesis, before this step. Differences in the oxidation of the three tetrazolium compounds could reflect different redox potentials and the presence of a complex of several oxidative enzymes.

Whatever is its biochemical basis, the test for the oxidation of tetrazolium salts permits a classification of the sporulation mutants. The physiological association between the coloration of TZ by old colonies and the formation of spores is supported by the following evidence: 1) young colonies of mutants (presumably containing growing bacteria) behave like wild type colonies, differences appear only at the time when wild type colonies develop the characteristic brown color and excrete proteases; 2) the phenotype SP^+TZ^- has not been observed; 3) the coincidence of the SP^- and TZ^- phenotypes is at least by an order of magnitude higher than would be expected for double mutants. One should also note the equal frequency of TZ^- phenotype among Sp^- and $Sp^\pm$ mutants, this argues against the role of an enzyme unrelated to sporulation in the tetrazolium test.

It is known that sporulating bacteria have a high QO_2 and contain a new NADH-oxidase, absent from several sporulation mutants (SZULMAJSTER, 1964; SZULMAJSTER and HANSON, 1965). More recently sporulation mutants deficient in glucose dehydrogenase (BOTT and DAVIDOFF-ABELSON, 1966), in succinic

dehydrogenase and in other enzymes (Freese and Fortnagel, 1967) have been isolated. Metabolic alterations of this kind could be at the basis of the mutant phenotypes in respect to TZ oxidation. It would be interesting to perform the tetrazolium tests on these mutants.

2. The Diversity of Proteolytic Activities

The occurrence of extracellular proteolytic enzymes in various microorganisms is rather frequent and in several cases more than one enzyme is produced by a single species (Davies, 1963; Hagihara, 1960; Pollock, 1962). Besides the well-known subtilysin, alcaline, neutral and acid proteases have been obtained from *B. subtilis* (McConn, Tsuru and Yasunobu, 1964; Rappaport, Riggsby and Holden, 1965; Tsuru *et al.*, 1966a, b). Evaluation of these results is difficult because of the diversity of the producer strains, often not identified unambiguously. In most cases, attention was given to the final recovery of one single enzyme after growing the bacteria for long periods (e.g. one week) in ill-defined culture media; under these conditions it is likely that if several proteolytic enzymes have been produced, only one survived inactivation, autolysis and mutual digestion. Results of Michel (1967) suggest that such phenomena do occur in cultures of sporulating *B. subtilis*. It is likely that proteases could be better studied in conditions where diffusion of the enzymes into the agar, soon after their production, may protect them.

In the present study proteolytic activities were observed on widely different substrates, like native and denatured proteins and elastin. Although several proteases are rather nonspecific (e.g. subtilysin, Ottesen and Spector, 1960), it is suggested, on the basis of both kinetic and genetic observations, that the various tests described here distinguish the activity of at least six distinct enzymes. This suggestion is based on the long delay observed between the appearance of various activities, and, principally, on the phenotypes of sporulation mutants. The existence of two complementary phenotypes, positive with one substrate and negative with the other and *vice versa*, is taken as a tentative proof of the presence of two distinct enzymes.

What is the specificity of these enzymes? Isolation and separation of the enzymes is necessary to answer this question, and at present we can only guess. Specific gelatinases are known (Mandl, 1961), and are likely candidates for the first activity (but probably some of the enzymes appearing later would also digest gelatin). Many proteolytic enzymes digest proteins with little or no tertiary structure, this seems to be the case with the enzyme(s) observed on denatured albumin and on casein. More difficult to interpret are the enzymes active on native albumin and hemoglobin. Finally a true elastase was described in *B. subtilis* (Mandl, 1961). Identification of the enzyme activities described here with specific proteases would be premature. (It should be emphasized that an enzyme, the activity of which is observed in our conditions on denatured albumin, does not need to be absolutely specific for this substrate. The only conclusion retained for the following discussion is that various mutants display different phenotypes. Thus, the expression *phenotype* could conveniently replace the words enzyme or protease without altering the conclusions.)

Since the best known proteases of *B. subtilis* are extracellular enzymes, this could be also true for most of the activities observed in this work. This is sug-

gested by the appearance of the halos around the colonies in most tests; however some of the latest activities (e.g. on native albumin and on elastin) could be due to intracellular enzymes, liberated by lysis.

In conclusion, proteolytic activities of sporulating *B. subtilis* cultures can be attributed to a complex of several exoenzymes. The tests described here allow the distinction of at least six factors including a gelatinase and an elastase, yet on this basis one cannot establish a clear one substrate-one enzyme relationship. Thus the description of a mutant as Cas^-, for instance, should be interpreted as a mutant lacking one or several enzymes that are, under these experimental conditions, the most active on casein. For simplicity, lack of one type of activity will be considered in the following discussion as the absence of one enzyme.

3. Functional Relationship Between Sporulation and the Synthesis of Proteases and Antibiotics

A relationship between the synthesis of Ab(St) and sporulation was clearly shown by previous work (Balassa, Ionesco and Schaeffer, 1963; Schaeffer *et al.*, 1965a): 1) no Sp^+Ab^- mutant was found; 2) all the Sp^+ revertants and transformants from Sp^- mutants were Ab^+; 3) transformation of an Sp^-_1 Ab^- mutant by the DNA of an unlinked mutant $Sp^-_3 Ab^-$ yielded only Sp^+Ab^+ recombinants. It was concluded therefore that the production of this antibiotic is related to a specific step in the sporulation process. Electron microscope evidence indicated that it was an early step (Schaeffer *et al.*, 1965a). Using similar methods, the same conclusion was reached concerning a factor that determines the rate of RNA and protein turnover (Balassa, 1964a, b). The role of sporulation in protease formation was also established in several laboratories in respect to a *B. subtilis* gelatinase (Spizizen, 1965), an enzyme digesting casein (Michel, 1967), a *B. licheniformis* protease (Bernlohr, 1964) etc. In the case of the proteolytic enzymes described in this paper, evidence for their connection with sporulation is based on the absence of the Sp^+Pr^- phenotype and on the high frequency of coincidence of the Sp^M and Pr^M phenotypes. It seems that the production of proteolytic enzymes is associated with sporulation by the same kind of relationship as the synthesis of antibiotics.

The significance of the intermediate ($\pm$) phenotypes is not clear. Colonies of oligosporogenous mutants could contain sporulating bacteria in a sufficient number to develop a weak activity; this was shown in the case of antibiotic production (Schaeffer *et al.*, 1965a) and proteases (Table 12). On the other hand, true asporogenous mutants with intermediate proteolytic activities have also been found, therefore the existence of mutants producing no spores but limited amounts of sporulation-associated substances is not excluded. It can be suggested that all these mutants are, in a broad sense of the word, regulatory mutants. Certain mutants develop sporulation-related biochemical characters much later than wild-type (Balassa, 1964b; Michel, 1967), although no delayed spore formation occurs.

4. Possible Physiological Roles of Proteases and Antibiotics in Spore Formation

a) Proteases. The most generally admitted physiological function of exocellular hydrolytic enzymes is to supply nutrients in an assimilable form. More specific intracellular functions could also be attributed to the proteases, especially

those acting on native or on insoluble proteins. The former are good candidates for the enzyme(s) responsible for the high rate of intracellular protein turnover (Balassa, 1964a), the latter could serve in the formation, growth and/or degradation of the protein coats of the spores during sporulation and germination. [It was also proposed that a protease would function as a key enzyme in the induction of sporulation by destroying a specific repressor (Schaeffer, Millet and Aubert, 1965).]

b) Antibiotics. Two kinds of functions could be imagined for these products. They might be excreted in order to inhibit growth of other bacterial species at a time when nutrients become limiting and "vital space" for the *B. subtilis* population is important. On the other hand, it was suggested that *Bacillus* polypeptide antibiotics would be incorporated into the spore coat (Bernlohr and Novelli, 1963). Although this observation was questioned (Snoke, 1964), it remains possible that the antibiotics are precursors or by-products in the formation of the cell wall or of the spore envelop.

The above suggestions imply that proteases and antibiotics are actually involved in and perhaps necessary for spore formation. A completely different situation should equally be envisaged. The genes involved in the production of these substances could be, for fortuitous or evolutionary reasons, under the control of the general regulatory system governing the activity of sporulation genes. In this case the sporulation-associated substances would not play any direct physiological role in spore formation, and the relationship would be purely regulatory.

III. One-Directional Pleiotropy and "Regulatory" Genes

1. Pleiotropic Action of Sporulation Genes

Mutations in any of at least several dozen genes can prevent normal spore formation. Sporulation is a succession of morphological events, divided into six clearly recognizable steps (Schaeffer *et al.*, 1965a); sporulation mutants may be blocked at any of these steps (Ryter, Schaeffer and Ionesco, 1966). When a mutant is blocked at an early step, no sign of any later morphological structure is visible, although general macromolecular metabolism proceeds normally. This suggests that if an early gene does not function, no function of later genes will be performed; in other words all the sporulation genes have a pleiotropic action exerted over other sporulation genes designated as "later" genes.

Available biochemical evidence supports this view. Many biochemical changes accompany sporulation in *Bacillus subtilis*; such changes do not occur in early sporulation mutants. This was shown for the synthesis of NADH-oxidase (Szulmajster, 1964), of an antibiotic (Balassa, Ionesco and Schaeffer, 1963), of the "turnover factor" (Balassa, 1964b) and of a protease (Michel, 1967).

In this paper, more than 500 mutants have been examined for ten sporulation-associated characters. The tests used here are very crude, but most likely they are measuring the presence or absence of distinct biochemical characters. If one takes into consideration all the tests, the number of phenotypes observed is fairly high. It is obviously impossible to analyze the meaning of the complete phenotype of each mutant. However, the following rules were observed: 1. The number of multiply deficient mutants is frequently much higher than expected from random coincidence. 2. The pattern of these deficiencies is not random:

certain phenotypes are predominant. 3. Pairs of characters display asymmetrical correlations. 4. These asymmetrical correlations permit, as predicted by the hypothesis of sequential gene activation, the ordering of several biochemical traits into a single linear order. Thus the pleiotropic interactions between genes are not reciprocal but occur only in one way; this can be described as a unidirectional pleiotropic system with the following order:

Gel — Cas, Alb — TZ7, Protam, Ab(BH) — Ab(St), Hgl — TZ2, AlN, El

When the same properties are ordered by the kinetics of their appearance (Results III-1), or by the frequency of deficient mutants (Results III-2), similar sequences have been found. Since the three ways of ordering are all based on the hypothesis of sequential gene activation but have been realized by independent methods, their similarity is strongly in favor of the original hypothesis. On the basis of these data, the following sequence can be suggested for the order in which the various sporulation-associated characters are involved in spore formation:

Gel — Cas, Alb — TZ7 — Protam, Ab(BH) — Ab(St) — Hgl — TZ2 — AlN — El.

This sequence is reflected in the phenotype of sporulation mutants, leading to unidirectional pleiotropic interactions between sporulation genes.

Such a conclusion indicates that the phenotype Gel should be associated with an early step of spore formation, while the elastase activity is related to a late step. In the simplest case, mutations in a very early gene will have the phenotype Gel^-El^-, mutations in intermediate genes will lead to Gel^+El^- bacteria, and late mutants will be Gel^+El^+. Preliminary electron microscopic observations (Balassa, unpublished) confirmed that the elastase activity is related to a late step, after the formation of the prespore.

2. The Significance of "Exceptional" Mutants

Despite the regular patterns just described, the phenotype of a non-negligible number of sporulation mutants does not fall in any of the expected categories (see Tables 11, 12). This is not surprising and should be expected *a priori* for several reasons: 1. Simple artifacts, such as misreading of the tests, reversion of a mutant on some plates, etc. would create disordered results. 2. The presence or absence of a character is not an all-or-none phenomenon; intermediate phenotypes are frequent. In such cases, the sensitivity of a test becomes an important factor; a mutant producing 10% of each of two enzymes may appear positive in one test and negative in another. This applies particularly to oligosporogenous mutants. 3. Both double mutants and "regulatory" mutants, in which the normal sequence of events is altered, might arise. 4. Some of the tests might reflect the interaction of several sporulation-associated enzymes (a protease and its activator, two members of the respiratory chain, etc.); in these cases the correlations predicted are more complex. 5. Finally, the morphopoietic pathway could be branched, therefore the pleiotropic sequence may be partially overlapping.

The results described here show that statistical analysis of the obtained mutant phenotypes can give some information on the nature of genetic interactions and of the differentiation pathway before more precise biochemical methods become available. Despite the exceptions, when asymmetrical correlations do arise, it seems difficult to attribute them to artifacts. More detailed

biochemical and genetic studies of the exceptional mutations will hopefully contribute to the understanding of spore formation.

3. Role of Regulatory Genes in Sporulation

Assuming that sequential gene activation occurs during the consecutive steps of sporulation, the genetic control of spore formation should involve a dozen or more genes with regulatory functions in respect to other sporulation genes. The role of regulatory genes in sporulation was suggested many times (SCHAEFFER *et al.*, 1965; BALASSA, 1966b; HALVORSON, 1965, etc.). LEVISOHN and ARONSON (1967) isolated *B. cereus* mutants simultaneously derepressed for protease production and for sporulation in certain growth conditions; it is not clear if the primary effect concerns sporulation itself or a normal metabolic pathway, perhaps involved in catabolic repression (SCHAEFFER, MILLET and AUBERT, 1965). The case of mutants derepressed for elastase production, reported above, is therefore particularly interesting. In these mutants sporulation starts normally and proceeds far beyond the initial steps, as judged from the production of antibiotics and proteases and from preliminary electron microscope observations. At a late stage, overproduction of an enzyme, normally associated with sporulation, blocks further steps. The exact nature of these mutations is not yet known and it is not clear if the "derepression" is due to the block of sporulation or *vice versa*, but the case falls under the definition of regulatory genes in the broadest sense.

It is not known how many genes are subject to the control of a regulatory gene in a single step of regulation. El^D mutants might be derepressed only for the elastase gene or for several genes functioning simultaneously (e.g. El and AlN).

Electron microscopic evidence (unpublished) strongly suggests that the block in El^D mutants occurs late in sporulation (step IV or step V). Future work should precise the kinetics of enzyme production by such mutants, the exact nature of the morphological block and the number of genes derepressed by a single mutation.

IV. The Mechanism of Genetic Control in Sporulation

Pleiotropic effects can arise from metabolic as well as genetic interactions. In the case of sporulation, little is known about the metabolic pathways. However the number of steps involved, the variety of the biochemical events considered and the very existence of exceptional mutants, not following this pathway, all favor the interpretation that in this case pleiotropy is due to genetic interactions. Further evidence for such a view comes from the observation that unstable messenger RNAs are synthesized in the process of spore formation (BALASSA, 1966a) and a fraction of these are qualitatively different at different steps of sporulation (DOI and IGARASHI, 1964; ARONSON, 1965). It can therefore be tentatively concluded that the unidirectional pleiotropy of sporulation genes is due to their interaction during sequential gene activation.

The best known mechanism controlling gene activity in bacteria is the one studied by JACOB and MONOD (1961) consisting in the interaction of a repressor protein with an operator gene which regulates the activity of several structural genes of an operon. There is no proof of the presence of such a system in differentiating organisms, it is however generally accepted that gene activation

during development could be explained by repressor-inducer interactions. Specific models have been presented by MONOD and JACOB (1961), and considered for sporulation by HALVORSON (1965). Such models are based on sequential gene activation. The existence of unidirectional pleiotropic effects favors this kind of models.

Sequential induction, based on a single metabolic pathway, could be another explanation for the observed pleiotropic effects. The variety of the products involved in sporulation, the existence of exceptional mutants and the fact that no intercellular complementation was described among sporulation mutants (SCHAEFFER *et al.*, 1965a) make this hypothesis rather unlikely.

Unidirectional pleiotropic interactions define a genetic relationship between a large number of sporulation genes. This is a regulatory interaction at a more complex level than operons (JACOB and MONOD, 1961) and regulons (MASS and McFALL, 1964). I propose to call *a sporulon* the group of genes which: 1. are involved in sporulation but inactive during vegetative growth, 2. are sequentially activated and repressed during spore formation and 3. are submitted to unidirectional pleiotropic interactions. As a generalization, genes would be considered to be part of *a differon*, if 1. they are inactive before a given morphogenetic pathway is induced; 2. they are sequentially activated and repressed during morphogenesis; 3. they regulate the activity of other, later genes of the same *differon* but are without effect on earlier genes. Although further proofs are required, it is the aim of this study to show that *unidirectional pleiotropy* may be used as a recognition sign and a tool to analyze genes of a *differon.*

Evidently, a sporulon (or differon) is a unit including several operons. It differs from the postulated systems of sequential induction in at least two respects: activation of a gene is accompanied by repression of the preceding genes and the effectors are not likely to be the metabolic products of the genes.

The linearity of the morphogenetic pathway may be an oversimplification. Each regulatory step could involve several genes, clustered in an operon or dispersed but forming a regulatory unit (regulon). Moreover, the morphopoietic pathway itself should converge at the beginning (induction) and the end (formation of a mature spore), but may form branches in between. Analysis of the patterns of exceptional mutants and the determination of the number of genes derepressed by a single El^{D}-type mutation will hopefully lead to a better understanding of the exact configuration of the sporulation pathway. This will require, however, biochemical characterization of the sporulation-associated phenotypes.

The concept of unidirectional pleiotropy might be used to distinguish *bona fide* sporulation mutants from mutations in the intermediary metabolism, or, more exactly, to recognize genes of the sporulon. One could reasonably suppose that a mutation affecting the intermediate metabolism would prevent the induction of the sporulation process rather than a later step. If it is true, mutants which are able to produce at least one of the exoenzymes or antibiotics associated with sporulation (e.g. gelatinase) should be true sporulation mutants. Of course, mutants negative for all the sporulation-associated characters could be either "metabolic" mutants or sporulation mutants blocked at a very early stage.

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