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Extracellular control of spore formation in *Bacillus subtilis*

(differentiation/cell density/pheromones/gene expression)

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ABSTRACT **Spore formation in the Gram-positive bacterium *Bacillus subtilis* has been classically viewed as an example of unicellular differentiation that occurs in response to nutritional starvation. We present evidence that *B. subtilis* produces an extracellular factor(s) that is required, in addition to starvation conditions, for efficient sporulation. This factor is secreted and accumulates in a cell density-dependent fashion such that cells at a low density sporulate poorly under conditions in which cells at a high density sporulate efficiently. Conditioned medium (sterile filtrate) from cells grown to a high density contains this extracellular differentiation factor (EDF-A) and stimulates spore formation of cells at low density under normal starvation conditions. EDF-A is heat-resistant, protease-sensitive, and dialyzable, indicating that it is at least in part an oligopeptide. Production of EDF-A is reduced or eliminated in *spo0A* and *spo0B* mutants, which are defective in many processes associated with the end of vegetative growth. Mutations in *abrB*, which suppress many of the pleiotropic phenotypes of *spo0A* mutants, restore production of EDF-A.**

Differentiation in prokaryotes often involves communication between cells. This communication is mediated by a variety of chemical signals or pheromones (1). In myxobacteria, multiple pheromones are involved in several stages of development (2, 3). Bioluminescence in *Vibrio* is regulated by production of an autoinducer. The autoinducer of *Vibrio fischeri* has been identified as a complex lactone (cited in ref. 1). Mating pheromones of *Streptococcus faecalis* are peptides produced by potential recipient cells, which elicit a response from the potential donor cells (4). Streptococcal species (*Streptococcus pneumoniae*) also secrete a small protein that is required to induce genetic competence (5). The most well-studied pheromone of *Streptomyces* is A factor, which is produced by *Streptomyces griseus* and is required for streptomycin biosynthesis and sporulation (6).

In contrast, sporulation in *Bacillus subtilis* has been classically viewed as a unicellular process in which individual cells respond to conditions of nutrient deprivation. Sporulation has been thought to occur in the absence of any intercellular communication. A factor has been described that, when added to cells in nonnutrient medium, stimulates sporulation of *Bacillus cereus* and *B. subtilis*. This "endogenous factor" or sporogen was isolated from cell extracts and may be involved in the intracellular regulation of sporulation (7–9). We present evidence that there is communication between cells of *B. subtilis* during sporulation and that this extracellular signaling is required for efficient sporulation.

Rapidly growing *B. subtilis* cells can be induced to differentiate into a dormant cell type, the endospore, upon deprivation of an essential nutrient, carbon, nitrogen, or phosphorous (see refs. 10–12 for reviews). Formation of the mature heat-resistant spore takes approximately 8 hr from the initial time of starvation (t_0). The sporulation process is characterized by numerous alterations in gene expression and a variety of physiological and morphological changes. The ordered progression of recognizable changes has been used to divide sporulation into six stages (stage 0, II, III, IV, V, and VI). Mutants that are unable to sporulate (Spo$^-$) are characterized by the stage at which sporulation is blocked (e.g. *spo0* or *spoII*).

Initiation of sporulation requires at least seven genes called *spo0* genes. A mutation in any one of these genes prevents the earliest of the biochemical and morphological changes associated with sporulation (13, 14). Historically, sporulation has been induced either by allowing rapidly growing cells to exhaust a nutrient broth medium or by a nutritional shift down—that is, by removing growing cells from a rich medium and resuspending them in a medium containing a poorly metabolizable carbon, nitrogen, or phosphate source. In addition to inducing sporulation, some starvation conditions induce the synthesis of several products that are not essential for spore formation but whose synthesis is impaired in some of the *spo0* mutants. For example, at the end of vegetative growth, wild-type cells produce extracellular proteases and antibiotics and become competent to take up exogenous DNA. *spo0A* mutants, the most pleiotropic of the *spo* mutants, are defective in all of these processes. Although some of the *spo0* genes affect the properties of growing cells, none are essential for vegetative growth.

Freese and colleagues (15, 16) have shown that under most, if not all, sporulation conditions, there is a drop in the intracellular concentrations of GDP and GTP. In addition, conditions that reduce the pools of GDP and GTP can induce sporulation, even in media containing excess nutrients (17). One way GTP pools can be reduced is with the drug decoyinine. Decoyinine is an inhibitor of GMP synthetase and thus blocks the conversion of XMP to GMP, which causes a drop in the concentrations of GDP and GTP. When added to rapidly growing cells, decoyinine can cause efficient sporulation, even in nutrient-rich media (18). However, this treatment does not induce many of the other changes (competence as well as extracellular protease and antibiotic production) associated with the end of vegetative growth (10, 19, 20). Thus, induction of sporulation with decoyinine provides a more well-defined system for the study of sporulation-specific events and eliminates many of the starvation-associated events not involved in sporulation.

While studying the effects of decoyinine on gene expression, we observed that sporulation was inefficient at low cell densities, indicating that starvation conditions *per se* were not sufficient to cause efficient sporulation. In this report, we show that *B. subtilis* produces an extracellular differentiation factor(s) that accumulates in the medium when cells reach high density and stimulates sporulation of cells at low density.

MATERIALS AND METHODS

Bacterial Strains. *B. subtilis* strains were derived from strain 168. The strain used for the cell density experiments

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Abbreviation: EDF-A, extracellular differentiation factor A.

was AG130, a *trp*$^+$ derivative of ZB308 (21). Other strains are indicated in figures and table legends.

Cell Growth and Sporulation. Cells were grown in S7 minimal medium (19), except that Mops was used at 50 mM rather than at 100 mM. Glucose and glutamate were added at 1% and 0.1%, respectively, to S7 minimal medium to make growth medium ($S7_{50}$ medium). Required amino acids were added at 40 μg/ml. Cells were first grown overnight at room temperature on Luria–Bertani (LB) solid medium (22), resuspended in growth medium ($S7_{50}$ medium), and used to inoculate a culture to an OD_{600} ≤0.03. Cultures were grown at 37°C in flasks in a shaking water bath for at least four doublings. Sporulation was initiated by the addition of decoyinine (U-7984, Upjohn) to a final concentration of 500 μg/ml. Addition of decoyinine caused the doubling time to increase from ≈60 min to ≈180 min. The amount of sporulation was determined after incubation for 10–16 hr at 37°C with aeration. Sporulation was essentially complete ≈9 hr after the addition of decoyinine, as the total number of spores did not significantly change between 9 and 24 hr. Samples were serially diluted in Spizizen salts (23), and the number of spores was measured as heat-resistant (80°C for 15 min) colony-forming units on LB plates. Viable cells were measured as total colony-forming units on LB plates. The % sporulation = [spores/ml)/(cells/ml)] × 100%.

β-Galactosidase Assays. Cells were grown as described above. Decoyinine was added at time t = 0 to induce sporulation, and 0.5-ml samples were taken for determination of β-galactosidase specific activity expressed in Miller units (1000 times the change in A_{420} per min per OD_{600} of 1 ml of culture) (22).

RESULTS AND DISCUSSION

Sporulation Is Inefficient at Low Cell Density. During the course of experiments in which spore formation was induced by the addition of decoyinine to growing cells, we noticed that the efficiency of sporulation was strongly affected by cell density. At high cell density (OD_{600} > 0.5, ≈10^8 cells/ml), sporulation was normal, but at low cell density (OD_{600} < 0.1), sporulation was very inefficient (Fig. 1). In these experiments, cells were grown to a high density, diluted to a lower density, and induced to sporulate by the addition of decoyinine. The percent sporulation varied from experiment to experiment and especially among strains, but the large effect of cell density on sporulation was reproducible. A similar effect of cell density on sporulation is apparent from data published by Vasantha and Freese (fig. 5b of ref. 24). They studied the role of manganese in sporulation and noted that the requirement for manganese was most apparent at high densities.

The efficiency of sporulation was also density-dependent when sporulation was induced by a resuspension technique (nutritional shift down). Cells were grown to a high density (OD_{600} = 0.7) in medium containing Sterlini–Mandelstam salts (25) supplemented with glucose and glutamate and were resuspended at various densities in resuspension medium (25) to induce sporulation. Resuspension of cells at low density (OD_{600} = 0.04) resulted in inefficient sporulation (0.02% spores), whereas resuspension at high density (OD_{600} = 0.7) resulted in efficient sporulation (39% spores). Thus, efficient sporulation is density dependent under at least two different starvation conditions.

Cells starved at low density were blocked at an early step in sporulation. In the presence of decoyinine, cells at low density did not make alkaline phosphatase (data not shown), an enzyme normally induced during sporulation at stage II. When examined microscopically, these cells did not appear to make an asymmetric septum, indicating a block at stage 0.

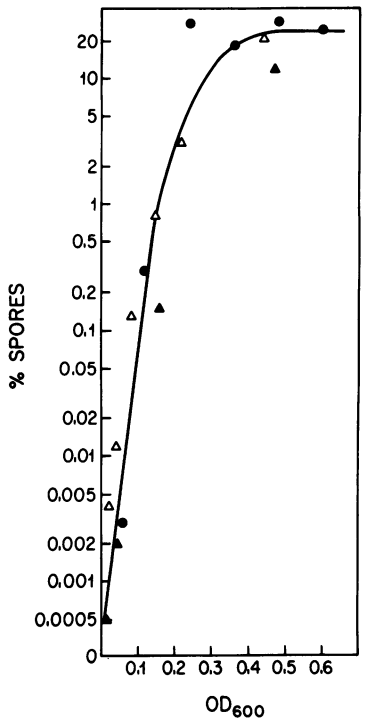

FIG. 1. Sporulation is cell density-dependent. Strain AG130 was grown to high density in $S7_{50}$ medium at 37°C and was diluted to lower densities. Starvation was initiated by the addition of decoyinine to 0.5 ml of cells in an 18-ml test tube. Samples were incubated at 37°C with aeration for 12–16 hr followed by determination of viable cells and spores. The percent sporulation is plotted as a function of cell density (OD_{600}) at the initial time of starvation (t = 0). Different symbols represent results from three independent experiments.

Expression of Sporulation-Induced Genes in Cells at High and Low Density. We measured the effect of cell density on the expression of three genes that are induced during sporulation. These are *cotA* (26) and *sspA* (27), which are normally induced at intermediate to late times during sporulation, and *spoVG* (21, 28), which is induced early during sporulation. The expression of each gene was measured using previously described *lacZ* fusions (21, 26–28). Because cells at low density were not sporulating, expression of most genes normally induced during sporulation was expected to be blocked. This was the case for *cotA–lacZ* and *sspA–lacZ*. Following the addition of decoyinine, expression of these genes was induced only in cells at high density and not in cells at low density (Fig. 2). Thus, the sporulation-specific events required to induce expression of these genes must be blocked at low cell density. In contrast, expression of *spoVG–lacZ* was induced following addition of decoyinine to cells at both low density and high density (Fig. 2). However, the induction of *spoVG* at low density was consistently different from that observed at high density; the maximal expression was usually one-third to one-half of that observed at high density. This effect resembles that of some *spo0* and *spoII* mutations on expression of *spoVG* (29). Taken together, these results on gene expression indicate that cells starved at low density can initiate some of the events required for sporulation, including induction of expression of *spoVG*, but they cannot sporulate efficiently, and expression of other sporulation-induced genes is blocked.

Extracellular Differentiation Factor Present in Conditioned Medium. The efficiency of sporulation of cells at low density could be stimulated by inducing sporulation in conditioned medium (Table 1). Conditioned medium was prepared by growing cells to high density (OD_{600} > 1.5), removing the cells by centrifugation, and filter-sterilizing (0.45-μm pore

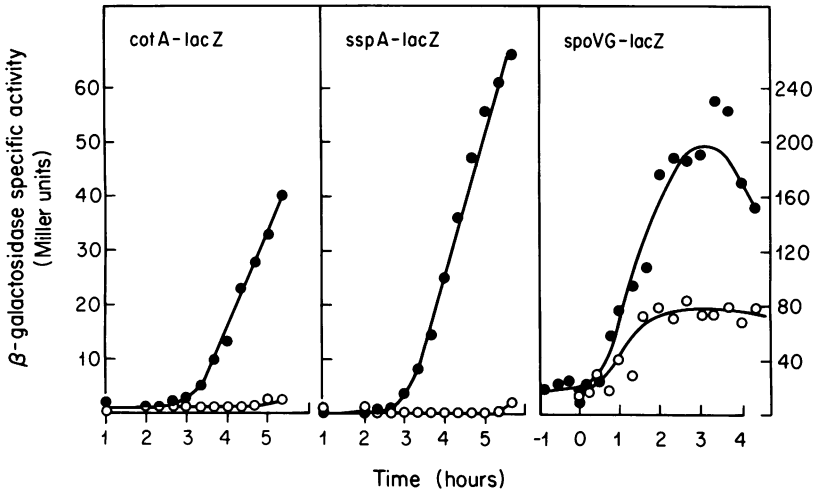

FIG. 2. Expression of sporulation-induced genes at high and low cell density. Strains containing *lacZ* fusions to the indicated gene were grown to a high density (OD_{600} = 0.5–0.7) and diluted to a low density (OD_{600} = 0.02–0.04). Decoyinine was added to both high and low density cultures to initiate starvation (t = 0), and samples were taken periodically for determination of β-galactosidase specific activity. The *cotA–lacZ* translational fusion was provided by S. Cutting (ref. 26 and S. Cutting and R.L., unpublished results). The *sspA–lacZ* fusion has been described (27) and was kindly provided by P. Setlow (University of Connecticut, Farmington, CT). The *spoVG–lacZ* fusion described previously (21, 28) was in strain AG130. ●, High density; ○, low density.

size) the supernatant fluid. The stimulation of sporulation by conditioned medium indicated that when cells reached high density an extracellular factor required for efficient sporulation had accumulated in the medium. The factor, or factors, causing this stimulation is called extracellular differentiation factor A (EDF-A). The presence of EDF-A significantly stimulated the frequency of sporulation of cells at low density (1.5% spores with EDF-A compared to 0.003% without), although the frequency was lower than that of cells at high density (36%).

We characterized EDF-A by treating conditioned medium in a variety of ways and then assaying for EDF-A activity. EDF-A is heat-stable (100°C 15 min) and is at least in part a small protein or oligopeptide. Treatment of conditioned medium with Pronase attached to agarose beads, followed by removal of the Pronase, destroyed EDF-A activity as assayed by the ability of the treated medium to stimulate cells at low density to sporulate (Table 2). This treatment did not inhibit sporulation in general, because cells at high density could still sporulate in the treated medium. Treatment of conditioned medium with agarose beads alone did not destroy EDF-A activity, indicating that the activity is truly Pronase-sensitive. In addition, EDF-A was dialyzable (Table 3). Dialysis experiments were done in two ways. First, conditioned medium was inside the dialysis tubing and was dialyzed against a 100-fold excess of fresh medium, which resulted in the loss of EDF-A activity. Second, fresh medium was inside the dialysis tubing and was dialyzed against conditioned medium, which resulted in the recovery of EDF-A inside the tubing. These results indicate that EDF-A is at least in part a heat-stable oligopeptide.

Attempts to purify EDF-A from conditioned medium have been unsuccessful. EDF-A flowed through both anion and cation exchange resins. Activity was lost following chromatography on the hydrophobic resin phenyl-Sepharose. EDF-A may be composed of multiple compounds. Consistent with this possibility was the observation that a 1:10 dilution of conditioned medium resulted in the loss of EDF-A activity.

Production of EDF-A Is Controlled by Genes Involved in Sporulation. Cells at low density can be stimulated to sporulate in the presence of cells at high density. By using genetically marked strains, individual *spo* mutants at high density were tested for the ability to produce EDF-A, as judged by their ability to stimulate wild-type cells at low density to sporulate. The wild-type cells were first grown to high density and then were diluted to low density, either in sterile medium or into the *spo* mutants at high density. Most of the *spo* mutants tested stimulated sporulation of wild-type cells at low density (Tables 4 and 5). In addition, expression of the *cotA–lacZ* fusion in cells at low density was restored when those cells were mixed with a *spoIIA* mutant (which did not contain a *lacZ* fusion) at high cell density (data not shown).

Two mutants were defective in production of EDF-A. *spo0A* and *spo0B*, the two *spo0* mutants defective in many of the processes associated with the end of vegetative growth, did not stimulate sporulation of wild-type cells at low density, indicating that these genes are required for production of EDF-A. Similar results were obtained with conditioned medium made from the *spo* mutants (data not shown). *spo0E* and *spo0F* mutants seemed to be partially defective in EDF-A production. They sometimes seemed to stimulate sporulation of wild-type cells at low density, but the results were variable.

Table 1. Sporulation of cells at low density is stimulated by conditioned medium

Cell density (OD_{600})	Conditioned medium	% spores
0.6	−	34
0.06	−	0.003
0.06	+	1.5

Cells were grown and treated with decoyinine as described in Fig. 1.

Table 2. Conditioned medium is inactivated by treatment with Pronase

Cell density (OD_{600})	Conditioned medium	Pronase treatment	% spores
0.52	−	−	23
0.052	−	−	0.006
0.052	+	−	3
0.052	+	+	0.005

Cells were grown and treated as described in Fig. 1. Conditioned medium (5 ml) was treated with Pronase attached to agarose beads (44 mg; ≈5 units of Pronase) (Sigma no. P4531) for 60 min at 37°C; the beads were removed by centrifugation, and the treated medium was filter-sterilized.

Table 3. Dialysis of conditioned medium

Medium	% spores
Fresh	0.0007
Conditioned	0.13
Conditioned dialyzed against fresh*	0.0007
Fresh dialyzed against conditioned†	0.09

Cells were grown to high density, diluted to low density (OD_{600} = 0.04) in the indicated medium, and treated as described in Fig. 1. After dialysis at 4°C for 12 hr, the medium inside the tubing was filter-sterilized and tested for EDF-A activity. Preparation of dialysis tubing included boiling, which may have altered the pore size.

*Ten milliliters of conditioned medium was inside the dialysis tubing and was dialyzed against 1 liter of fresh medium. The dialysis tubing had an approximate molecular weight cutoff of 3500.

†Four milliliters of fresh medium was inside the dialysis tubing and was dialyzed against 400 ml of conditioned medium. The dialysis tubing had an approximate molecular weight cutoff of 10,000. Similar results were obtained with dialysis tubing with an approximate molecular weight cutoff of 1000.

The defect in EDF-A production is not the only reason that *spo0A* and *spo0B* mutants do not sporulate. A mutant that was Spo$^-$ because of a defect in EDF-A production would be expected to sporulate when EDF-A is supplied. *spo0A* and *spo0B* mutants were not stimulated to sporulate either in the presence of other cells producing EDF-A or in conditioned medium containing EDF-A. In addition, mutations in *abrB*, which suppress many of the pleiotropic phenotypes of *spo0A* and *spo0B* mutants but not the sporulation defect (33, 34), restored production of EDF-A (Tables 4 and 5). If it was only the defect in production of EDF-A that was causing the Spo$^-$ phenotype of *spo0A* or *spo0B* mutants, then *spo0A abrB* and *spo0B abrB* double mutants would be phenotypically Spo$^+$, but they remain Spo$^-$.

In addition to EDF-A production, a variety of functions associated with the end of vegetative growth are controlled by *spo0A* and *abrB*. In addition to blocking the expression of most sporulation genes and preventing sporulation, *spo0A* mutations block the normal production of extracellular proteases and antibiotics and the acquisition of genetic competence. Mutations in *abrB* suppress, or partially suppress, all of the pleiotropic phenotypes, except the sporulation defect (33, 34). Even the expression of some sporulation genes, such as *spoVG*, is restored by *abrB* mutations (21). Thus, production of EDF-A is controlled in a manner analogous to production of other products associated with the end of vegetative growth. Although EDF-A could be one of the many peptide antibiotics produced by *B. subtilis*, we have no evidence to indicate that this is the case.

EDF-A could be produced in one of two ways. It could be encoded by a structural gene, like the mating factors of *Streptococcus* (4) and *Saccharomyces* (35). Alternatively, it could be synthesized enzymatically (nonribosomally) in a manner analogous to some peptide antibiotics (36). Characterization of mutants altered in EDF-A production should help elucidate how EDF-A is produced and regulated.

Table 4. Stimulation of sporulation of cells at low density by various *spo* mutants

Cells added	Wild-type spores, no. × 10^{-4} per ml
None	1
spo0A	5
spo0A abrB	400
spo0H	100
spo0J	100

Wild-type cells were grown to high density and diluted to low density (OD_{600} = 0.03) by dilution into fresh medium or the indicated *spo* mutant, which was at a high density (OD_{600} > 0.6). Starvation was initiated by the addition of decoyinine as described in Fig. 1.

Table 5. EDF-A production by *spo* mutants

spo mutant	EDF-A production
spo0A	−
spo0B	−
spo0E	±
spo0F	±
spo0H	+
spo0J	+
spo0K	+
spo0A abrB	+
spo0B abrB	+
spoIIA	+
spoIVC	+

EDF-A production was determined by the ability of the indicated mutant to stimulate sporulation of wild-type cells at low density as indicated in Table 4. The *spo* alleles tested included *spo0A12*, *spo0A204*, *spo0B136*, *spo0E11*, *spo0F221*, *spo0H81*, *spo0J93*, *spo0K141*, *spoIIA1*, and *spoIIA69* (30); *spo0HΔHind* (31); *spo0H::cat* (J. Healy and R.L., unpublished results); and *spoIVC*::Tn*917* ΩHU215 (32). The *abrB* alleles included *abrB23* (33), *abrB703* (21), and *abrB*::Tn*917* (P. Zuber, M. Mahariel, and R.L., unpublished results).

Although EDF-A appears to be a type of pheromone, it is formally possible that our defined minimal medium (we have tested three different types of defined medium) contains an inhibitor of sporulation and that EDF-A is an antagonist of the putative inhibitor. Although this possibility seems unlikely, it cannot be eliminated until the mechanism by which EDF-A acts is determined.

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