JOURNAL OF BACTERIOLOGY, Oct. 1993, p. 6321–6327
0021-9193/93/196321-07$02.00/0

Vol. 175, No. 19

Characterization of a New Sporulation Factor in *Bacillus subtilis*

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Received 23 November 1992/Accepted 27 July 1993

We report the existence and partial purification of sporulation factor, which stimulates sporulation of *Bacillus subtilis* at low cell density. Proline or arginine is required for stimulation under the conditions of our assay. Sporulation factor is a small heat-stable substance produced by the cells during exponential growth phase. It is required in small amounts and is resistant to various proteolytic agents. Several *spo* mutants were tested for the ability to produce functional sporulation factor. All of these mutants produce factor and do not sporulate in the presence of factor from wild-type cells. Sporulation factor is not involved in the induction of α-amylase synthesis at the initiation of sporulation.

When starved for a carbon or nitrogen source, cells of *Bacillus subtilis* are induced to form endospores. Sporulation can also be triggered by phosphate starvation under some conditions. Spores formed by *B. subtilis* are dormant structures which are structurally and biochemically distinct from the vegetative cell. Characteristics of the spore include refractility and resistance to heat, desiccation, and organic solvents. The process of sporulation has been described in detail elsewhere (reviewed in reference 15).

Although sporulation seems to occur relatively synchronously, it has generally been viewed as a unicellular process without cell-to-cell interaction. However, there is some evidence of communication between cells during sporulation. Srinivasan and Halvorson reported the presence of an "endogenous factor" involved in sporogenesis of *Bacillus cereus* (20, 21). The factor, termed sporogen, was found to induce sporulation in *B. cereus*. Grossman and Losick have reported the existence of EDF-A (extracellular differentiation factor A) in *B. subtilis* (5). This differentiation factor is a small protein or oligopeptide which stimulates sporulation of cells at low density. In *Streptomyces griseus*, there is a low-molecular-weight compound required for spore formation called factor A (7). The presence of factors involved in stimulating sporulation in these systems and conditions led us to the discovery of an extracellular sporulation factor (SF), described in this report. This finding, in turn, provides further evidence of signalling between prokaryotic cells.

MATERIALS AND METHODS

Strains. *B. subtilis* 168 (*trpC2*) was used as the standard laboratory strain. Asporogenous *spo* mutants were all derived from the parent *B. subtilis* 168 (14). WLN-4 and WLN-11 are isogenic strains derived from *B. subtilis* 1A289 (*aroI906 amyE metB5 sacA321*), which was obtained from the Bacillus Genetics Stock Center. Strains used to determine specificity of SF were *B. subtilis* BR151 (*lys3 metB5 trpC2*), *Bacillus globigii*, *B. cereus* 569 (*ant* Str^r Tc^r *cry*$^+$) (2), and *Escherichia coli* JM83 [*ara* Δ(*lac-proAB*) *rpsL* φ80 *lacZ*Δ*M15*) (26).

Media. Chemically defined sporulation medium (CDSM) (6), Mandelstam-Sterlini resuspension medium (RS) (22), and S-7 medium (4) have been described elsewhere. Cells were plated on peptone yeast broth agar (GIBCO) for measurement of sporulation frequency.

Sporulation assay. Liquid CDSM cultures were inoculated with cells grown overnight on CDSM plates and grown to 100 Klett units (no. 66 red filter). The cells were then washed once in a volume of RS equivalent to that of the original culture, resuspended in an equal volume of RS, and then diluted 10^3-fold in RS (final concentration, ~5×10^5/ml). To 10 ml of this cell suspension, *B. subtilis* spent culture fluid (SCF; see below) and/or amino acids were added as specified in the tables and figure legends. Cells were then shaken for 24 h at 37°C. Cells were plated for viable count, heat shocked at 80°C for 20 min, and plated for spore count. Uninoculated CDSM without glucose was generally used as a negative control. Variability of sporulation frequency was seen for samples used as negative controls (0.004 to 0.31%; e.g., compare Tables 1 and 4); however, SCF-treated samples always sporulated at a significantly higher frequency. The absence of glucose in the *B. subtilis* SCF has been confirmed (by glucose oxidase reaction [9]).

Preparation and extraction of SCF. SCF was prepared by growing cells in CDSM at 37°C to T_{22} (22 h after the end of log-phase growth), unless otherwise noted in the figure legends, and removing the cells by filtration (pore size, 0.45 μn). SCF was frozen, lyophilized, and extracted twice with equal volumes of either acetone, ethyl acetate, methanol, or ethanol. Solvents were then evaporated, and the extracted material was dissolved in distilled water. Material which remained after extraction (nonextracted residue) was also resuspended in distilled water. All resuspended samples were sterilized by autoclaving at 121°C for 20 min. Methanol-extracted SCF was used in some experiments as indicated in the figure legends.

Protease treatment. Pronase and proteinase K treatments were performed by dissolving methanol-extracted SCF in 0.01 mM Tris (pH 7.4), adding enzyme to 1 mg/ml, and incubating the mixture at 37°C for 1 h. Chymotrypsin and *B. subtilis* type VIII protease treatments were performed by

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dissolving methanol-extracted SCF in distilled water, adding enzyme to 1 mg/ml, and incubating the mixture at 37°C for 1 h. CNBr treatment was performed by dissolving methanol-extracted SCF in 10% (vol/vol) CNBr and incubating the mixture at 37°C for 1 h.

Gel permeation chromatography. Separation was performed by using a 35- by 1-cm column of Sephadex G-25 beads and a Gilson Minipuls II pump set at 150. Elution buffer was 10 mM Na_2HPO_4, and 1-ml fractions were collected and autoclaved. Optical density at 280 nm (OD_{280}) was determined by a Bausch & Lomb Omniscribe.

TLC. Thin-layer chromatography (TLC) was performed by using 20- by 20-cm Kieselgel 60 F_{254} silica gel plates (VWR). Material was visualized with UV light. Plates were allowed to develop in various solvent systems until good separation was achieved. Separate bands were scraped off the plate and extracted twice in methanol. The methanol was allowed to evaporate, and extracted material was resuspended in distilled water and autoclaved.

Separation using SEP-PAK C-18 cartridges. A SEP-PAK cartridge with a nonpolar C-18 packing (Waters Associates) was used to separate material by polarity. The cartridge was prewet with methanol, and 2 ml of sample was then added. The void volume was collected, and material of interest was eluted with increasing concentrations of methanol. Void volumes and eluted fractions were dried by evaporation, brought up to original volume with distilled water, and autoclaved.

HPLC. High-pressure liquid chromatography (HPLC) was performed by using a Beckman model 332 gradient liquid chromatography and an Ultrasphere-OPS C_{18} column (4.6 mm by 15 cm; Altex). Sample was eluted with either a 0 to 30% acetonitrile–0.15 M KCl or a 0 to 50% methanol–0.15 M KCl eluting system. In both cases, the flow rate was 1 ml/min. OD_{214} was measured by a Beckman 160 absorbance detector. Fractions corresponding to absorbance peaks were collected, lyophilized, resuspended in distilled water, and autoclaved.

Analysis of α-amylase synthesis. Measurement of α-amylase synthesis and sporulation in strains WLN-4 and WLN-11 were performed as described by Nicholson and Chambliss (12) except that methanol-extracted SCF (2%, vol/vol) was substituted for decoyinine.

RESULTS

Requirements of the assay. When cells of *B. subtilis* were grown in CDSM, washed and diluted in RS, and shaken overnight, they failed to sporulate significantly (Table 1, no additions). *B. subtilis* SCF, taken at the end of the exponential growth phase (T_0) and extracted with methanol (see Materials and Methods), also failed to enhance sporulation. Proline, histidine, or arginine at 40 μg/ml also failed to stimulate sporulation and may inhibit it, but not necessarily. The effect of the addition of these amino acids on sporulation may simply reflect variability in the assay. However, SCF plus proline or arginine stimulated sporulation significantly (Table 1), whereas SCF plus histidine did not. Thus, in addition to constituents of the RS medium, either proline or arginine and SF present in the SCF were required for efficient stimulation under the conditions of the assay. Amino acid pools were used to show that no other amino acids stimulated sporulation significantly, with or without SCF. When cells were grown in CDSM, washed and resuspended in an equal volume of RS, and shaken overnight (undiluted), they sporulated efficiently (>30%) in the absence and presence of added SCF and proline (not shown). At high cell density, arginine or proline (or some derivative) may be present in sufficient amounts as a by-product of cellular metabolism or cell lysis. Alternatively, the cells may not require these amino acids for efficient sporulation at high cell density.

TABLE 1. Requirements for sporulation

Addition(s) to assay[a]	% Sporulation[b]
None	0.31
SCF	0.31
Proline	<0.01
SCF + proline	86
Histidine	<0.01
SCF + histidine	<0.01
Arginine	<0.01
SCF + arginine	59
Inosine	0.12

[a] *B. subtilis* test cells were treated with *B. subtilis* SCF (1%, vol/vol), amino acids (40 μg/ml), or inosine (0.002 mM) as indicated (see Materials and Methods). SCF was taken at T_0 and extracted with methanol.
[b] Spore titer divided by viable cell titer multiplied by 100.

The effect of increasing amounts of proline on sporulation was tested (Fig. 1). Percent sporulation reached an optimum at 10 μg/ml and then fell rapidly, reaching a basal level by 80 μg/ml. The function of proline is not known, but at these concentrations (10 μg/ml), it is not acting as a major carbon or energy source. These results indicate that SF (plus proline or arginine) is not simply acting by blocking an inhibitor of sporulation present in the media, since sporulation is not stimulated at high proline concentrations.

The effect of increasing amounts of SF is shown in Fig. 2. Sporulation increased dramatically from 0 to 10 μl of SCF added to 10 ml of test cells and then leveled off (Fig. 2). Concentration of the factor must be high enough in the spent media such that only small amounts are required for maximum effects in the assay.

Specificity of SF. SCFs of various related and unrelated organisms were tested for the ability to stimulate sporulation of *B. subtilis* 168 and to be stimulated to sporulate by either their own SCF or *B. subtilis* 168 SCF (Table 2). SF from *B. subtilis* 168 enhanced sporulation in *B. cereus* and possibly *B. subtilis* BR151. It also stimulated sporulation of *B.*

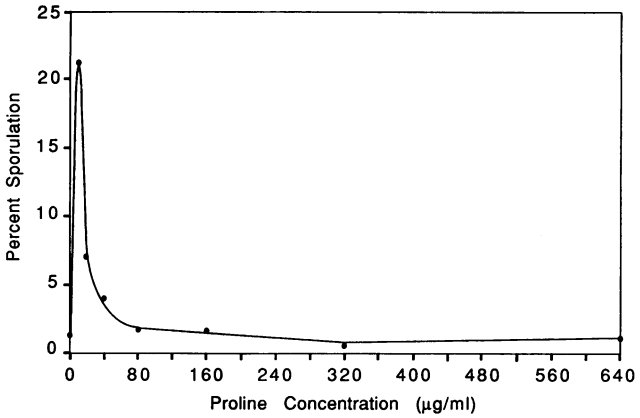

FIG. 1. Effect of proline concentration on sporulation frequency was measured by using the standard amount of methanol-extracted SCF (1%, vol/vol) and increasing concentrations of proline.

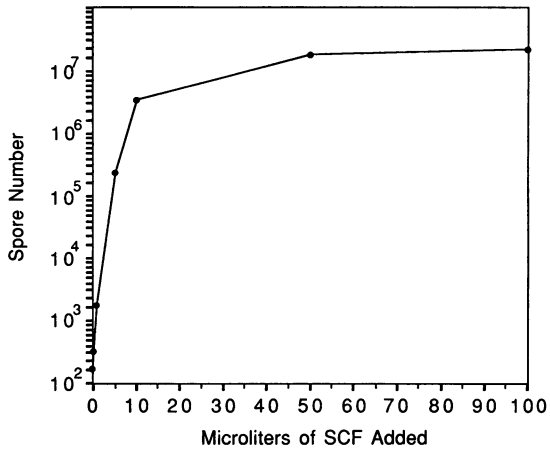

FIG. 2. Dose effects of SF. Increasing amounts of SCF were added to 10 ml of test cells plus 10 μg of proline per ml and assayed for SF activity.

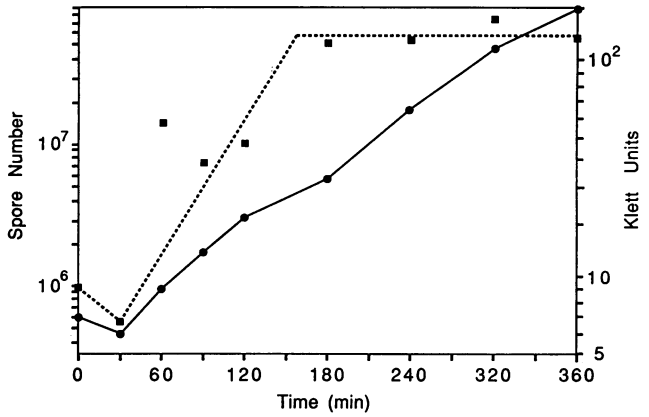

FIG. 3. Onset of factor production. SCF taken from *B. subtilis* culture at various times after inoculation into CDSM was methanol extracted and assayed for SF activity. The number of spores produced in the assay (■) is plotted versus the time at which the filtrate was taken. Turbidity of the culture (●) at that time is also plotted. SCF was added to the assay at 1% (vol/vol), and proline was present at 10 μg/ml.

globigii (data not shown). *B. subtilis* 168 was stimulated by SCF from *B. subtilis* BR151 but not by SCF from *B. cereus*, *B. globigii*, or *E. coli*. The possibility that SCFs from *B. globigii* and *B. cereus* contained factors inhibiting the action of SF was examined by adding spent media from *B. subtilis* 168 with *B. globigii* and *B. cereus* SCFs, respectively, and testing for inhibition of sporulation. *B. globigii* and *B. cereus* SCFs did not inhibit the activity of *B. subtilis* 168 SF (Table 2).

Production of SF. Onset of SF production was studied by taking SCF of *B. subtilis* at various times, extracting it with methanol, and assaying it for SF activity. Production of SF occurred from the beginning of exponential growth (Fig. 3). The plateau seen after 160 min reflects the inability of the assay to measure more than 80 to 100% sporulation. Dilution of the latter samples showed that production continued throughout exponential growth (data not shown).

Analysis of *spo* mutants for production and response to SF. Various sporulation mutants (*spo0A*, *spo0B*, *spo0E*, *spo0F*, *spo0H*, *spo0J*, *spo0K*, *spoIIIB*, *spoIVC*, and *spoA12*) were tested for the ability to produce a functional SF and for the ability of SF from wild-type cells to overcome their sporulation deficiency. All *spo* mutants tested were found to produce functional SF, and none were able to sporulate in the presence of SF from wild-type cells (data not shown).

Effects of SF on α-amylase production. SF was tested for its effects on α-amylase synthesis and sporulation under glucose-repressing conditions to determine whether it influences the induction of other processes that are associated with the onset of sporulation. α-Amylase was chosen because its synthesis is turned on in normally growing *B. subtilis* at T_0, coincident with the onset of sporulation, and its production could be examined in isogenic strains carrying wild-type *amyR1* (WLN-4) and *amyR1:gra-10* (WLN-11), a mutation conferring glucose resistant α-amylase synthesis. These strains were tested for α-amylase production with no additions, decoyinine (an inhibitor of GMP synthetase which is able to circumvent glucose repression of sporulation in *B. subtilis* [10]) at 250 μg/ml, and SCF (2%, vol/vol). In wild-type *B. subtilis* (WLN-4), synthesis of α-amylase is repressed in the presence of 2% glucose, both in the absence and in the presence of decoyinine (14) (Table 3). However, in the *amyR1:gra-10* strain (WLN-11), α-amylase synthesis is substantially enhanced in the presence of decoyinine (14)

TABLE 2. SF specificity[a]

Tester strain	Type of SCF	Spore titer	Viable cell titer	% Sporulation
B. subtilis 168	None[b]	2.2×10^3	4.7×10^6	0.05
	B. subtilis 168	4.8×10^7	7.0×10^7	69
	B. cereus	1.2×10^4	1.5×10^7	0.08
	E. coli	2.6×10^3	2.6×10^6	0.1
	B. globigii	1.3×10^5	1.3×10^7	1
	B. subtilis BR151	1.7×10^7	1.7×10^7	100
B. subtilis BR151	None	1.0×10^2	3.7×10^6	0.003
	B. subtilis 168	1.8×10^5	4.7×10^7	0.38
B. cereus	None	4.7×10^3	2.0×10^5	2.4
	B. cereus	2.3×10^6	3.0×10^6	77
	B. subtilis 168	2.0×10^6	1.7×10^6	118
B. subtilis 168	*B. subtilis* 168 and *B. cereus*[c]	1.1×10^7	3.1×10^7	35
	B. subtilis 168 and *B. globigii*[c]	3.7×10^7	3.6×10^7	103

[a] Test cells of the indicated type were treated with 1% (vol/vol) SCF from the indicated organism and proline (10 μg/ml).
[b] Instead of SCF, 1% (vol/vol) material similarly prepared starting with uninoculated medium (CDSM lacking glucose) was added.
[c] SCF (1%, vol/vol) from each organism was added.

TABLE 3. Ability of the SF to induce α-amylase synthesis

Strain	Differential rate of α-amylase synthesis[a]		
	Control	+Decoyinine	+SF
WLN-4 (wild type)	13	17	10
WLN-11 (*amyR1:gra-10*)	32	183	29

[a] Decoyinine- or methanol-extracted SCF was added to early-exponential-phase (30 Klett units) cultures grown in CDSM plus 2% glucose and 40 μg of proline per ml. The differential rate of synthesis was determined for the first hour after the additions. During this time, the control and SF-treated cultures grew normally. Decoyinine severely inhibited growth.

(Table 3). Addition of SCF to either WLN-4 or WLN-11 had no effect on α-amylase synthesis (Table 3). Thus, SF is not sufficient to turn on α-amylase synthesis, even in WLN-11, under these conditions, nor did it overcome glucose repression of sporulation (data not shown).

Heat stability and treatment with peptide-cleaving agents. SF was found to be heat stable. Autoclaving at 121°C for 20 min did not result in any detectable loss of activity (data not shown). Methanol-extracted SCF was subjected to treatment with various protein- and peptide-cleaving agents (see Materials and Methods for incubation conditions) and assayed for SF activity. The factor was found to be resistant to CNBr, proteinase K, chymotrypsin, *B. subtilis* type VIII protease, and pronase (Table 4).

Extraction using organic solvents. Partial purification of the factor was attempted by several methods. SCF was extracted with various organic solvents (see Materials and Methods). Both the extracted material and the nonextracted residue (pellet) were tested for the ability to stimulate sporulation (Table 5). The highest SF activity was found in the methanol extract and ethanol pellet. No significant activity was found in either the extract or pellet of acetone or ethyl acetate. SF activity may be destroyed by being subjected to these reagents. UV and visible scans of the methanol-extracted material gave large peaks below 250 nm and at 279 nm and smaller peaks at 423, 455, and 683 nm (data not shown).

Gel permeation chromatography of methanol-extracted SCF. Gel permeation chromatography was performed on both methanol-extracted SCF and methanol-extracted uninoculated (unspent) CDSM, using a column containing Sephadex G-25 beads (see Materials and Methods). OD of the fractions was read, and SF activity was assayed (Fig. 4). Fractionated SCF gave a large OD_{280} peak in fractions 13 to 33 and significant factor activity in fractions 24 and 25, whereas uninoculated CDSM gave a small peak absorbing at 280 nm in fractions 13 through 33 and no significant factor activity. Both SCF and CDSM gave a large OD_{280} peak beyond fraction 35, but neither had significant SF activity in those fractions. Fractionated SF was less stable than unfractionated SF and was not deemed useful for further purification.

TABLE 4. Treatment of SF with protein- and peptide-cleaving agents

Addition to assay[a]	Spore titer	Viable cell titer	% Sporulation
CDSM (−glucose)	1.7×10^2	3.8×10^6	0.004
SCF			
Untreated	2.4×10^7	4.5×10^7	53
CNBr treated	1.6×10^7	3.1×10^7	52
Proteinase K treated	1.8×10^7	2.6×10^7	69
Chymotrypsin treated	2.0×10^7	3.0×10^7	67
B. subtilis type VIII protease treated	2.2×10^7	3.3×10^7	67
Pronase treated	1.9×10^7	3.4×10^7	56

[a] Cells were treated with 1% (vol/vol) each indicated material and 10 μg of proline per ml. Methanol-extracted SCF was subjected to the indicated cleaving agent as described in Materials and Methods.

TABLE 5. Extraction of SCF with organic solvents

Addition to assay[a]	Spore titer	Viable cell titer	% Sporulation
CDSM (−glucose)[b]	7.0×10^2	2.8×10^7	0.003
Untreated SCF	9.0×10^6	8.1×10^7	11
Acetone extract	2.9×10^5	4.1×10^7	0.71
Acetone residue	2.4×10^5	6.2×10^7	0.39
Ethyl acetate extract	1.2×10^4	4.2×10^7	0.03
Ethyl acetate residue	9.1×10^3	8.4×10^7	0.01
Methanol extract	1.1×10^7	8.0×10^7	14
Methanol residue	3.5×10^5	4.3×10^7	0.81
Ethanol extract	1.7×10^2	1.4×10^7	0.001
Ethanol residue	1.2×10^6	1.0×10^7	12

[a] Cells were treated with 1% (vol/vol) each indicated material, prepared as described in Materials and Methods, plus 40 μg of proline per ml. SCF was taken at T_{22}.
[b] Untreated, uninoculated medium.

TLC of SCF. TLC was performed on methanol-extracted SCF and methanol-extracted CDSM, using silica plates with various solvents as developing systems (see Materials and Methods). Chloroform-methanol (9:1) gave three distinct bands of material, as visualized under UV light, for both SCF and unspent media. These bands were collected and tested for SF activity (Table 6). Fraction 2 of the extracted SCF gave significant activity. UV and visible spectra of fraction 2 from SCF and fraction 2 from unspent media gave similar spectra with peaks absorbing below 220 nm in both cases.

Other solvent systems which did not yield separate bands or gave bands which lacked any SF activity included 79% *n*-butanol–6% acetic acid–15% distilled water (system 1),

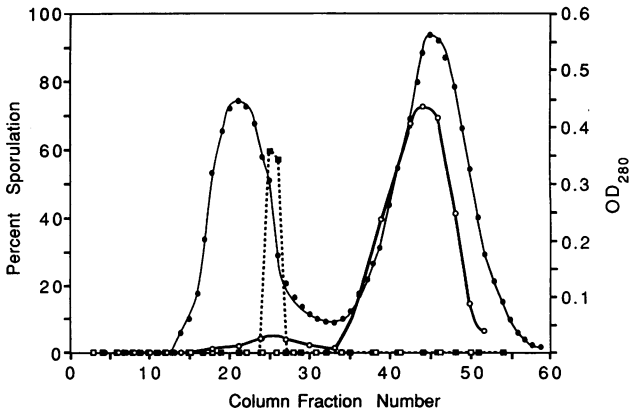

FIG. 4. Sephadex chromatography of SCF. Gel permeation chromatography was performed on methanol-extracted SCF (closed symbols) or uninoculated CDSM lacking glucose (open symbols), using a Sephadex G-25 column (see Materials and Methods). Ten milliliters of SCF was loaded, and 1-ml fractions were collected. Five milliliters of CDSM lacking glucose was loaded, and 2-ml fractions were collected. Fractions were assayed for SF activity (□, ■), and OD_{280} was measured (○, ●).

TABLE 6. TLC of SCF

Fraction used in assay[a]	Spore titer	Viable cell titer	% Sporulation
CDSM[b]			
Fraction 1	1.0×10^2	1.5×10^6	0.007
Fraction 2	2.6×10^2	2.2×10^7	0.001
Fraction 3	6.0×10^2	5.1×10^6	0.01
SCF			
Fraction 1	1.2×10^3	8.0×10^6	0.02
Fraction 2	5.2×10^6	2.4×10^7	22
Fraction 3	6.0×10^2	3.9×10^6	0.02

[a] Methanol-extracted SCF (or CDSM lacking glucose) was fractionated by TLC using chloroform-methanol as described in Materials and Methods. Cells were treated with 1% (vol/vol) each fraction and 10 μg of proline per ml.
[b] Fraction 1 is at the origin, fraction 2 is at the interface between the loading area and running zone, and fraction 3 runs slightly behind the solvent front.

90% acetic acid–10% distilled water (system 2), dimethylformamide (system 3), 80% chloroform–20% acetone (system 4), and 80% saturated ammonium sulfate–20% acetone–2% isopropanol (system 5). Solvent systems 1 to 4 are recommended for separating peptides; system 5 is recommended for separating nucleotides (1).

Separation of SCF on C-18 columns. SCF samples were passed through SEP-PAK C-18 cartridges in an attempt to further purify SF activity. Methanol-extracted SCF and unspent media were fractionated on the C-18 columns by using increasing concentrations of methanol as the eluent (see Materials and Methods). Void volumes and methanol-eluted fractions were tested for SF activity (Table 7). Significant activity was present only in the 25 and 50% methanol fractions of SCF. Attempts to further purify SF by HPLC (see Materials and Methods) were unsuccessful.

DISCUSSION

Sporulation is induced in liquid media by nutritional shiftdown, whereby cells growing exponentially in a rich medium are transferred to a poor medium. Sporulation under these conditions is density dependent (25). Cells starved at low density initiate some of the events necessary for sporulation but are blocked at stage 0, the onset of the sporulation process (5). Addition of SF to cells at low density allows them to proceed past this block and sporulate normally. This provides for a model system in which SF can be studied.

TABLE 7. Fractionation of SCF on a SEP-PAK C-18 column

Additions to assay[a]	Spore titer	Viable cell titer	% Sporulation
SCF			
1st ml of void volume	7.0×10^2	1.5×10^7	<0.01
2nd ml of void volume	$<10^2$	5.7×10^6	<0.01
25%[b] methanol eluate	6.1×10^7	1.5×10^8	41
50% methanol eluate	1.7×10^7	8.5×10^7	20
75% methanol eluate	7.0×10^5	1.5×10^8	0.47
100% methanol eluate	$<10^2$	6.0×10^6	<0.01
CDSM			
1st ml of void volume	$<10^2$	5.7×10^6	<0.01
2nd ml of void volume	$<10^2$	8.5×10^6	<0.01
25% methanol eluate	2.0×10^2	2.8×10^7	<0.01
50% methanol eluate	5.4×10^4	1.4×10^8	0.01
75% methanol eluate	2.0×10^2	4.5×10^7	<0.01
100% methanol eluate	1.0×10^2	1.8×10^7	<0.01

[a] Methanol-extracted SCF (or CDSM lacking glucose) was fractionated on a SEP-PAK C-18 column as described in Materials and Methods. Cells were treated with 1% (vol/vol) each indicated material and 40 μg of proline per ml.
[b] Methanol percentage used to elute fraction from column.

Results presented in this report show there is an extracellular SF in *B. subtilis* which stimulates sporulation under the proper conditions. Grossman and Losick have reported that EDF-A is produced by cells grown to high density and stimulates sporulation of cells at low density (5). Although both SF and EDF-A are extracellular and stimulate sporulation of *B. subtilis* under similar conditions, they are most likely not the same substance. SF is pronase resistant and is produced by all *spo* mutants tested, including *spo0A*. EDF-A is pronase sensitive, and *spo0A* mutants are defective in its production. These findings suggest the production of multiple extracellular factors for efficient sporulation, as proposed by Grossman and Losick (5). EDF-A was identified as a factor that stimulates sporulation of cells that are artificially induced to sporulate by the addition of decoyinine. EDF-A apparently does not stimulate sporulation under the conditions of our assay or is lost in our manipulations.

Conditions for SF activity reveal several facts regarding its action. Viable cell number generally increased about 10-fold during the assay (from $\sim 5 \times 10^6$ to $\sim 5 \times 10^7$/ml) with and without SF addition; however, cells sporulate efficiently only upon the addition of SF. A slight growth requirement need not be surprising in light of Mychajlonka and Slepecky's finding that DNA synthesis is required for microcycle sporulation of *B. subtilis* (11). It is assumed that glutamate (0.2% in RS) is being used as the primary carbon and energy source under our assay conditions. The role of arginine or proline is unknown. The low concentrations (10 to 40 μg/ml) of these amino acids in the assay preclude their use as a major source of carbon or energy. It is interesting that in *E. coli*, breakdowns of arginine and proline share an intermediate step not shared by histidine (16). The metabolism of these compounds is also closely related to that of glutamate. Stimulation of sporulation by SF may be involved with glutamate metabolism.

Extensive homology between the deduced gene products of *spo0A* and *spo0F* and several regulatory proteins in other prokaryotes has been noted (24). These regulatory proteins belong to a family of two-component systems involved in sensing environmental stimuli (18). The *spo0A* gene product shows homology to the effector component and appears to function in the sporulation process as a key transcriptional regulator which can serve as a repressor or activator, depending on the target gene on which it acts (23). Recently, Burbulys et al. have hypothesized that *spo0A* and *spo0F* are part of a phosphorelay system which includes *kinA* and *spo0B*, with *spo0A* being activated as the ultimate phosphoacceptor (3). An attractive possibility is that SF acts as one of several (or many) signalling factors that function through this phosphorelay system to bring about efficient sporulation.

Partial purification and characterization reveal several properties of SF. Retention by Sephadex G-25 medium indicates that SF has a molecular weight of 1,000 to 5,000. Heat stability and protease resistance make extensive secondary structure or enzymatic activity unlikely. Binding to the SEP-PAK column indicates a nonpolar character. Methanol-extractable SF is hydrophilic, and its behavior on a SEP-PAK C-18 column (Table 7) and on TLC plates (Table 6) indicates a polar character. Methanol-extractable compounds are probably not nucleic acids or nucleotides. Some possibilities as to the chemical identity of SF include a small polypeptide, phospholipid, lipopolysaccharide, or sugar.

There is no indication that SF is inosine, which has been reported to be a sporogen (19). Inosine failed to stimulate sporulation under our assay conditions (Table 1).

The possibility that SF is a short polypeptide or oligopeptide is especially attractive because *B. subtilis* is known to produce many polypeptides of unknown function. Although the factor is resistant to several peptide-cleaving agents, it may be a short polypeptide containing no sites for these agents. SF does not appear to be analogous to heat-stable A-factor of *Myxococcus xanthus*, which has been shown to be a mixed set of amino acids and small peptides that are predicted to act in fruiting body development and spore formation by specifying a required minimum cell density (8). SF differs from heat-stable A-factor in two ways. SF does not have a threshold level requirement for activity, and SF appears to be a specific factor of determined size, as indicated by gel permeation chromatography, rather than a conglomerate of peptides and amino acids.

Many sporulation-deficient (*spo*) mutants of *B. subtilis* have been isolated for which the basis of their deficiency is unknown. The possibility that some of these mutations are involved in SF production was explored by testing various *spo* mutants for the abilities both to produce a functional SF and to respond to factor from wild-type cells. All mutants tested were able to produce functional SF but failed to sporulate in the presence of factor from wild-type cells.

It is noteworthy that SF is produced during exponential growth phase. Sporulation is a process that is triggered by nutrient deprivation and therefore is thought not to begin until the end of log-phase growth. It is likely that other factors or processes, which do not occur until the end of growth, are required for efficient sporulation in addition to the presence of SF. This is supported by the fact that SF does not stimulate sporulation under all conditions (i.e., glucose-repressing conditions).

Our data argue against the idea that sporulation is normally triggered when SF reaches a threshold concentration. Under the conditions of our assay, addition of very small amounts of SCF result in efficient sporulation. Also, there is a direct relationship between SF concentration in the assay and the amount of sporulation, indicating the absence of a threshold (Fig. 2).

In *B. subtilis*, the synthesis of α-amylase (an extracellular enzyme involved in starch breakdown) is activated at T_0, temporally coincident with the onset of sporulation (17). Like sporulation, α-amylase synthesis is repressed by excess glucose (17). Synthesis of α-amylase from *amyE* is partially regulated by transcription from the *amyR1* promoter region (13). Activation at T_0 and glucose-mediated repression of α-amylase production are decoupled by *gra-10*, a *cis*-acting mutation in *amyR1* (12). Addition of decoyinine to a *gra-10* strain (WLN-11) causes immediate activation of α-amylase synthesis, as well as stimulating sporulation by greater than five orders of magnitude (14). However, when SF was added at a high concentration to WLN-11, neither α-amylase production nor sporulation was induced (Table 3). Excess SF (20 times higher than in the standard assay) was added because cells were present at a much higher concentration in this experiment than in the normal sporulation assay. We conclude that SF is not involved in the activation of α-amylase synthesis under the conditions tested; however, it still may be involved in expression of other functions initiated at T_0.

SF produced by *B. subtilis* exhibited some species specificity. It is not surprising to find some cross-utilization of SF by other sporulating bacilli, and as one would expect, *E. coli* does not produce SF. There is obviously some species specificity because filtrates from *B. cereus* and *B. globigii* do not stimulate sporulation in *B. subtilis* 168 (Table 2). It is surprising that the reverse is not true. Apparently, some factors are more general (or some receptors are less specific) than others. It is especially surprising that *B. subtilis* BR151, a derivative of *B. subtilis* 168, may not be stimulated by filtrate from the latter.

ACKNOWLEDGMENTS

We are grateful to Miriam M. Susskind for helpful discussion and critical reading of the manuscript and to Jo Handelsman for use of HPLC equipment and technical assistance.

This work was supported by NIH grant 34324 to G.H.C. C.W. is the recipient of a UW—Madison CALS assigned fellowship.

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