Sporulation in *Bacillus subtilis*. Theoretical and Experimental Studies in Continuous Culture Systems

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SUMMARY

A theoretical treatment of growth and sporulation of *Bacillus subtilis* Marburg strain 168 in continuous culture is given. Sporulation is considered as a probability event, and a specific rate constant for its initiation in vegetative cells is introduced. The processes of growth and sporulation have been assumed to compete with each other in the vegetative organism. Equations are given which allow calculation of the rate constants characterizing growth and sporulation in steady-state continuous-flow systems. The relationship between the specific rate of initiation of sporulation and the growth rate of vegetative cells was determined with glucose-limited cultures and was approximately linear; the spore initiation rate increased with decreasing growth rate. This result was used to obtain equations relating the incidence of refractile spores to the dilution rate of a continuous culture maintained in a steady state. A number of possible models of the process of spore initiation have been considered; two were consistent with the linear relationship between spore initiation rate and growth rate determined by experiment.

INTRODUCTION

Continuous cultivation techniques have been applied by numerous workers to the study of spore or cyst formation by bacteria. Málek (1952) and Macura & Kotková (1953) investigated the growth phases of *Azotobacter* in multistage continuous cultures; similar techniques were employed by Málek, Chaloupka, Vosyková & Vinter (1953) to study sporulation in *Bacillus pumilis*. Sporulation of *Bacillus cereus* has been used by Řičica (1969) as an example of product formation in multistage continuous cultures. In single stage chemostats a number of attempts to study sporulation have been made, for example, in *Bacillus megaterium* (Aubert, Millet & Castoriadis-May, 1961; Aubert, Millet & Schaeffer, 1965); in *Bacillus subtilis* (Kerravala, Srinivasan & Halvorson, 1964; Dawes & Mandelstam, 1969; Dawes, Kay & Mandelstam, 1969); in *Clostridium thermosaccharolyticum* (Hsu & Ordal, 1969). Ierusalimskii (1958) and Ierusalimskii & Rukina (1959) introduced a procedure whereby organisms growing in continuous culture could be viewed microscopically and thereby studied the reversibility of sporulation of clostridia and bacilli.

Despite this interest in spore formation in continuous culture, we are not aware of any attempt to develop a theoretical treatment of bacterial differentiation in continuous culture analogous to those carried out for vegetative bacterial growth, e.g.

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Monod (1950), Novick & Szilard (1950), Herbert, Elsworth & Telling (1956), Herbert (1959, 1961), among others. This neglect may be due partly to the greater complexity of the system and partly to practical problems which have been encountered in continuous cultivation of spore-forming organisms. These include the selection of asporogenous mutants (Aubert *et al.* 1961), the failure of some spore-forming bacteria to attain a steady state (Kerravala, *et al.* 1964) or the germination of all but about 10% of the spores formed in single stage systems (E. O. Powell, personal communication).

This paper examines sporulation in continuous cultures in which growth is a function of the concentration of a single growth-limiting substrate; the possible dependence of sporulation on growth rate is investigated. The treatment is based on proposed models of sporulating systems; these models are evaluated in terms of experimental data obtained from steady state continuous culture systems.

THEORY

General remarks concerning sporulation

To describe formally a culture of bacteria undergoing spore formation it is first necessary to consider the nature of the sporulation process and to categorize the various populations of bacteria present in a culture undergoing both vegetative growth and sporulation.

Initiation of vegetative organisms to sporulation is thought to occur when certain nutrients necessary for growth become depleted (Büchner, 1890; Knaysi, 1945; Grelet, 1946, 1952, 1957). However, Aubert *et al.* (1961) and Schaeffer, Millet & Aubert (1965) showed that, in certain bacilli, sporulation occurred to some extent in minimal media, even when the bacteria were growing exponentially at their maximum rate. This observation led Schaeffer *et al.* (1965) to propose that initiation of a bacterium to spore formation could be considered as a probability event, an hypothesis confirmed by experiments with continuous culture in which Dawes & Mandelstam (1969) found that bacteria were initiated to form spores with a probability dependent on the dilution rate.

Once initiated to spore formation the organisms pass through a sequence of morphological and biochemical changes including the production of protease, antibiotic, alkaline phosphatase, glucose dehydrogenase and dipicolinic acid and the appearance of refractility and heat resistance of the spores, etc. (Murrell, 1967; Mandelstam, 1969).

Microbiologists, particularly those interested in continuous culture, describe cultures in terms of 'organism' as the basic unit; that is, in terms of 'recognizably separate entities' (Powell, 1956). For a sporulating system, however, there is no *a priori* argument for adopting the organism level as the unit for quantitative representation of initiation to spore formation. In fact, members of the genus *Bacillus* can be found either as unicellular organisms, or as organisms containing more than one cell (up to multicellular filamentous forms). We have found that the majority of *Bacillus subtilis* (Marburg strain 168), under the conditions of continuous culture specified in this paper, are unicellular, some are bicellular and few occur as filamentous forms. Under favourable conditions every cell can form a spore (see Robinow, 1960) regardless of the number of cells in each organism. Under less favourable conditions

cells within a filamentous organism may behave differently with respect to initiation of sporulation.

For these reasons the cultures have been described in terms of cell as the sporulating unit in this paper.

Cell populations present in sporulating systems

To analyse a sporulating bacterial culture, we assume it consists of three populations: vegetative cells (population X) which have not been initiated to form spores and are undergoing the normal process of cell division (all members of X are assumed competent to form spores under appropriate conditions); population Y , which consists of cells that have become initiated to form spores, but which have not reached that stage of development in the sporulation sequence which is arbitrarily used as the criterion of spore formation (in this study the criterion is the appearance of refractility; once initiated, cells are presumed incapable of further division and do not contribute to increases in cell numbers); population Z , which have reached refractility or which have developed further (this class contains all free spores in addition to cells containing observable spores).

We use n_x , n_y and n_z to denote the numbers per unit volume of culture of the cell types X , Y and Z , respectively.

The spore initiation process

Little is known of the mechanism of initiation to sporulation of vegetative cells, particularly those in a growing culture. The theory developed in this paper is based on a treatment of spore initiation which resulted in the simplest mathematical description. Other feasible models (not given here) have been treated and lead to similar expressions.

Vegetative cells are treated as a single population which can undergo one of two competing processes: growth or sporulation

in which ν and κ are first-order rate constants. Once a cell has entered the spore formation sequence, we assume it always forms a mature spore at some later time and that there are no cells in the culture which are undergoing neither vegetative growth nor spore formation.

Next, the probability of initiation of a cell to sporulation before it divides is defined by ϕ . As would be expected, the relationship of the probability (ϕ) to the rate constants (ν and κ) depends upon the details of the particular treatment of the process of spore initiation chosen, although different models lead to similar expressions to that derived from the simple model outlined above, i.e.

$$\phi = \frac{\kappa}{\nu + \kappa}. \quad (1)$$

Kinetics of growth and initiation to sporulation

The growth of non-sporulating bacteria in terms of cell number n_x is usually described by the rate equation

$$\frac{dn_x}{dt} = \nu n_x, \quad (2)$$

where ν is the specific number growth rate.

Aubert *et al.* (1961) showed that bacteria formed spores during exponential growth, and that spores increased in proportion to the cell population. Thus a first order specific rate constant κ for the process of initiation of vegetative cells to sporulation may be defined such that:

$$\text{Rate of cell initiation to spore formation} = \kappa n_x. \quad (3)$$

Since we assume that growth and initiation are exclusive, the rate of increase of vegetative cells in a sporulating culture is given by

$$\frac{dn_x}{dt} = (\nu - \kappa) n_x. \quad (4)$$

Here ν is the specific growth rate of the cells if no sporulation were taking place. Increase in cell numbers is still exponential, but the effective growth rate is now $(\nu - \kappa)$.

Kinetics of spore germination

Most studies of the germination kinetics of spores have been carried out in cultures where an agent promoting germination has been applied suddenly (see Sussman & Halvorson, 1966). In such systems, Woese & Morowitz (1958) found that germination can be approximately represented by first order kinetics. In the chemostat, however, spores are continually exposed to any germinant which may be present, and are heterogeneous with respect to germination rate (see experimental section).

For a system composed of n spore fractions, each fraction Z_i germinating with first-order rate constant α_i , and comprising a proportion λ_i of the total spore population, the rate equation for the change in total spore concentration, n_z , at time t , due to germination is

$$\frac{d}{dt} [n_z(t)] = - \left[\sum_{i=1}^n \alpha_i \lambda_i(t) \right] n_z(t). \quad (5)$$

In a steady-state chemostat culture the proportion λ_i will be constant at a given dilution rate and an overall first-order rate constant α can be used to describe germination, i.e.

$$\alpha = \left[\sum_{i=1}^n \alpha_i \lambda_i \right]. \quad (6)$$

The values of α vary to some extent with dilution rate since the λ_i values vary with dilution rate.

Spore germination leads not only to a loss of spores from the culture, but contributes towards the vegetative cell population. There is a time lag between commencement of the germination process and outgrowth with the formation of a vegetative cell. This means that the rate of increase of vegetative cells at time t is not given by $\alpha n_z(t)$, but by $\alpha n_z(t - t_g)$ multiplied by a factor describing the loss of spores from the culture during the preceding time interval t_g (where t_g is the interval between the commencement of germination and cell outgrowth). For analysis of steady-state chemostat cultures it is reasonable to neglect this correction for washout since we found that at high dilution rates germination did not significantly contribute to any increase of vegetative cells, while at low dilution rates, where αn_z is significant, the correction for t_g is not important.

Growth and sporulation in the chemostat

The theoretical aspects of bacterial growth in single-stage homogeneous continuous cultivation systems given by Herbert *et al.* (1956) have been extended in the following treatment to the case of spore-forming bacteria.

Changes in the concentration of cell species. The completely mixed continuous culture system consists of a suitable vessel in which the culture is maintained at constant volume v . Sterile growth medium is fed into the vessel at constant flow rate f , and the vessel contents stirred so that there is uniform and rapid mixing. The dilution rate D is defined by

$$D = \frac{f}{v}. \quad (7)$$

The time dependence of the vegetative cell (number) concentration, n_x , is given by

$$\frac{dn_x}{dt} = (\nu - \kappa n_x) - Dn_x + \alpha n_z. \quad (8)$$

The number of initiated cells at time t , $n_y(t)$, is related to the values of κ and n_x during the previous time interval $t - t_0$ to t , since there is a finite time interval between initiation of sporulation and appearance of a recognizably refractile spore. This time interval t_0 we call the sporulation time. At time $t - \tau$, the rate of initiation to sporulation from equation (3) is

$$\kappa(t - \tau) n_x(t - \tau).$$

Of those cells initiated at time $t - \tau$, a fraction will wash out of the culture before reaching time t , i.e. before the cells reach an 'age from initiation' of τ . This fraction of cells washed out is given by the exponential factor

$$\exp\left(-\int_{t-\tau}^t D(\xi) d\xi\right).$$

Combining these last two expressions, at time t the number of initiated cells with ages between τ and $\tau + d\tau$ is

$$\kappa(t - \tau) n_x(t - \tau) \exp\left\{-\int_{t-\tau}^t D(\xi) d\xi\right\} \cdot d\tau$$

and $n_y(t)$ is given by:

$$n_y(t) = \int_0^{t_0} \kappa(t - \tau) n_x(t - \tau) \exp\left\{-\int_{t-\tau}^t D(\xi) d\xi\right\} d\tau. \quad (9)$$

Finally, the equation governing n_z , the number concentration of entities recognizable as spores, is required. Cells are not recognized as containing spores until they have reached an age from initiation of t_0 , hence the rate balance equation for $n_z(t)$ is given by

$$\frac{dn_z}{dt} = \kappa(t - t_0) n_x(t - t_0) \exp\left\{-\int_{t-t_0}^t D(\xi) d\xi\right\} - Dn_z - \alpha n_z. \quad (10)$$

Changes in the concentration of substrate. Monod (1942) showed, for non-sporulating bacteria, the existence of an empirical relationship between the rate of bacterial growth and the rate of substrate utilization:

$$\frac{dn}{dt} = -y \frac{ds}{dt}, \quad (11)$$

where n is the concentration of bacteria and y is known as the yield constant. This yield constant describes the rate of consumption of substrate by cells undergoing vegetative growth (Monod, 1950; Herbert *et al.* 1956).

In a culture undergoing sporulation as well as vegetative growth, growth-limiting substrate is undoubtedly consumed by cells which have been initiated to spore formation. However, the rate of consumption of substrate by initiated cells will depend on the age τ of the initiated cells. A function $\mathcal{C}(\tau)$ describing the rate of consumption of substrate by initiated cells $N_y(t, \tau) d\tau$ with ages between τ and $\tau + d\tau$ can be defined by letting the rate of consumption of substrate be

$$\mathcal{C}(\tau) N_y(t, \tau) d\tau. \quad (12)$$

From this, the total rate of change of substrate concentration by initiated cells is

$$\frac{ds}{dt} = D(s_R - s) - \frac{1}{y} \cdot \frac{dn_x}{dt} - \int_0^{t_0} \mathcal{C}(\tau) \kappa(t - \tau) n_x(t - \tau) \exp \left\{ - \int_{t-\tau}^t D(\xi) d\xi \right\} d\tau. \quad (13)$$

The steady state

In the following equations the practice of Herbert *et al.* (1956), who use a tilde to denote steady-state values, will be followed.

When steady-state conditions obtain, $(d/dt)(n_x)$, $(d/dt)(n_z)$ and (ds/dt) are zero, and ν , κ , and D are time invariant, and equations (8), (9) and (10) simplify respectively to

$$\nu - \kappa = D - \alpha \frac{\tilde{n}_z}{\tilde{n}_x}, \quad (14)$$

$$\tilde{n}_y = \frac{\kappa \tilde{n}_x}{D} [1 - \exp(-Dt_0)], \quad (15)$$

$$\tilde{n}_z = \frac{\kappa \tilde{n}_x}{D + \alpha} \exp(-Dt_0). \quad (16)$$

The total cell concentration, n_T , at steady state is given by:

$$\tilde{n}_T = \tilde{n}_x + \tilde{n}_y + \tilde{n}_z = \tilde{n}_x \left[1 + \frac{\kappa}{D} \left\{ 1 - \exp(-Dt_0) \right\} + \frac{\kappa}{D + \alpha} \exp(-Dt_0) \right]. \quad (17)$$

Finally, an equation relating substrate concentration to species concentrations can be obtained from equation (13) if the function describing the rate of consumption of substrate by initiated cells is known. These aspects will not be developed further in this paper.

An observable characteristic of sporulating systems is the number of refractile spores as a fraction (θ) of the total population present in the culture, defined by

$$\theta = \frac{n_z}{n_x + n_y + n_z}. \quad (18)$$

From equations (14), (15), (16) and (18), the following relations can be derived;

$$\tilde{\theta} = \frac{\kappa \exp(-Dt_0)}{D + \kappa + \alpha \left[1 + \frac{\kappa}{D} \left\{ 1 - \exp(Dt_0) \right\} \right]}, \quad (19)$$

$$\kappa = \frac{D\tilde{\theta}(D + \alpha)}{D[\exp(-Dt_0) - \tilde{\theta}] - \alpha\tilde{\theta}[1 - \exp(-Dt_0)]}, \quad (20)$$

$$\nu = D + \kappa \left\{ 1 - \frac{\alpha \exp(-Dt_0)}{D + \alpha} \right\}. \quad (21)$$

The probability of initiation of a vegetative cell to spore formation before it can undergo cell division has been assumed to be given by equation (1). Experimentally it is possible to measure $\bar{\theta}$, α and D , and t_0 can be obtained by examining the transient variation of θ with time on making a shift in dilution rate, e.g. Dawes *et al.* (1969). Thus application of equations (20), (21) and (1) allows experimental evaluation of κ , ν and ϕ , without the use of any assumptions concerning the detailed dependence of κ and ν on the substrate concentration, s , provided $\bar{\theta}$ values can be measured at particular dilution rates. However, in order to predict values for $\bar{\theta}$, κ , ν , and ϕ given D alone, it is necessary to determine the detailed dependence of κ on ν or of κ and ν on s . Moreover, these relationships must be available in order to begin to predict n_x , n_y , n_z , n_t and s as functions of D .

Variation of growth rate and spore initiation rate with substrate concentration. Some assumptions must now be made regarding factors affecting the specific rate constants ν and κ . We assume that both ν and κ depend only on the concentration of the limiting substrate at time t , and are not affected by the prevailing levels of n_x , n_y or n_z , or by the previous history of the culture. We further assume that the specific rate constant for the increase of cell numbers, ν (which describes the rate of division of the bacteria and not the rate of increase of vegetative cell numbers in the sporulating population), varies with the limiting substrate concentration as

$$\nu = \nu_m \left(\frac{s}{K_s + s} \right), \quad (22)$$

as was first demonstrated by Monod (1942) for non-sporulating systems; ν_m is the maximum specific growth rate of the bacteria at saturation levels of the limiting substrate, and K_s is a saturation constant.

It therefore remains to determine the variation of κ (or ϕ) with either ν or s . It has been shown that κ and ν can be obtained experimentally by measuring θ values at various dilution rates. It is therefore possible to determine empirically the variation of κ with ν and hence κ with s from equation (22).

METHODS

Organism. *Bacillus subtilis* (Marburg strain 168), auxotrophic for indole or tryptophan was stored as a spore suspension.

Continuous cultivation. The composition of the growth medium was as follows: 2.78 mM-glucose, 4.75 mM-sodium citrate, 0.1 mM-tryptophan, 9.5 mM-NH₄Cl, 20 mM-KH₂PO₄, 1 mM-NaNO₃, 1 mM-Na₂SO₄, 1 mM-KCl, 2 mM-CaCl₂, 0.0036 mM-FeCl₃, 0.041 mM-MgCl₂, 0.1 mM-MnCl₂; the pH was adjusted to 7.0 with NaOH. Growth under these conditions was limited by the concentration of glucose. Cultivation was carried out at 37° in a conical vessel of approximately 500 ml. capacity. The culture (about 100 ml.) was stirred by rapid aeration (1 l. min.⁻¹ of sterile-filtered humidified air). Problems of evaporation of the culture were avoided by saturation of the inlet air with water vapour. Sterile medium was fed into the vessel by means of a peristaltic pump. In order to maintain a near-continuous inflow of medium at low flow rates, an air-lock and a fine bore inlet were added to the feed line between the pump and inlet.

Inoculation of the chemostat was carried out as described previously (Dawes *et al.* 1969), and the culture allowed to reach equilibrium at various dilution rates. The

attainment of a steady state was determined by observing the turbidity of the culture at 600 nm. and by measuring the fraction of refractile spores in the culture.

Estimations. The incidence of refractile spores (θ) as a fraction of the total population was determined under the phase-contrast microscope, using cell samples taken from the chemostat and fixed immediately after sampling with formaldehyde. Samples were taken at intervals of about one generation time until there was no change in spore count; for these samples, sufficient cells were counted to include at least 100 spores: this corresponds to a 10% counting error.

The time taken for an initiated cell to form a refractile spore (t_0) was determined by observing the effect of a sudden decrease in dilution rate of the chemostat system, as described previously (Dawes *et al.* 1969).

No suitable technique was available for estimation of the specific germination rate (α) of spores under chemostat conditions. However, germination rates were obtained for various spore fractions (resolved by equilibrium density gradient centrifugation of a sample from a steady-state culture) by suspending the spores in the supernatant of a steady-state chemostat culture and determining at intervals thereafter the fraction of spores which appeared phase-dark in the phase-contrast microscope. This supernatant was obtained by rapidly filtering 50 ml. of culture through a membrane filter (Millipore, 0.65 μ pore size). From these data, plots of

$$-\ln \frac{n_z(t = t)}{n_z(t = 0)}$$

against time t were constructed for the various fractions and their germination rates determined from the slopes. The overall germination rate for the spore population was obtained from these rates and the proportions of spores present in each fraction using equation (6).

From equations (20), (21) and (1) it can be seen that small errors in measurement of dilution rates could lead to much larger errors in estimation of the parameters, κ , ν and ϕ . For this reason the flow rate of medium was obtained by measuring the time taken for 25 ml. of culture to flow from the chemostat. At least three series of measurements were taken at each steady state. The volume of culture in the chemostat vessel was measured directly after each experiment.

The maximum growth rate constant (ν_m) was determined by observing the rate of increase of bacterial mass (estimated by turbidity measurements) from a low inoculum into the chemostat vessel containing fresh sterile medium. Rates were estimated at relatively low bacterial concentrations (less than 0.1 mg. ml.⁻¹), the average of three determinations was taken as ν_m .

Turbidity of the culture was measured at 600 nm. using a SP 600 spectrophotometer. Measurements of pH, using an EIL 2320 pH meter, were taken as soon as possible after sampling from the culture.

Contaminants and asporogenous mutants were detected by plating samples of culture on various minimal-media plates. *Bacillus subtilis* 168 requires tryptophan for growth, hence contaminants were readily observed on tryptophanless minimal-media plates. Asporogenous mutants were detected as white colonies on minimal-agar plates containing tryptophan: the parent organism formed brown colonies after several days incubation at 37°.

Three spore fractions were resolved from a steady-state culture by density-gradient

centrifugation. An exponential gradient of Urografin was prepared over the density range 1.0 to 1.25, and the cells and spores obtained from a steady-state continuous culture sample (50 ml.; $D = 0.12 \text{ h}^{-1}$) by centrifugation were suspended in 1 ml. of the culture supernatant and were layered onto the gradient. Fractions were then obtained by centrifugation at 45,000g for 30 min.; one fraction pelleted due to its greater density ($> 1.25 \text{ g.cm.}^{-3}$) and was found to contain free spores on microscopic examination. Two other fractions containing spores were obtained in the gradient and found to consist of spores included in lytic cells (mixed with germinating phase-dark spores) and spores included in nonlytic cells.

Fig. 1. Sporulation in steady-state continuous culture of *Bacillus subtilis* 168. A continuous culture of *B. subtilis* 168 was allowed to reach steady state under glucose limitation at a dilution rate of 0.11 h^{-1} . Measurements of bacterial concentration (●) and refractile spore incidence (○) were taken at intervals over a 24 h. period, and were not found to differ significantly from straight lines of zero slope.

RESULTS

Stability of continuous cultures of Bacillus subtilis 168

Steady-state glucose-limited continuous cultures of *Bacillus subtilis* 168, undergoing sporulation as well as vegetative growth, were obtained over the range of dilution rates from 0.05 h^{-1} to 0.39 h^{-1} . These steady states were not maintained indefinitely with respect to spore formation, since asporogenous mutants appeared spontaneously and began to replace the sporulating organism to a significant extent after the steady state had been established for about 15 days.

Figure 1 gives an example of a steady-state culture (in which the dilution rate, D , was 0.11 h^{-1}) before the observable appearance of mutants; measurements were taken over a 24 h. period of the incidence of refractile spores (θ) and the turbidity of the culture. In general, therefore, experiments were not continued for longer than a week.

Parameters for treatment of data obtained from continuous cultures

Germination rate (α). Spores formed in continuous cultures were found, by examination, to be heterogenous with respect to their germination characteristics. Three spore fractions of differing densities were obtained and germination rates for these fractions each suspended in filtrate from a steady-state culture were obtained, as described in Methods. The plots of

$$-\ln \left[\frac{n_z(t)}{n_z(0)} \right] \text{ versus } t$$

are given in Fig. 2, the slopes of the straight lines in these plots were taken as estimates of the germination rates for the various spore fractions; these are given, together with the density range of the spores and the proportion of spores in each fraction, in Table 1.

Hence, as 'young spores' (those in nonlytic cells) germinate at faster rates than 'old spores', the specific germination rate for the population in the chemostat is not

Fig. 2. Rates of germination of various spore fractions. Three spore fractions resolved from a chemostat spore population by equilibrium density-gradient centrifugation (see text) were each suspended in the rapid membrane filtrate of a steady-state culture and the refractile spore incidence determined at hourly intervals. The plots suggested in the text for estimating first-order germination rates for each of these fractions are given for: ● spores from the most dense fraction; ▲ spores from the intermediate density fraction, and ■ from the least dense fraction. $n_z(t)$ is the number of refractile spores per unit volume at time t .

constant for all dilution rates since it can be shown that the ratio of 'young spores' to 'old spores' varies with dilution rate. However, as was discussed in the theoretical section, the correction for germination is not important at higher dilution rates. Hence the value calculated for the above low dilution rate sample from the data in Table 1 and equation (6) of $\alpha = 0.05 \text{ h.}^{-1}$ has been taken as a reasonable approximation for

representation of the system. All data were treated using a range of α values in order to determine the effect of variation of this parameter on the interpretation of results.

The time for the formation of refractile spores (t_0). The effect of imposing a sudden decrease in the dilution rate (from 0.4 h^{-1} to 0.05 h^{-1}) on the incidence of refractile spores in a steady-state chemostat culture is shown in Fig. 3. The incidence of refractile

Fig. 3. The time for the formation of a refractile spore, t_0 . At zero time the dilution rate of a steady-state culture at an initial value of 0.42 h^{-1} was changed to 0.05 h^{-1} , and the incidence of refractile spores (O) followed with time. At 3 h. a sudden change in the spore incidence began which was complete at 5 h. The average time for the change to occur (4 h.) has been taken as the time (t_0) for the formation of a refractile spore.

Table 1. *The variation of germination rate of spore fractions from a continuous culture sample*

Three fractions of the total spore population of a steady-state continuous culture sample ($D = 0.117 \text{ h}^{-1}$) were obtained by equilibrium density gradient centrifugation. The first order germination rate for each fraction suspended in the filtrate of a steady state continuous culture ($D = 0.123 \text{ h}^{-1}$) were obtained. Methods have been given in the text.

Type of spore	Density of spores (g. cm. ⁻³)	Germination rate (h. ⁻¹)	Proportion of spores
Spores included in non-lytic cells	1.17 to 1.22	0.092	0.39
Spores included in lytic cells	1.23 to 1.25	0.042	0.19
Spores free from parent cells	> 1.25	0.024	0.42

spores began to increase 3 h. after the change, and reached a new steady-state value approximately 5 h. after the change. The average time for the increase in refractile spores (4.0 h.) has been taken as the average time for formation of a refractile spore after initiation of a vegetative cell.

The maximum growth rate constant (ν_m). For balanced growth under batch conditions, the mass growth rate and the number growth rate, ν , must have the same value,

Table 2. *The effect of dilution rate on spore incidence in steady-state continuous culture of Bacillus subtilis 168*

The continuous culture apparatus and experimental details are described in the text. Measurements were taken at each dilution rate until steady state was reached, and each row of figures refers to a different steady state. Six different chemostat runs were necessary to obtain this data, and the values obtained from each run are denoted by the superscripts a, b, ...f.

Dilution rate (D) h. ⁻¹	Refractile spore fraction (θ)	pH of culture pH units	Turbidity of culture 600 nm.
0.054 ^c	0.3060	7.9	1.18
0.055 ^a	0.3310	7.9	1.03
0.102 ^e	0.1995	7.7	1.00
0.111 ^b	0.1820	7.9	1.16
0.115 ^b	0.1750	7.9	1.04
0.116 ^c	0.1880	—	—
0.123 ^d	0.1690	7.8	1.05
0.132 ^d	0.1219	7.8	1.02
0.161 ^a	0.1190	7.9	0.98
0.194 ^e	0.0750	—	0.94
0.222 ^b	0.0675	7.5	0.86
0.257 ^d	0.0418	7.5	0.91
0.261 ^b	0.0501	7.4	0.86
0.269 ^d	0.0440	7.2	0.90
0.280 ^c	0.0347	7.6	0.94
0.299 ^c	0.0262	7.7	0.96
0.303 ^d	0.0295	7.6	0.91
0.307 ^d	0.0307	7.6	0.85
0.380 ^f	0.020	7.4	0.74
0.387 ^b	0.013	7.2	0.68

Table 3. *Calculated data for steady-state cultures of Bacillus subtilis 168*

Each row of figures refers to the values for the variables calculated from equations (1), (20) and (21) and the data in Table 2 for each different steady state.

Dilution rate (D) h. ⁻¹	Specific growth rate (ν) h. ⁻¹	Specific initiation rate (κ) h. ⁻¹	Probability of initiation (ϕ)
0.054	0.099	0.0721	0.422
0.055	0.107	0.0844	0.440
0.102	0.156	0.0698	0.309
0.111	0.165	0.0679	0.291
0.115	0.169	0.0673	0.284
0.116	0.178	0.0762	0.300
0.123	0.181	0.0705	0.280
0.132	0.174	0.0496	0.222
0.161	0.217	0.0644	0.229
0.194	0.237	0.0477	0.167
0.222	0.272	0.0547	0.167
0.261	0.311	0.0527	0.145
0.257	0.296	0.0414	0.123
0.269	0.314	0.0481	0.133
0.280	0.318	0.0399	0.111
0.299	0.331	0.0335	0.092
0.303	0.341	0.0393	0.103
0.307	0.347	0.0422	0.108
0.380	0.423	0.0437	0.094
0.387	0.415	0.0287	0.065

Hence the mean value (of three observations) obtained for the maximum mass growth rate as described in the Methods section (0.487 h^{-1}) was taken as the maximum growth rate constant, ν_m .

Quantitative data for steady-state cultures at various dilution rates

The steady-state data obtained from six separate runs in the same chemostat are summarized in Table 2, and the calculated values of κ , ν and ϕ for each steady-state sample are given in Table 3 and are plotted against the dilution rate (D) in Fig. 6, 7 and 8. Fig. 5 gives the values of θ against D . For calculation, the values $\alpha = 0.05 \text{ h}^{-1}$ and $t_0 = 4.0 \text{ h.}$ were used. A plot of κ versus ν is given in Fig. 4. This was found to be approximately linear.

Regression analysis gave the relationship

$$\kappa = 0.091 - 0.143\nu. \quad (23)$$

Fig. 4

Fig. 5

Fig. 4. Variation of the rate of sporulation initiation with growth rate of *Bacillus subtilis* 168 in steady-state continuous culture. Values for the rate of sporulation initiation (κ) and the growth rate (ν) obtained experimentally from glucose-limited continuous cultures at different steady-state dilution rates were plotted against each other. The continuous line is that obtained from a linear regression analysis of κ on ν , and is represented by equation (23).

Fig. 5. The relationship between the refractile spore incidence at steady state in a continuous culture and dilution rate. Data for the refractile spore incidence given in Table 2 are plotted against dilution rate for comparison with the theoretical relationship predicted from equations (19), (21) and (23). Open circles represent experimental points, each point represents the refractile spore incidence in the culture after a particular steady state had been established. The continuous line represents the theoretical curve.

Test of the theoretical treatment

In addition to the calculation of κ , ν and ϕ values using the observed values for α and t_0 , various combinations of α values (from 0 h^{-1} to 0.1 h^{-1}) and t_0 values (from 3.0 h. to 5.0 h.) were used in treatment of the data in Table 2 in order to evaluate the

effect of errors in measurement of these parameters on the interpretation. If an α value of 0.005 h^{-1} or less is used in the calculation of κ and ν , the resulting values of κ are almost constant over the range of dilution rates employed. This situation is, however, unlikely since germinating spores have been observed in steady-state cultures by phase-contrast or electron microscopy in numbers which indicate that the α value must be close to that used to calculate the data in Table 3, i.e. $\alpha = 0.05 \text{ h}^{-1}$.

Fig. 6

Fig. 7

Fig. 6. The quantitative relationship of the rate of sporulation initiation to dilution rate of a steady-state continuous culture of *Bacillus subtilis* 168. Values for the sporulation initiation rate (κ) calculated from the experimental data as described in the text are plotted against dilution rate (D). The open circles correspond to measurements taken on a culture after a particular steady state had been established (see Table 3). For comparison, the theoretical curve obtained from equations (21) and (23) is given as the continuous line.

Fig. 7. The quantitative relationship of the growth rate to the dilution rate of a steady-state continuous culture of *Bacillus subtilis* 168. Values for the growth rate (ν) calculated from the experimental data as described in the text are plotted against the dilution rate (D). Each open circle corresponds to a particular steady state, and the theoretical curve obtained from equations (21) and (23) is given as the continuous line.

Prediction of θ , κ , ν and ϕ values. With knowledge of the two constants arising from the linear relationship between κ and ν , it is possible to predict θ , κ , ν and ϕ values, given the dilution rate D , without knowing the values for either the yield constants for substrate utilization or the saturation constant for growth rate (K_s) using equations (21) (23), (19) and (1). By substitution of equation (23) in equation (21), ν can be obtained as a function of D and the constants α and t_0 , and from this the variation of κ , θ and ϕ follow directly from the above equations.

Theoretical curves for θ , κ , ν and ϕ against D have been obtained from $\alpha = 0.05 \text{ h}^{-1}$, $t_0 = 4.0 \text{ h}$, $\nu_m = 0.487 \text{ h}^{-1}$, and using equation (23). These have been plotted in Fig. 5 to 8 together with the experimental data. It can be seen that good quantitative agreement exists between the theoretical predictions and the experimental values.

Fig. 8. The quantitative relationship of the probability of initiation of a vegetative cell to sporulation to the dilution rate of a steady-state continuous culture of *Bacillus subtilis* 168. Values for the probability of spore initiation (ϕ) calculated from the data of Table 2 as described in the text are plotted against the dilution rate (D). Open circles correspond to particular steady states, and the continuous line represents the theoretical curve obtained from equations (1), (21) and (23).

DISCUSSION

In the sporulating system, it is clear that a single yield constant will not, in principle, be sufficient to describe the substrate utilization of the very heterogeneous population. A theoretical basis for a description of substrate utilization in a sporulating culture has been briefly discussed, although in the absence of good data concerning substrate levels prevailing in the culture vessel, these aspects of the problem were not pursued further. The concentration of limiting substrate was not measured due to the difficulty of obtaining accurate data at low dilution rates; such a difficulty was encountered in experiments with glycerol limitation in chemostat cultures of *Aerobacter cloacae* (Herbert *et al.* 1956). We must state at this point that this lack of data about substrate concentrations led us to assume (without direct experimental verification) that Monod's relation, equation (22), describes the dependence of growth rate (ν) on the substrate concentration (s). This assumption does not affect the conclusions drawn concerning the linear dependence of the specific rate of spore initiation on growth rate, nor is it implicit in the treatment of the second model of spore initiation which appears in the following discussion. Nor is this assumption required for prediction of the observed variation of κ , ν , θ and ϕ with dilution rate. The assumption has been made in the first model of spore initiation.

A new and important parameter in the present treatment is the specific rate constant κ , which describes the process of initiation of vegetative cells to spore formation. Assumptions which simplify the relation of κ to growth and probability of sporulation were chosen in order to keep the mathematics simple, and those assumptions leading to more complicated mathematics added nothing.

With all these assumptions (all of which can be criticized) we have shown that for *Bacillus subtilis* 168 cultures under the conditions specified here, the relationship between the spore initiation rate κ and the cell division rate ν is approximately linear and is of the form of equation (23)

$$\kappa = a - b\nu,$$

where $a = 0.091 \text{ h}^{-1}$ and $b = 0.143$.

Models of the spore initiation process

The empirical relationship found between the spore initiation rate and the growth rate provides a method for testing models of the spore initiation process. We have found two very different models of spore initiation to predict the observed behaviour and these are set out briefly below.

Model 1. Repression model. There have been several suggestions made in the literature that sporulation in bacilli is controlled by the level of intracellular metabolite(s), see Schaeffer *et al.* (1965), Mandelstam (1969), Hsu & Ordal (1969). Schaeffer *et al.* (1965) drew an analogy between sporulation control and catabolite repression. The following repression system is analogous to the mechanism of control of the synthesis of an inducible enzyme postulated by Jacob & Monod (1961). Its postulates are:

(i) Spore formation is initiated by a repression system in which an 'effector' metabolite (E) reacts with a target, T_1 (say a protein)

and the molecule $T_1 E$ reacts with a second target T_2 (say a site on the chromosome)

(ii) Both reactions are reversible and in equilibrium.

(iii) The concentration of the effect or metabolite is a function of the limiting substrate concentration, s . The concentrations of the 'targets' ($[T_1]$ and $[T_2]$) do not vary with s .

(iv) The probability of initiation of a cell to spore formation is proportional to the fraction of T_2 target sites unoccupied at any particular instant, i.e.

$$\phi = \frac{[T_2]}{[T_2] + [T_1 T_2 E]}.$$

(v) The concentration of effector metabolite $[E]$ is proportional to the limiting substrate concentration, s .

By straightforward application of these postulates the probability ϕ can be shown to vary with substrate concentration as

$$\phi = \frac{a + b}{a + bs},$$

where a, b are constants derived from the concentrations of the targets and the equilibrium constants for the reactions postulated in (i) and $b = 1$.

From this, by means of equations (1) and (22), the linear relationship, equation (23), of κ to ν can be derived.

Model 2. Initiation occurs at constant frequency in cells containing nonreplicating chromosomes. This model was suggested by Professor J. Mandelstam (personal communication) as an interesting alternative to repression models. Its postulates are:

(i) Cells can be initiated to spore formation only when they contain nonreplicating

chromosomes. (This assumption leaves open the question of what control mechanism prevents expression of the spore genes when DNA-replication is occurring.)

(ii) The 'time gap' between successive rounds of DNA replication increases linearly with generation time for generation times in excess of a minimum DNA-replication time τ_{rep} . Initiation occurs at constant rate in cells containing nonreplicating chromosomes.

Using these postulates it can be shown that

$$\kappa = \gamma(1 - 1.44\tau_{\text{rep}}\nu),$$

i.e. a linear relationship of the form (equation (23)):

$$\kappa = a - b\nu.$$

If this model were correct, the constants (a and b) determined empirically would enable one to estimate the minimum DNA-replication time τ_{rep} . From the data in the experimental section τ_{rep} would have a value of 1.09 h. (cf. 0.7 h. for *Escherichia coli* found by Lark, 1966, and Helmstetter & Cooper, 1968).

Neither of the above models may in fact represent the actual process of spore initiation. At the moment, evidence obtained from other studies tends to support hypotheses based on the involvement of 'inhibition' of sporulation by small molecule metabolites. For example, growth limitation of bacilli in continuous culture by means of an auxotrophic requirement or an essential ion leads to much lower incidences of spores than that found under conditions of carbon or nitrogen limitation (Aubert *et al.* 1965; Dawes & Mandelstam, 1969). However, it is possible that 'time gaps' between DNA replications vary differently with growth rate when different substrates limit growth. No conclusive evidence in support of either hypothesis (or any other) is currently available. The results presented here do, however, provide a method for testing the validity of hypotheses concerning the control of sporulation initiation.

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