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Conditions controlling commitment of differentiation in *Bacillus megaterium*

(development/sporulation/nutrient effects)

ELISABETH B. FREESE, PETER COONEY, AND ERNST FREESE

National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Department of Health, Education and Welfare, Bethesda, Maryland 20014

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ABSTRACT **The developmental stage at which cells of *Bacillus megaterium* are committed to continue differentiation, i.e., sporulation, depends on both the previous growth medium and the new medium to which cells are transferred for the commitment test. The latest "stage of no return," after which cells continue differentiation, no matter how rich in nutrients the medium, is reached as soon as the forespore is completely surrounded by a double membrane.**

In both uni- and multicellular organisms, cells begin structural and functional differentiation at about the time at which they have stopped their multiplication. At some stage of their development they become "committed" to continue differentiation even when they are exposed to a new environment in which they would originally have continued to duplicate. The seemingly sudden development of this commitment with respect to a given environment has created the impression that a single cellular process may trigger the commitment with respect to all possible environments in which cells could otherwise duplicate. This trigger could involve the production or destruction of one cell component [e.g., a protease (1) or an altered RNA polymerase (2)]; alternatively, it could be represented by the last and in some respect asymmetric cell division which often occurs when cells are exposed to conditions under which they begin differentiation (3, 4). We will show in the following that this notion of a single trigger is incorrect at least for the sporulation of bacilli, because the developmental stage at which commitment occurs depends on the media from and to which cells are transferred. Nevertheless, a developmental stage exists beyond which no known combination of compounds supporting cell replication can arrest the sporulation process; but this stage occurs later than the last (asymmetric) septation and is represented by the complete enclosure of the forespore cell with a double membrane through which active transport presumably cannot proceed.

MATERIALS AND METHODS

Bacillus megaterium ATCC 19213 was used because its refractile spores can be easily observed. For each experiment, spore samples were heat-shocked at 75° for 10–15 min, grown on nutrient agar plates for 24 hr at 30°, and shaken at 30° in ½ strength nutrient broth before inoculation into the growth media. Nutrient sporulation medium was used as described previously (5), or it was supplemented with 0.5% casein hydrolysate (of Nutritional Biochemicals Corp.) (NSMC) or with 0.5% sucrose (NSMS). Minimal medium (MS) was the same as that used by Greene and Slepecky (6), but contained as carbon source 0.1% of carbohydrates or 0.2% of aspartate or glutamate, neutralized by KOH. To determine the frequency of commitment, the cultures were diluted 5-fold into a new medium at different times during development, shaken at 30°, and at t_{10} (of the undiluted control culture) samples were frozen and stored at −20°. After thawing (within 3 days), the titer of refractile spores was determined in a Petroff-Hausser chamber, using a Zeiss phase-contrast microscope.

To determine the morphological stage at which commitment occurred, cell samples were taken from the original culture at different times, fixed with glutaraldehyde, postfixed with osmium tetroxide, and stained with uranyl acetate and lead citrate as described previously (7). For each sample 300 to 700 longitudinally sectioned cells were analyzed under the electron microscope (Philips EM201).

RESULTS

Sporulation after cell transfer to a fresh medium

When cells of *Bacillus megaterium* were grown in a nutrient sporulation medium (NSM), their titer increased exponentially up to a time t_0 after which the rate of cell multiplication gradually declined to zero, as can be seen by the change in the optical density at 600 nm (OD_{600}) (Fig. 1). In a minimal sporulation medium, the transition between the exponential cell increase and the nongrowing (developmental) phase is more abrupt but otherwise the timing of spore development is similar (6). After t_0, developmental changes occur, leading to the formation of intracellular particles (spores) which are refractile (bright in phase contrast), causing a slight increase in the OD_{600}, and which later develop resistance to organic solvents and heat. After their release from the mother cells, these spores can germinate and then resume growth in the proper medium. The increase in the titer $[P_0(t)]$ of phase-bright spores, still located in their mother cell, is shown in Fig. 1. All cells capable of forming phase-bright spores had done so by t_{10}, i.e., 10 hr after t_0. We shall designate time t_d as the time during development at which the cells are exposed to new culture conditions, e.g., owing to dilution in a fresh medium. At that time t_d, a certain fraction of the cells [frequency $c(t_d)$] are committed to continue their development in the new medium; all or some of the noncommitted cells resume growth and later, upon nutritional exhaustion of the dilution medium, again start the sporulation process. The titer $[P(t_d,t)]$ of phase-bright spores observed at time t increased, therefore, in two rounds. The first round, resulting from the sporulation of cells committed at the time t_d, occurred at about the time at which cells would have sporulated originally, while the second round occurred when the cells that had resumed growth spo-

Abbreviations: MS, minimal medium; NSM, nutrient sporulation medium; NSMC, NSM + casein hydrolysate; NSMS, NSM + sucrose; t_n, time (in hours).

rulated too. This is shown in Fig. 1 for a 5-fold dilution of cells in NSM (at t_3). Transfer of cells to fresh medium at later times t_d produced an increasing frequency of committed cells; since the fraction of uncommitted cells was then smaller, the lag between the first and the second round of sporulation increased correspondingly. It is apparent from Fig. 1 that the time t at which the titer of phase-bright spores is measured can be chosen so that the first round of sporulation is complete, whereas, the contribution of the second round of sporulation is negligible. At that time [e.g., t_{10}], the titer $[P(t_d,t_{10})]$ of phase-bright spores in the diluted culture would be equal to the titer in the original culture, multiplied by the frequency of commitment $[c(t_d)]$ and divided by the dilution factor [$D = 5$ in our case]:

$$P(t_d,t_{10}) = c(t_d) \cdot P_0(t_{10})/D$$

Instead of recording separately the titers of phase-bright spores for the experimental and the control culture, we will plot more conveniently the ratio:

$$r(t_d,t_{10}) = P(t_d,t_{10})/(P_0(t_{10})/D)$$
$$[= c(t_d) \text{ if second round is negligible}]$$

This "sporulation ratio" is equal to the frequency $[c(t_d)]$ of committed cells, provided that the committed cells in the diluted culture have sporulated at t_{10} to the same extent as the original culture and that the effect of the second round of sporulation can be disregarded. Our results show that at t_{10} both assumptions are justified (Fig. 1).

Relation of commitment to membrane development

To determine the timing of commitment with respect to transfer to different media, cells were grown in NSM and at different times of development were 5-fold diluted in four different media, i.e., the minimal salt medium MS + sucrose, the original medium NSM, and two richer media NSM + casein hydrolysate (NSMC) and NSM + sucrose (NSMS). Fig. 2 (broken lines) contains a plot of the sporulation ratios observed with the four different transfer media. Cells were committed early with respect to transfer to MS + sucrose, later with respect to NSM and latest with respect to NSMC or NSMS. These sporulation ratios, representing the commitment frequencies $c(t_d)$, were compared to the frequencies of different stages of prespore membrane development which cells had reached in the original NSM medium at time t_d. For this purpose, samples of the cells (in the original NSM medium) had been taken at different times t_d for a statistical evaluation under the electron microscope. For this analysis of ultra-thin sections, earlier definitions of the stages of membrane development during sporulation were used (8–10). Since stage III proved to be critical, it was necessary to subdivide it into three sub-stages. The stages distinguished here are shown in Fig. 3 and sketched in Fig. 4. During stages O and I, cells contain no visible prespore membrane and show either no distinct DNA pattern (stage O) or a distinct axial filament (stage I). These two stages were not always distinguishable and were therefore combined. Stage II cells contain a developing or completed asymmetric septum, which in contrast to cell division septa is very thin (containing only small amounts of cell wall material). Stage IIIa starts with a slightly curved prespore membrane and ends when the membrane has engulfed the prespore compartment by 50%. During stage IIIb the engulfment has proceeded beyond 50% up to almost complete

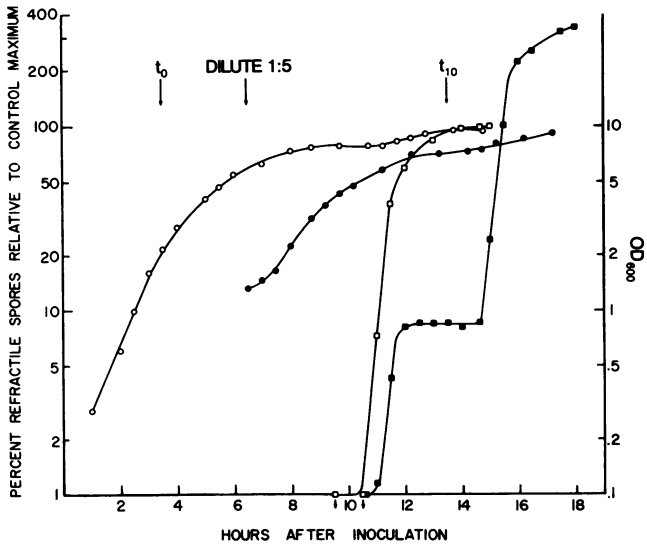

FIG. 1. Growth and sporulation of *Bacillus megaterium* grown in NSM and of cells diluted 5-fold in fresh NSM at t_3. Circles = growth (measured by OD_{600}); squares = sporulation (percentage of refractile spores). Hollow symbols = original culture (without dilution); full symbols = diluted culture.

enclosure of the prespore cell. Only when the engulfment was complete, producing an isolated forespore whose membrane was no longer attached to the mother cell, was a developmental stage called IIIc. The beginning of stage IV, during which the cortex develops between the double membrane (9), can not be clearly distinguished from IIIc; but this was not important here, since only the cumulative frequencies of developmental stages were used. The "cumulative frequency" of a stage (written as the stage abbreviation followed by a + sign) is defined as the frequency of that stage and all later developmental stages together. For example, the cumulative frequency IIIc+ includes all cells in which the membrane has completely engulfed the prespore, plus any later stages of spore development.

Fig. 2 shows the results obtained from the statistical analysis of sporulation stages for cells grown in NSM. The frequency of cells at stages O/I decreased while the cumulative frequencies of stage II+ and of the three stages IIIa+, IIIb+, and IIIc+ increased sequentially. Eventually, almost all (94%) of the cells produced asymmetric septa and later stages (i.e., stage II+). Since Fig. 1 indicated that only 80% of the cells later contained phase-bright spores, a small fraction of the cells apparently aborted their development at some time after prespore septation. It is instructive to compare the times at which cells developed different sporulation stages with the times at which they were committed with respect to different transfer media. If portions of the culture were 5-fold diluted, at different times t_d, in MS + sucrose, commitment occurred before any prespore septum had been formed in the original culture. If portions of the culture were transferred to NSM, commitment occurred slightly after the time of septation. But if the transfer was made into the richest media, NSMC and NSMS, commitment clearly coincided with the cumulative frequency [IIIc+] of cells that had reached at least stage IIIc of development. This commitment with respect to transfer from NSM to NSMC (or NSMS) was the latest commitment observed with respect to any nutrient combination.

The same type of analysis was performed for cells grown in and transferred to various other media. When cells were

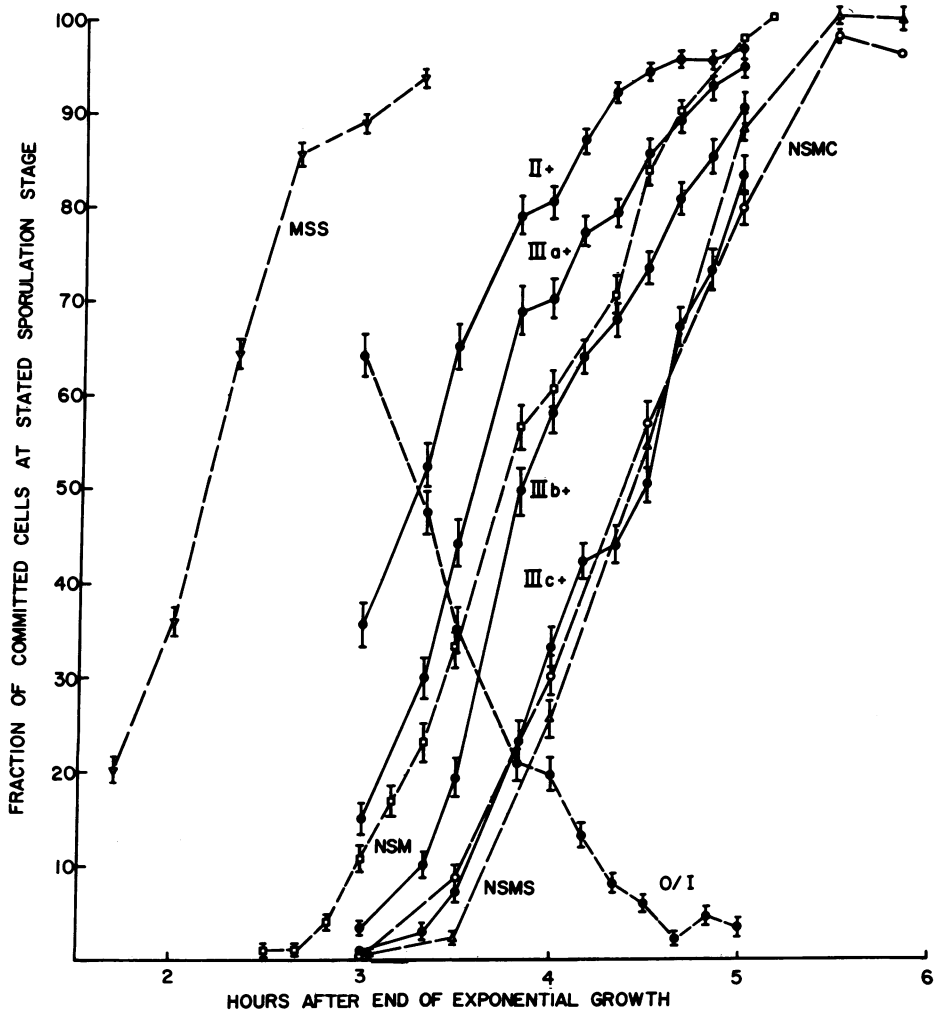

FIG. 2. Comparison of commitment times with membrane stages of sporulation. *B. megaterium* was grown in NSM. Sporulation ratios $r(t_d,t_{10})$ were determined at t_{10} in cultures 5-fold diluted at different times (t_d) in four different media: ▽- -▽, MSS; □- -□, NSM; ○- -○, NSMC; △- -△, NSMS. In the original culture, the morphological stages of sporulation were observed in thin sections under the electron microscope (for definition of stages see Figs. 3 and 4). The cumulative frequency of cells having developed to the given stage (Fig. 4) or beyond is shown by the solid (broken for O/I) curves (full symbols). Vertical bars = standard deviation $[F(1 - F)/N]^{1/2}$ with F = frequency of cells of given property and N = total number of cells inspected. MSS = MS + sucrose; NSMS = NSM + sucrose; NSMC = NSM + casein hydrolysate.

grown in MS + sucrose, commitment with respect to transfer to MS + sucrose again occurred about 1 hr before the cells had formed prespore septa. Other experiments, not utilizing electron microscope examinations, have shown that commitment with respect to transfer to MS + glucose or glycerol occurred at the same time as that with respect to sucrose; commitment with respect to MS + aspartate or glutamate occurred still about 1 hr earlier (Cooney and Freese, in preparation). The commitment with respect to dilution in NSMC coincided approximately with the time at which cells had formed prespore septa (stage II+). Finally, when cells were grown in half strength NSM containing yeast extract (0.05%) and glucose (0.1%), cells were committed with respect to 5-fold dilution in the same medium at the septation stage (II+) and with respect to transfer to that medium containing also casein hydrolysate (0.5%) and glucose (0.2%) at the engulfment stage (IIIb+).

DISCUSSION

For many species of *Bacillus* it has been observed that dilution in fresh medium or addition of nutrients can interupt the sporulation process up to a certain time of development after which the cells are committed to continue their differentiation (7, 11–14). Since usually only one medium was thoroughly tested, commitment appeared to occur at a certain time of development, in agreement with the trigger idea. However, it was already surprising that the observed commitment occurred at different times of development in different bacilli. Furthermore, in *B. subtilis* at least two times of commitment had been observed, one before septation (at t_0) with respect to glucose addition (15) and the other at stage III of development with respect to dilution in fresh nutrient sporulation medium (12).

Our results show clearly that the developmental stage at which cells are committed with respect to media changes depends on both the medium in which the cells are initially grown and on the medium to which they are transferred for the commitment test. Thus, one can state that the better the cells were adapted to utilize a complex medium, with respect to the entry and metabolism of the different nutrients, the later they were committed. Transfer to any minimal medium always produced early commitment. Transfer to a rich medium (NSMC) showed later commitment, which still oc-

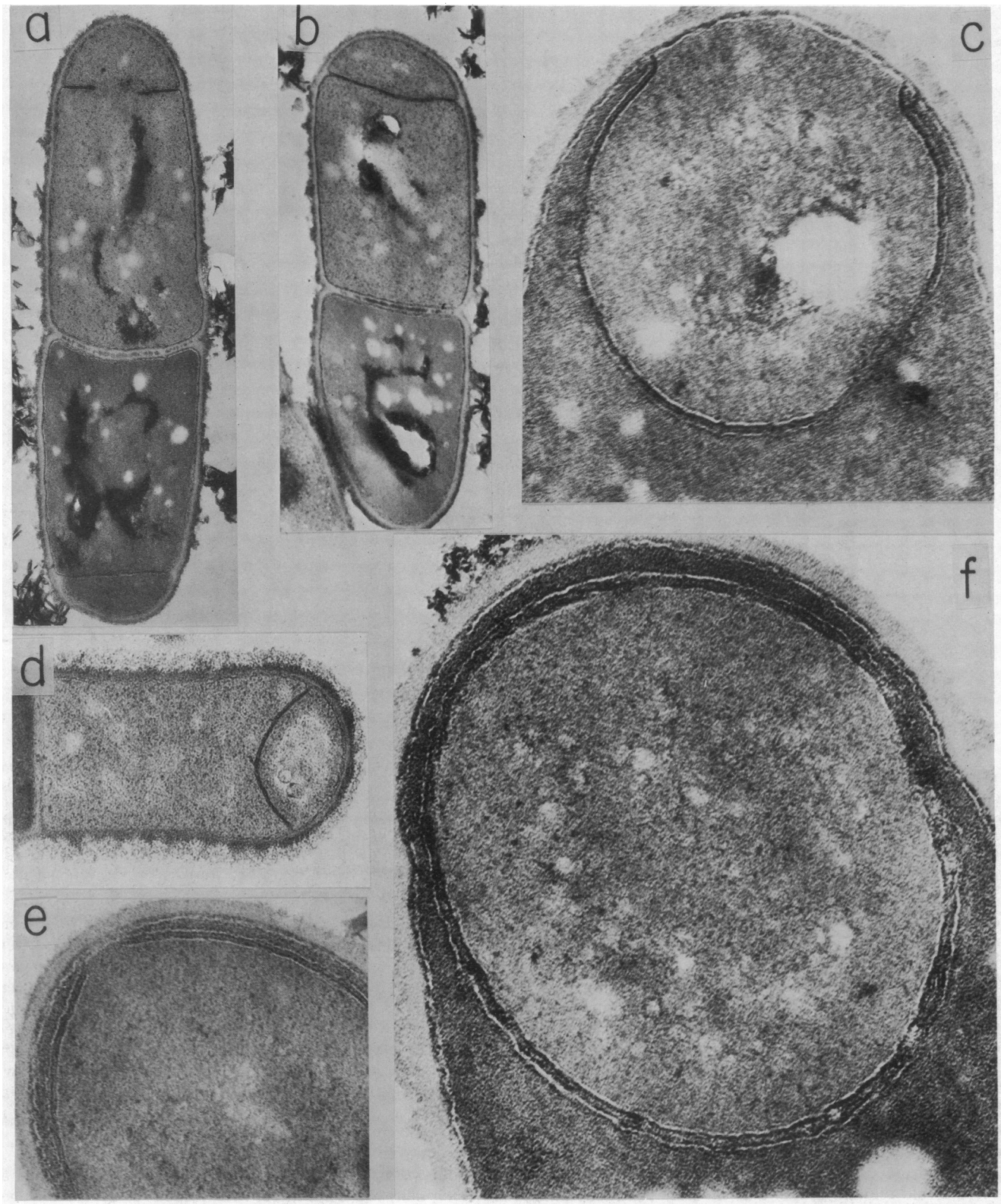

FIG. 3. Electron micrographs of cells at different stages of sporulation. *a,b* = stage II; *d* = IIIa; *c,e* = IIIb; *f* = IIIc. Magnifications: *a,b,d* = ×30,000; *c,e* = ×90,000; *f* = ×120,000.

curred earlier when the cells were transferred from a minimal medium (MS + sucrose) than from a rich medium (NSM).

The sequential development of commitment with respect to different nutrients must reflect a sequential change of cellular properties, such as decrease of the different transport mechanisms for nutrients, inactivation of certain enzymes needed to convert one metabolite into another, etc. In *B. subtilis*, the specific activity of the glucose–phosphoenolpyruvate transferase system, which is responsible for glucose

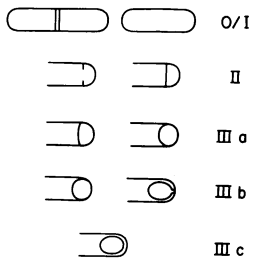

FIG. 4. Definition of the morphological stages used.

uptake and phosphorylation, has indeed been shown to decrease during the time during which cells become committed with respect to glucose addition (15). The mechanisms responsible for the commitment of *B. megaterium* with respect to different carbon sources remain to be investigated.

Since commitment with respect to any single compound occurs early, before prespore septation, the combination of different compounds is clearly more efficient in supplying the cells with all metabolites needed to stop development and to enable the resumption of growth (in all or some cells). This higher nutritional efficiency of a combination of compounds is demonstrated already by the higher growth rate of vegetative cells in a complex medium rather than a minimal medium. This effect is apparently enhanced during the developmental period at a time at which most compounds may be poorly transported and metabolically only slowly interconvertible; the supply of many compounds can then provide all needed metabolites (at intracellular concentrations sufficient to support growth) up to a stage of development at which the supply of only one or two such compounds is insufficient.

There is no apparent relationship between the development of commitment with respect to single or multiple nutrients and the membrane septation and engulfment. However, the latest commitment time, which was observed with respect to transfer from a rich (NSM) to a very rich medium (NSM + casein hydrolysate or NSM + sucrose), coincided with the closure of the prespore double membrane. As has been pointed out earlier (15), the engulfment process shows that the two membranes of the forespore would actively transport in opposite directions; consequently, after the prespore has been completely engulfed, active transport through both membranes should be no longer possible (unless one membrane develops pores) and substrates should enter the forespore cell only by the slow process of facilitated transport. This picture agrees with our observation that the sporulation process can no longer be stopped by any combination of nutrients after the forespore is completely engulfed by the double membrane.

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