Properties of a *Bacillus subtilis* Mutant Able to Sporulate Continually During Growth in Synthetic Medium

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Several mutants of *Bacillus subtilis* were isolated which sporulate continually during exponential growth in glucose medium. The *spdA1* mutation, responsible for the continual sporulation of one of the mutants, mapped near *thr*. When an exponentially growing culture of a strain containing *spdA1* was maintained at essentially constant turbidity, 5% of the viable cells contained heat-resistant spores. The continual sporulation depended on the stringent response since it was absent in *spdA relA* double mutants. Genetic and biochemical analysis indicated that the continual sporulation of *spdA1* strains was associated with a lower specific activity of pyruvate carboxylase, which limited the rate of oxaloacetate synthesis from glucose via pyruvate and thereby the supply of compounds depending on the citrate cycle, especially aspartate. Therefore, the mild stringent response caused by the *spdA1* mutation seems to result from a partial deficiency of aspartyl-tRNA which may exert its sporulation-initiating effect during a limited time interval in each growth cycle. A mutant blocked in fumarase activity (*citG*) behaved similarly. It grew only slowly in glucose medium because much of the limiting oxaloacetate was wasted for the excretion of fumarate. The mutant produced little aspartate and sporulated at a high frequency in glucose medium, even in the presence of glutamate; the sporulation was again prevented by aspartate or malate or by introduction of the *relA* marker into the strain.

INTRODUCTION

Sporulation of *Bacillus subtilis* is usually studied under conditions where most cells enter the sporulation development within a time span of less than 1 h. Such conditions occur automatically at the end of growth in certain (sporulation) media; they can be initiated by the transfer of cells from a rich to a poor growth medium, and they can be induced by the addition of inhibitors of purine synthesis or by the use of auxotrophs causing partial deprivation of guanine nucleotides. The onset of this massive sporulation was found to be correlated with a decrease in the concentration of GTP (and GDP), whereas other nucleotides increased under some sporulation conditions and decreased under other sporulation conditions (Lopez *et al.*, 1979; Freese, 1981; Freese *et al.*, 1981). The transfer of cells from a rich to a poor medium results in a (transient) deficiency of amino acids which produces the stringent response encompassing the increase of two highly phosphorylated guanine nucleotides and the decrease in the synthesis of RNA and other macromolecules (Gallant, 1979). Relaxed (*relA*) mutants have been used to demonstrate that the stringent response is not necessary for sporulation, e.g. in nutrient sporulation medium (Rhaese & Groscurth, 1976; Nishino *et al.*, 1979; Lopez *et al.*, 1981*a*). However, other conditions of nutrient deprivation, such as the step-down from a medium containing most amino acids to one containing only glutamate (Sterlini & Mandelstam, 1969), or partial deprivation of any one amino acid, cause sporulation by evoking the stringent response, which includes the decrease in GTP concentration (Lopez *et al.*, 1981*a, b*; Ochi *et al.*, 1981).

In certain media which enable only slow growth, a small fraction of cells can continually enter the sporulation development process at a frequency that depends on the composition of the

medium; this was shown in cultures whose turbidity (Schaeffer *et al.*, 1965) or growth-limiting supply of a nutrient (Dawes & Mandelstam, 1970) was kept essentially constant. The biochemical mechanism of this sporulation control has not been studied; it may be related to the fact that sporulation can be initiated by the stringent response to partial amino acid deprivation (Ochi *et al.*, 1981).

Several mutants have been reported which can sporulate in a medium containing excess glucose (Kunst *et al.*, 1974; Takahashi, 1979). It is not clear from the literature, however, whether this sporulation occurred continually or only after the end of growth; the latter would indicate that the depletion or accumulation of some compound may cause this sporulation. We have obtained several strains that produce high spore titres in synthetic medium containing excess ammonium, glucose and phosphate, in which the standard and most other strains of *B. subtilis* produce only few spores. The mutant cells enter into sporulation not only after the end of growth but at a high frequency throughout exponential growth. Because this continual sporulation can be prevented by the addition of glutamate or other compounds associated with the citrate cycle, the cause of this phenomenon can be studied in more depth. We describe here the genetic and biochemical properties of one of these mutations (termed *spdA1*). The continual sporulation caused by the *spdA* mutation was limited to (*rel*⁺) strains showing the stringent response. Our results demonstrate that an enzyme change which is so minor that it does not noticeably affect cell growth can have a major effect on differentiation.

METHODS

Organisms, media and spore titre. The strains of *B. subtilis* used are listed in Table 1. Synthetic medium contained 10 mM-(NH₄)₂SO₄, 5 mM-potassium phosphate buffer (pH 7.0), 100 mM-morpholinopropanesulphonate (pH adjusted to 7.0 by KOH), 2 mM-MgCl₂, 0.7 mM-CaCl₂, 50 µM-MnCl₂, 5 µM-FeCl₃, 2 µM-thiamin·HCl, 1 µM-ZnCl₂, 20 µg L-methionine ml⁻¹ and 50 µg L-tryptophan ml⁻¹; 55 mM-D-glucose (glucose medium) or other carbon sources were added as stated. Nutrients required by auxotrophic mutants were added at final concentrations of 30–50 µg ml⁻¹ for amino acids and 1 mM for hypoxanthine. Acid production by mutants was revealed on plates containing bromocresol purple and CaCO₃, prepared according to Carls & Hanson (1971).

Cells were precultured on Tryptose Blood Agar base (TBAB; Difco) plates for 8–15 h. They were suspended in synthetic medium; the *A*₆₀₀ was measured at an appropriate dilution (*A*₆₀₀ below 0.8), and the cells were inoculated into the growth medium to give a calculated *A*₆₀₀ of about 0.001. The cultures, occupying 1/10 or less of an Erlenmeyer flask, were shaken in a reciprocating shaker at 120 r.p.m., 37 °C. Growth was followed by measuring the *A*₆₀₀ in a Gilford spectrophotometer. The viable cell titre (*V*) and heat-resistant spore titre (*S*) were determined by diluting the culture in 0.1 M-potassium phosphate buffer pH 6.5, containing 1 mM-MgCl₂ (PM buffer), and plating both immediately, and after 20 min incubation at 75 °C, on TBAB plates. In some experiments, in which we felt it was important to observe even only partially developed spores, we measured the octanol-resistant spore titre. In this method, 5 µl octanol was added to 1 ml culture, and the tube left for 5 min at 37 °C. After dilution with PM buffer the cells were plated on TBAB plates (Cooney *et al.*, 1977).

Amino acid determinations. Cell extracts were prepared for amino acid analysis by the amino acid analyser as described by Whiteman *et al.* (1980). To demonstrate that the large amount of glutamate observed was actually L- and not D-glutamate, L-glutamate was also assayed enzymically using glutamate dehydrogenase and NADH₂ in glycine/hydrazine buffer according to Bernt & Bergmeyer (1971).

In some experiments employing a medium containing glutamate, the amount of glutamate remaining in the culture medium was determined after centrifugation of a 1.3 ml sample for 2 min in an Eppendorf microfuge. A 1 ml volume of the supernatant was adjusted to pH 9.0 with NaOH and was then freeze-dried to remove most of the ammonia. The dried material was solubilized in 1.0 ml H₂O.

Gene transfer by transformation or transduction. To prepare DNA for transformation, cells growing exponentially in 100 ml glucose medium containing 20 mM-glutamate plus any auxotrophic requirements were harvested at an *A*₆₀₀ of 1.0 and washed with 40 ml Tris/HCl (50 mM, pH 7.8) containing 50 mM-EDTA (Tris/EDTA). The cells were suspended in 5 ml Tris/EDTA and incubated with 100 µg lysozyme ml⁻¹ at 37 °C for 20 min. Protease K (50 µg ml⁻¹ final concentration) and SDS (0.5%, w/v final concentration) were added, and the mixture was incubated for 10 min at 37 °C. Phenol (5 ml), saturated with Tris/EDTA, was added and thoroughly mixed with the extract using a Pasteur pipette. This mixture was mixed with 1 ml chloroform/isoamyl alcohol (25:1), to dissolve lipids and produce a clear interface, and then centrifuged at 10000 g for 20 min. The top layer was removed and mixed with 1/9 vol. 4 M-NaCl. Ethanol (2 vol.) was layered on top, and DNA threads were collected on a glass rod and then washed with 70% ethanol. After removal of most of the ethanol, the DNA was dissolved in 1.5 ml 100 mM-Tris/HCl containing 10 mM-EDTA, pH 8.0. When the DNA was dissolved, an equal volume of 4 M-NaCl was

Table 1. Origin and characteristics of the *Bacillus subtilis* strains used

Strain	Genotype*	Origin†
60001	<i>trpC2</i>	Spizizen 168
60015	<i>metC7 trpC2</i>	E. Nester, SB 26
60198	<i>OrdA1</i>	Whiteman <i>et al.</i> (1980), 60009 + UV
60813	<i>citD metC7 trpC2</i>	60015 + NTG
61423	<i>citG trpC2</i>	Carls & Hansen (1971), 1A8
61469	<i>lys-3 metB104 trpC2</i>	F. Young, BR151
61473	<i>hisA1 leuA8 lys-1 metB purB thrA5 trpC2</i>	T. Iijima, HLL 3g
61639	<i>metC7 purH1 spdA1 trpC2</i>	Freese <i>et al.</i> (1979a), 60015 + EMS
61684	<i>GuaC3 metC7 purH1 spdA1 trpC2</i>	61639 + EMS
61705	<i>aroI906 purB33 dalA1 trpC2</i>	Dedonder <i>et al.</i> (1977), QB928
61708	<i>gltA292 trpC2</i>	Dedonder <i>et al.</i> (1977), QB922
61709	<i>aroD120 lys-1 trpC2</i>	Dedonder <i>et al.</i> (1977), QB935
61711	<i>hisA1 thr-5 trpC2</i>	Dedonder <i>et al.</i> (1977), QB917
61716	<i>metB10 purH1 trpC2</i>	Tf of 61469 by 61639
61852	<i>lys relA1 trpC2</i>	J. Gallant, BR17
61964	<i>metC7 spdA1 trpC2</i>	Tf of 61639 by 60198
62017	<i>ilvBdel1 kauA1 purH1 relA1</i>	Tf of 61885 by 61676
62092	<i>metC7 OrdA1 trpC2</i>	Tf of 61639 by 60198
62109	<i>hisA1 spdA1 trpC2</i>	Tf of 61711 by 61639
62156	<i>citG metC7 spdA1 trpC2</i>	Tf of 61639 by 61423
62159	<i>GuaC3 metC7 spdA1 trpC2</i>	Tf of 61684 by 60813
62160	<i>citD GuaC3 metC7 spdA1 trpC2</i>	Tf of 61684 by 60813
62163	<i>citG ilvBdel1 kauA1 relA1</i>	Tf of 62017 by 61423
62172	<i>hisA1 leuA8 lys-1 metB purB spdA1 trpC2</i>	Td of 61473 by 61639
62173	<i>hisA1 leuA8 lys-1 metB purB trpC2</i>	Td of 61473 by 61639
62177	<i>trpC2</i>	Reversion of 61423
62222	<i>hisA1 lys-1 metB purB spdA1 trpC2</i>	Td of 62172 by 61852
62223	<i>hisA1 lys-1 metB purB relA1 spdA1 trpC2</i>	Td of 62172 by 61852
62225	<i>hisA1 lys-1 metB purB relA1 spdA1 trpC2</i>	Td of 62172 by 61852

* Standard *B. subtilis* gene symbols are used (Henner & Hoch, 1980). For unmapped mutations the first letter of the gene symbol is capitalized. *pur* indicates a block in the common purine pathway; such a mutant can grow with adenine, guanine, or hypoxanthine as a purine source. *GuaC3* is an unmapped mutation that produces a deficiency in guanosine monophosphate reductase (T. Endo & E. Freese, unpublished). *OrdA1* is an unmapped mutation that produces a deficiency of the inducible L-ornithine : 2-oxoacid aminotransferase (Whiteman *et al.*, 1980). *spdA1* is the mutation investigated here, which causes continual sporulation in glucose medium.

† Abbreviations: EMS, NTG, and UV indicate mutagenesis by ethyl methanesulphonate, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and ultraviolet radiation, respectively. Tf denotes transformation by DNA and Td denotes transduction of the first strain by a PBS1 phage lysate obtained from the second strain.

added and the solution was kept at 4 °C. To transform a given marker into a recipient strain (by congression), donor DNA (50 µg ml⁻¹ final concentration) was added to a culture of bacteria auxotrophic for the property to be selected against and the mixture incubated according to Iijima *et al.* (1977). After 2 h, the cells were plated on selective medium, and colonies possessing the desired marker were isolated.

For transduction by phage PBS1, a phage lysate was prepared from the donor strain grown in 1.75% (w/v) Antibiotic Medium 3 (Difco); the lysate was treated with 10 µg DNAase ml⁻¹ for 30 min at 37 °C. Recipient strains were grown in Antibiotic Medium to an A_{600} of 1.0. A 3 ml volume of the culture and 3 ml PBS1 lysate were mixed and incubated for 20 min at 37 °C. The cells were washed with PM buffer and plated on selective medium which contained: glucose medium, 20 mM-glutamate, 0.001% (w/v) yeast extract and auxotrophic requirements as appropriate. Cells not treated with PBS1 phage were also plated on the selective medium to measure the reversion frequency of the selected marker. To identify the *spdA* marker, isolated transformants were grown in glucose medium to the end of exponential growth or early into the stationary phase; if the frequency of phase-bright spores, observed under the phase-contrast microscope, was higher than 10%, the isolate was scored as *spdA*.

Selection and identification of transformants or transductants. The *OrdA* property (see Table 1) was identified by slow growth of cells on plates containing glucose medium in which (NH₄)₂SO₄ was replaced by 10 mM-Na₂SO₄ and 2 mM-ornithine as nitrogen source; *OrdA* mutants grew well if the ammonium-free medium contained 2 mM-glutamate. The deficiency of 2-oxoglutarate dehydrogenase (*citD*) was identified by acid production on bromocresol purple plates.

Deficiency of fumarase (*citG*) was determined by the inability of strains to grow on aspartate as sole carbon source (synthetic medium containing 20 mM-potassium aspartate and appropriate auxotrophic requirements). The

reversion to *citG*⁺ was revealed by the loss of the extensive acid production on bromocresol purple plates. Fumarase activity was also assayed in cell extracts according to Hanson & Cox (1967).

The *rel*⁺ or *rel* property was ascertained by measuring the incorporation of [³H]uracil into TCA-precipitable material. Cells were cultured in glucose medium containing glutamate (20 mM), histidine (30 µg ml⁻¹), lysine (50 µg ml⁻¹) and hypoxanthine (0.5 mM). When the *A*₆₀₀ reached 1, the culture was diluted with fresh medium containing [³H]uracil [0.1 µCi ml⁻¹ (3.7 kBq ml⁻¹) and 15 µM final concentration] to an *A*₆₀₀ of 0.15. After shaking for 5 min the culture was divided into two equal portions, one of which received granaticin (final concentration 0.2 µg ml⁻¹). Every 5 min for 40 min, 0.5 ml of each culture was mixed with 0.5 ml ice-cold 10% (w/v) TCA. After 2 h in the cold, the precipitate was filtered on to a membrane filter (pore size 0.45 µm) and washed twice with 4 ml ice-cold 5% (w/v) TCA; the radioactivity of the collected material was determined in a scintillation counter (LKB 1210) using Aquasol (New England Nuclear) as the scintillation fluid.

Enzyme assays. To prepare cell extracts, cells were cultured in glucose medium containing appropriate auxotrophic requirements. When the *A*₆₀₀ reached 1, 35 ml of culture was centrifuged at 10000 *g* for 10 min at 4 °C. The cells were washed with 0.05 M-Tris/HCl (pH 7.2) and suspended in 0.9 ml of this buffer. Lysozyme (100 µl; 1 mg ml⁻¹) was added, and the mixture left at 37 °C for 20 min. The lysate was centrifuged at 15000 *g* for 20 min to remove cell debris. The supernatant was used for enzyme assays. Protein was measured according to Kalb & Bernlohr (1977).

Pyruvate carboxylase was assayed spectrophotometrically at 20 °C (Diesterhaft & Freese, 1973). The reaction mixture contained 50 mM-Tris/HCl (pH 7.5), 5 mM-MgCl₂, 1 mM-NADH, 35 mM-NaHCO₃, 2 mM-ATP, 0.2 mM-acetyl-CoA and 50 µg malate dehydrogenase ml⁻¹. The reaction was started by the addition of 2 mM-pyruvate and followed by the decrease in *A*₃₄₀. The specific activities are expressed in nmol NADH consumed (mg protein)⁻¹ min⁻¹.

Pyruvate dehydrogenase was assayed at 20 °C as described for 2-oxoglutarate dehydrogenase by Reed & Mukherjee (1969). The reaction mixture contained 50 mM-potassium phosphate buffer (pH 6.5), 1 mM-MgCl₂, 1 mM-NAD, 0.5 mM-EDTA, 2 mM-glutathione, 0.1 mM-coenzyme A, and 0.2 mM-thiamin pyrophosphate. The reaction was initiated by the addition of 1 mM-sodium pyruvate, and the rate of increase in *A*₃₄₀ was measured. The specific activities are expressed in nmol NAD reduced (mg protein)⁻¹ min⁻¹.

RESULTS

Growth and sporulation of strains containing the spdA1 mutation

Among the purine mutants whose sporulation had been examined in synthetic medium containing excess (55 mM) glucose (glucose medium) with purine supply limiting (Freese *et al.*, 1979*a*), we found some strains which sporulated continually even in the presence of excess (1 mM) hypoxanthine. We do not know whether the mutations (termed *spd*) causing such continual sporulation often arise spontaneously or whether they occurred due to some inadvertent selection. The typical growth and sporulation behaviour of such a strain (61639) *purH spdA1* is shown in Fig. 1(*a*) (only the relevant gene symbols are mentioned in the text). Following inoculation of glucose medium containing 1 mM-hypoxanthine at a low *A*₆₀₀ (0.001), the heat-resistant spore titre increased throughout growth and thereafter. Apparently, in every cell generation a certain fraction of cells was initiated to enter the sporulation process. Neither glucose nor hypoxanthine was used up at the end of the experiment. In contrast, the parent (strain 60015) produced far fewer spores and most of these only after growth had stopped (Fig. 1*b*).

Addition of glutamate to the glucose medium suppressed sporulation of the *spdA1* mutant until glutamate had been used up (the glutamate decrease is shown in the inset to Fig. 1*a*). With 20 mM-glutamate, sporulation remained suppressed throughout the experiment. This amount of glutamate also suppressed the small increase in the spore titre of our standard strain (60015; Fig. 1*b*).

The approximate map location of the *spdA1* marker was determined by transducing the 'kit' strains provided by Dedonder *et al.* (1977) for mapping purposes with phage PBS1 grown in strain 61639. The *spdA1* marker was linked most closely to the *thr* marker (63 out of 76 *thr*⁺ transductants were *spdA*) and more loosely to *hisA* (17/99) and *citG* (26/50). For a map of the auxotrophic markers see Henner & Hoch (1980).

The continual sporulation observed in strain 61639 did not depend on the purine requirement of the *purH* mutant. When the *purH* marker of strain 61639 was transformed into another strain (61469), the resulting strain (61716) produced only 7.3×10^4 spores ml⁻¹ after 12 h in glucose

Fig. 1. Growth and sporulation of the *spdA1* mutant (61639) and its parent (60015) without and with glutamate. (a) Cells of strain 61639 were inoculated into 100 ml glucose medium supplemented with 1 mM-hypoxanthine to give an A_{600} of 0.001. When the A_{600} reached 0.05, the culture was divided into five portions which received: no addition (●), or glutamate at 2 mM (○), 5 mM (△), 10 mM (▲) or 20 mM (□) (final concentrations). The A_{600} (top) was monitored and viable cell titre (middle) and heat-resistant spore titre (bottom) were determined at the indicated times. The numbers on the curves indicate the initial glutamate concentrations. The glutamate concentrations remaining in the cultures at different times are shown in the inset. (b) The standard strain (60015) was cultured in glucose medium as above. When the A_{600} reached 0.04, the culture was divided into two portions which received: no addition (●) or 20 mM-glutamate (□).

medium containing 1 mM-hypoxanthine (grown as in Fig. 1). A similar spore titre (2.1×10^4 ml⁻¹) was produced when the medium contained 20 mM-glutamate. Similarly, when the *purH* marker of strain 61639 was removed by transformation with DNA of a *pur*⁺ strain (60198), all 10 isolated and purified *pur*⁺ transformants sporulated in glucose medium equally as well as strain 61639; addition of 1 mM-hypoxanthine, 1 mM-adenine or 1 mM-guanosine did not significantly affect the sporulation frequency. One of these *pur*⁺ strains, 61964 (*spdA1*), was used for most of the following experiments.

We also assessed the ability of the *spdA1* marker to cause continual sporulation by maintaining a culture of strain 61964 at an essentially constant A_{600} for 17 h. For this purpose the culture was diluted twofold in glucose medium whenever the A_{600} reached 0.4. The titre of heat-resistant spores per viable cell initially increased rapidly and then remained almost constant at a value of about 5×10^{-2} (Fig. 2). The viable cell titre reached a maximum and then decreased very slowly, probably because an increasing fraction of the cells entered spore development and thus no longer divided. Under the same growth conditions, the spore titre of a culture of strain 60015 remained lower and that of a culture of strain 60001 remained much lower, although both values increased slowly (Fig. 2).

The continual sporulation of *spdA1* strains in glucose medium could be prevented not only by glutamate but also by other compounds providing carbon to the citrate or the arginine cycle.

Fig. 2. Sporulation of the *spdA1* mutant (61964), its parent (60015) and Spizizen's 168 strain (60001) in cultures maintained at a constant A_{600} . Strains 60001 (○), 60015 (△) and 61964 (●) were grown in glucose medium to an A_{600} of 0.4. A 5 ml sample of each culture was mixed with 5 ml prewarmed glucose medium, and mixtures were shaken until the A_{600} reached 0.4 again. This dilution and growth was repeated every 90 min for 14.5 h; the A_{600} of the cultures was thereby kept between 0.2 and 0.4. The A_{600} (top) was always measured before dilution. The viable cell titre (middle) and spore titre (bottom) were determined every 90 min.

This is shown for strain 61964 (*spdA1*) in Table 2; other amino acids (such as alanine, isoleucine, methionine, valine, etc.), purines or pyrimidines had no effect (legend to Table 2).

Conceivably, the compounds suppressing sporulation did not prevent its initiation but interfered with some later stage of the sporulation process. To examine this possibility, strain 61964 was grown in glucose medium containing 20 mM-glutamate and, at an A_{600} of 0.05, either decoyinine (400 μ M) or mycophenolic acid (200 μ M) was added to induce sporulation (Mitani *et al.*, 1977; Freese *et al.*, 1979b). With both inducers a massive increase in the spore titre was observed 6–8 h after their addition. At t_{10} the spore titre was 2.1×10^5 ml $^{-1}$ without inducer, but 1.9×10^8 ml $^{-1}$ with decoyinine and 2.3×10^8 ml $^{-1}$ with mycophenolic acid. Similar induction of sporulation was obtained in cultures in which continual sporulation was prevented by arginine, aspartate, citrate, glutamine, malate, or ornithine (each at 10 mM). These results indicate that the compounds suppressing the continual sporulation interfered with the initiation of sporulation and not with later spore development.

*Genetic and biochemical approaches to determine the basis of the *spdA1* mutation*

The above results had shown that a compound metabolically associated with the arginine or the citrate cycle was required to suppress the continual sporulation resulting from the *spdA1* mutation. We separated the two cycles by a mutation (*OrdA1*) which produced a deficiency in ornithine:2-oxoacid aminotransferase and thereby prevented the conversion of ornithine to glutamic γ -semialdehyde and glutamate (Whiteman *et al.*, 1980). The *OrdA1* mutation was transformed (by congression) into strain 61639, producing the *OrdA1 spdA1* strain (62092).

Table 2. *Effect of various compounds on the sporulation of the spdA1 strain 61964*

Strain 61964 was cultured in 350 ml glucose medium from $A_{600} = 0.005$ to $A_{600} = 0.05$. Samples (10 ml) were then transferred into 125 ml flasks containing one of the stated compounds. After 10 h further incubation, the viable cell titre and spore titre were determined; the former was between $1.7 \times 10^8 \text{ ml}^{-1}$ and $1.1 \times 10^9 \text{ ml}^{-1}$. Addition of D-glucose, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , L-valine, L-isoleucine, L-alanine, L-homoserine, L-methionine or acetate (each at 10 mM), glycine, L-phenylalanine or L-tryptophan (each at 5 mM), putrescine (2 mM), or adenine, guanosine, hypoxanthine, uracil or thymine (each at 1 mM) did not affect sporulation (spore titres between $1.5 \times 10^8 \text{ ml}^{-1}$ and $4.8 \times 10^7 \text{ ml}^{-1}$).

Compound	Concentration (mM)	$10^{-5} \times$ Spore titre ml^{-1}
No addition	—	1300.0
L-Glutamate	10	0.8
L-Glutamine	10	7.5
L-Aspartate	10	2.4
L-Ornithine	10	2.3
L-Citrulline	10	17.0
L-Arginine	10	2.5
L-Histidine	10	14.0
DL-2,6-Diaminopimelic acid	10	95.0
L-Lysine	10	60.0
L-Proline	5	42.0
L-Malate	10	2.7
Citrate	10	3.3
Succinate	10	360.0
Pyruvate	10	400.0
2-Oxoglutarate	10	3.2

When this strain was transferred from glucose medium to the same medium containing 10 mM-ornithine instead of $(\text{NH}_4)_2\text{SO}_4$ as nitrogen source, it grew only slowly (doubling time of 330 min), whereas it grew well with glutamate as nitrogen source (doubling time 70 min). Strain 61964 (*spdA1*), obtained in the same transformation procedure, grew well on both glutamate and ornithine (doubling time 70 min) because it had not received the *OrdA1* mutation. In common with the results shown in Fig. 1, glutamate and citrate increased the rate of growth and suppressed the continual sporulation of both strains, whereas ornithine or arginine had this effect only for the *Ord⁺* strain 61964. In the *OrdA1* mutant (62092), ornithine suppressed the continual sporulation only slightly and arginine did not suppress it at all (Table 3). Thus the compound controlling the continual sporulation must be associated with the citrate cycle rather than the arginine cycle.

To locate the sporulation-controlling compound more accurately, a deficiency in 2-oxoglutarate dehydrogenase (*citD* gene of strain 60813) was introduced by congression into a strain containing *spdA* (61684). In the resulting *citD spdA1* transformant (62160), continual

Table 3. *Comparison of sporulation of the spdA1 strain (61964) and the OrdA1 spdA1 strain (62092) in the presence of different compounds*

Cells were cultured in glucose medium as detailed in Table 2. When the A_{600} was 0.05, the stated compounds were added (10 mM final concentration). The viable cell titre (*V*) and heat-resistant spore titre (*S*) were determined 10 h later.

Addition (10 mM) to glucose medium	Strain 61964 (<i>spdA1</i>)		Strain 62092 (<i>OrdA1 spdA1</i>)	
	$10^{-5} \times$ Spore titre ml^{-1}	<i>S/V</i>	$10^{-5} \times$ Spore titre ml^{-1}	<i>S/V</i>
None	1400	3.0×10^{-1}	2400	3.2×10^{-1}
Citrate	7.6	5.2×10^{-4}	7.6	5.4×10^{-4}
Glutamate	1.2	1.5×10^{-4}	3.8	3.0×10^{-4}
L-Ornithine	0.4	2.5×10^{-5}	130	1.2×10^{-2}
L-Arginine	93	7.1×10^{-3}	1700	2.3×10^{-1}

Table 4. *Comparison of growth and sporulation of the spdA1 strain (62159) and the citD spdA1 strain (62160) in the presence of different compounds*

Cells were cultured in glucose medium as detailed in Table 2. When the A_{600} reached 0.05, one of the stated compounds (sodium salt) was added. The A_{600} was monitored and 10 h after the additions, the viable cell titres (between 4.5×10^7 and 1.2×10^9 ml⁻¹) and octanol-resistant spore titres were determined.

Addition (10 mM) to glucose medium	Strain 62159 (<i>spdA1</i>)		Strain 62160 (<i>citD spdA1</i>)	
	$10^{-5} \times$ Spore titre ml ⁻¹	Doubling time (min)	$10^{-5} \times$ Spore titre ml ⁻¹	Doubling time (min)
None	2700	65	1800	90
Malate	70	48	43	50
Citrate	15	45	750	95
2-Oxoglutarate	7	45	730	90
Aspartate	7	45	33	45
Glutamate	7	45	860	75

sporulation was still suppressed by aspartate or malate, whereas citrate, glutamate, or 2-oxoglutarate produced only a weak effect (Table 4). Another transformant (62159), isolated in parallel but not containing the *citD* mutation, still showed the suppression of sporulation by all of the above compounds (Table 4). Apparently, the compound controlling continual sporulation was one of the metabolites in the citrate cycle between succinyl-CoA and oxaloacetate, or was derived from such a metabolite. Such a derived compound is aspartate, which in turn serves as a precursor for a number of amino acids. To determine whether the production of any particular amino acid was significantly altered in the *spdA* strain, cells of strains 61964 and 60015 growing in glucose medium at an A_{600} of 1 were extracted with formic acid and the amino acid composition of the extract was determined in the amino acid analyser. Compared with strain 60015, the amino acid concentrations in strain 61964 were somewhat lower for aspartate (37%) and methionine (41%), and higher for alanine (72%), glutamine (80%), proline (100%) and valine (330%). The other amino acid concentrations were similar in the two strains. The concentration of glutamate was always much higher than that of any other amino acid; it was slightly higher (94 nmol per A_{600} unit) in strain 61964 than in strain 60015 (85 nmol per A_{600} unit). To determine whether the large glutamate peak represented L- or D-glutamate, we also measured L-glutamate enzymically; 95 and 87 nmol per A_{600} unit was found in strains 61964 and 60015 respectively. Thus most (or all) of the intracellular glutamate was in the L-form.

To examine whether valine, the amino acid whose concentration was particularly high in the *spdA* strain 61964, might be responsible for the increased sporulation frequency of this strain, the standard strain (60015) was grown in glucose medium with different amounts (up to 10 mM) L-valine. The spore titres, measured 12 h after the A_{600} reached 0.5, were all between 1.4×10^5 and 1.7×10^5 ml⁻¹, regardless of the valine concentration. Therefore, the increased valine concentration did not appear to be responsible for the increased sporulation frequency of the *spdA* mutant.

Effect of a fumarase mutation and assay of pyruvate carboxylase

Additional information was obtained by using the *citG* mutation which disables the production of fumarase. The *citG* strain (61423) grew at a low rate in glucose medium (Fig. 3) and excreted large quantities of fumarate as shown by acid production on bromocresol purple plates. The growth rate in glucose medium increased with the addition of 20 mM-2-oxoglutarate, citrate, glutamate, malate, or aspartate, in this sequence (only the results for citrate and malate are shown in Fig. 3). Apparently, the *citG* mutant converted so much of its limiting oxaloacetate (produced from glucose) into the excreted fumarate that it could not produce enough aspartate. Added glutamate (and its precursor citrate) probably counteracted this drain by feed-back inhibition and because glutamate (or 2-oxoglutarate) represses citrate synthase and aconitase (Hanson & Cox, 1967). This was confirmed by amino acid analysis of cell extracts. When strains

Fig. 3. Comparison of growth rates of *citG* strain (61423) and *citG spdA1* transformant (62156). The cells were cultured in glucose medium containing 20 mM-glutamate. At an A_{600} of 0.2, the cells were collected on membrane filters and resuspended in prewarmed glucose medium without glutamate. Each cell suspension was divided into three portions which received: 20 mM-potassium citrate ($\triangle$ --- $\triangle$, strain 62156; $\bigcirc$ --- $\bigcirc$, strain 61423), 20 mM-potassium malate ($\triangle$ — $\triangle$, strain 62156; $\bigcirc$ — $\bigcirc$, strain 61423), or no addition ($\blacktriangle$ — $\blacktriangle$, strain 62156; $\bullet$ — $\bullet$, strain 61423).

were grown to an A_{600} of 1 in glucose medium containing 20 mM-glutamate and then extracted, the *citG* mutant cells (61423) contained 83 pmol glutamate per A_{600} unit but only 0.5 pmol aspartate per A_{600} unit, whereas the standard strain (60015) contained 86 pmol glutamate per A_{600} unit and 2.5 pmol aspartate per A_{600} unit. Thus, even in the presence of glutamate, aspartate was scarce.

Since the presence of both the *citG* and *spdA* mutations in one strain might reveal more clearly with which metabolic reaction the *spdA* mutation interfered, we introduced the *citG* mutation into the *spdA1* strain (61639) by congression. The resulting *citG spdA1* strain (62156) grew even more slowly than the *citG* strain (61423) in glucose medium with or without citrate (Fig. 3), but in the presence of aspartate, glutamate (result not shown) or malate, this strain grew at the same rate as the *citG* strain. The results suggested that the *spdA1* mutation decreased the rate at which glucose could supply oxaloacetate. Therefore, we examined the growth on pyruvate.

Our standard strain (60015) grew distinctly faster than the *spdA1* mutant (61964) in synthetic medium with pyruvate (100 mM) as sole carbon source. To our surprise, the original *trpC* mutant of Spizizen (60001) grew on pyruvate even faster. All three strains grew at the same rate (doubling time 80 min) in synthetic medium plus 100 mM-glucose or malate (results not shown). Because the metabolism of pyruvate was limited, we measured the specific activity of pyruvate carboxylase in these three strains and also in related strains. These activities were compared with the extent of sporulation in glucose medium. As the results in Table 5 show, strain 60001 had the highest pyruvate carboxylase activity and the lowest frequency of sporulation. Strain

Table 5. *Correlation of pyruvate carboxylase activity and sporulation in glucose medium*

Strain	Pyruvate carboxylase*		Sporulation†	
	Specific activity	%	Glutamate added (mM)	Spore titre ml ⁻¹
60001 (<i>trp</i>)	43.2	100	0	5.7×10^2
			20	1.0×10^3
60015 (<i>trp met</i>)	24.9	58	0	6.7×10^6
			20	7.5×10^3
61964 (<i>trp met spdA1</i>)	18.4	43	0	1.3×10^8
			20	7.7×10^4
61711 (<i>trp his thr</i>)	35.5	82	0	6.1×10^4
			20	2.9×10^3
62109 (<i>trp his spdA1</i>)	17.3	40	0	8.9×10^7
			20	1.2×10^5

* Pyruvate carboxylase activity [nmol NADH consumed (mg protein)⁻¹ min⁻¹] was assayed in cultures grown in glucose medium plus required supplements. When the A_{600} reached 1.0, extracts were prepared and assays were performed as described in Methods.

† The strains were cultured as detailed in Table 3 in glucose medium plus 30 µg histidine ml⁻¹ for strains 61711 and 62109, and 20 µg threonine ml⁻¹ for strain 61711. When the A_{600} reached 0.05, the cultures were split into two portions and glutamate (20 mM final concentration) was added to one of them. The heat-resistant spore titres were determined 12 h after glutamate addition. The viable cell titres were between 1.2×10^8 and 2.3×10^9 ml⁻¹.

60015 had only about 60% of this activity and sporulated better. The two strains carrying the *spdA1* mutation had only 40% of the pyruvate carboxylase activity of strain 60001 and produced a high spore titre (about 10^8 ml⁻¹) in glucose medium. In all strains, the spore titre remained low if the medium also contained 20 mM-glutamate (Table 5).

In contrast to pyruvate carboxylase, the specific activities of pyruvate dehydrogenase were similar in strain 60001 and the *spdA1* strain 61964 (specific activities of 59.6 and 56.4 respectively).

The involvement of the stringent response

The above results suggested that strains containing the *spdA1* mutation were partially deficient in the production of oxaloacetate from glucose and consequently may contain slightly reduced concentrations of derived compounds such as aspartate. This suggested that the continual sporulation might be caused by a partial stringent response and would therefore be limited to *rel*⁺ strains. To examine this possibility, we performed several genetic crosses which combined *relA* and *spdA1* mutations in one strain. The presence of the relaxed (*relA*) genes in a strain was verified by the continued uracil incorporation into acid-precipitable material following the addition of granaticin. Granaticin evokes the stringent response in *rel*⁺ strains because it inhibits the acylation of leucyl-tRNA (Ogilvie *et al.*, 1975; Lopez *et al.*, 1981a; Lopez *et al.*, 1981b). As we did not have a reliable direct biochemical assay of the presence of the *spdA1* mutation, we obtained our results from reciprocal crosses. The marker *spdA1* was transduced into a *relA* strain, and *relA* into an *spdA1* strain; in both cases a genetically linked marker was selected for. For the former cross, we first constructed (by congression) a *relA citG* strain (62163) because the *citG* marker could be easily scored by acid excretion, and was linked to the *spdA1* marker as was shown above. This strain was then transduced by PBS1 phage grown in the *spdA1* strain (61639). Fifty *cit*⁺ transductants (isolated as acid non-producers on colour test plates and also confirmed as being able to grow on aspartate as sole carbon source) all produced less than 0.1% spores 18 h after the cells had been inoculated into glucose medium (at an A_{600} of about 0.005) from plates containing glucose medium, 20 mM-glutamate and 50 µg ml⁻¹ isoleucine and valine). In contrast, the *spdA1* strain (61964) produced more than 50% spores. According to the earlier mapping results, half of these 50 transductants should have received the *spdA1* marker.

For the reverse transfer of the *relA* marker into an *spdA1* strain, we first constructed a *leu spdA1* strain (62172) because *leu* is linked to *relA* (Smith *et al.*, 1980). This strain was then

Table 6. Comparison of growth and sporulation character of *spdA1* and *spdA1 relA* strains

Cells were cultured from an initial A_{600} of 0.001 in glucose medium plus 30 μg histidine ml^{-1} , 50 μg lysine ml^{-1} and 1 mM-hypoxanthine for the first five strains, and 50 μg leucine ml^{-1} for 62172 and 62173. The viable cell titre (V) and heat-resistant spore titre (S) were measured 12 h after the culture passed an A_{600} of 0.05. Doubling times were measured in synthetic medium without glucose but containing 100 mM-potassium pyruvate, 30 μg histidine ml^{-1} , 50 μg lysine ml^{-1} and 0.5 mM-hypoxanthine.

Strain	Genetic character			$10^{-5} \times$ Spore titre ml^{-1}	S/V	Doubling time in pyruvate (min)
	<i>leu</i>	<i>relA</i>	<i>spdA1</i>			
62172	—	+	—	3000	3.8×10^{-1}	ND
62173	—	+	+	0.6	6.2×10^{-5}	ND
62222	+	+	—	1900	3.3×10^{-1}	185
62223	+	—	—	0.2	5.0×10^{-3}	185
62225	+	—	—	0.2	2.0×10^{-4}	185
61964	+	+	—	1300	2.0×10^{-1}	185
60001	+	+	+	0.006	4.0×10^{-7}	90

ND, Not determined.

Table 7. Involvement of *citG* in the induction of sporulation

The strains were cultured from an initial A_{600} of 0.005 in glucose medium plus 20 mM-glutamate (and isoleucine and valine, each at 50 μg ml^{-1} , for strain 62163). Octanol-resistant spore titres were determined 12 h after the cultures had passed an A_{600} of 0.05. The viable cell titres were between 2.3×10^8 and 2.6×10^9 ml^{-1} .

Strain	Genetic character		Addition (20 mM) to glucose medium	Octanol-resistant spore titre ml^{-1}	Doubling time (min)
	<i>citG</i>	<i>relA</i>			
61423	—	+	None	9.1×10^7	60
			Aspartate	8.0×10^1	40
			Malate	5.0×10^1	45
			2-Oxoglutarate	2.9×10^4	60
62163	—	—	None	6.0×10^4	75
62177	+	+	None	1.4×10^2	45

transduced by PBS1 phage grown in a *relA* strain (61852) and *leu*⁺ strains were isolated. Thirty-five transductants, tested for sporulation in glucose medium, fell into two classes; 28 strains sporulated well whereas the remainder did not. One of the strains which sporulated well (62222) and two of the strains which did not (62223, 62225) were tested for their relaxed properties by measuring the rate of uracil incorporation. The results in Table 6 show that the strains containing the *spdA1* marker sporulated well when they were stringent (*rel*⁺) but not when they were relaxed (*relA*). Thus the combination of *relA* and *spdA1* prevented the expression of the *spdA1* property regardless of the genetic background of the strain used.

In common with the *spdA1* strains, the *citG* strain (61423) sporulated at high frequency in glucose medium, but it sporulated even in the presence of glutamate (Table 7); a revertant of *citG* (62177) did not show this effect. The sporulation was prevented by the addition of aspartate or malate, both of which increased the growth rate of the *citG* strain. This extensive sporulation resulted from the stringent response because when the *citG* mutation was transformed into a *relA* strain, the resultant *citG relA* strain (62163) did not sporulate in glucose medium (with or without glutamate; Table 7).

DISCUSSION

The *spdA1* mutation conveyed to strains capable of the stringent response (*rel*⁺ strains) the remarkable property that cells growing in glucose medium entered sporulation development at every generation at a high probability. When the A_{600} of a culture of the *spdA* strain (61964) was

kept within a narrow range, the spore frequency reached 5% and then increased only very slowly, which indicates that it was close to the equilibrium value (Fig. 2). While a committed cell of *B. subtilis* needs 4–5 h to produce a spore, the non-committed cells continue to multiply. As the doubling time (of the mixture of multiplying and committed cells and finished spores) was about 1.5 h, it took a committed cell about three doubling times to produce a heat-resistant spore. Thus about $2^3 \times 5\% = 40\%$ of the cells in the culture were sporulating, contained spores or were free spores. [More accurately, the fraction of sporulating cells and spores in a growing population in which an equilibrium between sporulation and dividing cells has been reached, is $p(1 + p)^{-1}$, where p is the probability that a cell enters sporulation within one generation time. This relationship holds if p is small compared to 1 and if germination is negligible. However, these details are unimportant for the comparison of the three strains.] For the other two strains in Fig. 2, the spore titre continued to increase; if one assumes that the highest S/V value observed was close to the equilibrium value, only 6×10^{-4} cells of our standard strain (60015) and 1.6×10^{-5} cells of the original Spizizen 168 strain (60001) would sporulate or be finished spores at equilibrium. The 40-fold difference between strains 60015 and 60001 shows that even 'standard' strains normally used for sporulation experiments differ in their continual sporulation ability while they are growing in the same synthetic medium containing excess ammonium, glucose and phosphate but no other carbon sources supplying the citrate cycle. This explains why in other studies in which the induction of sporulation was measured, different background (non-induced) spore titres were observed when different strains and growth conditions were used (Freese *et al.*, 1979*a*; Lopez *et al.*, 1981*b*; Ochi *et al.*, 1981).

The continual sporulation of *spdA* strains resulted from a partial stringent response since it was absent in all *spdA relA* mutants produced by transforming the *spdA1* marker into a *relA* strain and vice versa. The amino acid whose partial deficiency was responsible for the frequent sporulation of *spdA1* mutants and also the elevated sporulation of strain 60015, as compared with strain 60001, was apparently aspartate, as was shown by the introduction of various mutations. Enzymic studies indicated that the specific activity of pyruvate carboxylase was particularly low in *spdA* mutants, and also lower in 60015 than in 60001. The activity of this enzyme, which is solely responsible for the synthesis of oxaloacetate from glucose (Diesterhaft & Freese, 1973), controls the rate of oxaloacetate synthesis in glucose medium and thus the synthesis of the amino acids derived from citrate cycle intermediates – in particular aspartate. The concentration of aspartate was slightly (37%) lower in the *spdA* mutant (61964) than in the standard strain (60015). Although this difference was not pronounced, the fact that the addition of aspartate or malate increased the growth rate of all strains indicates that the supply of these carbon sources was growth-limiting. Glutamate addition also increased the rate of growth but this was probably due to an indirect effect, because the intracellular glutamate concentration was always very high. Addition of glutamate is known to cause feed-back inhibition of its own synthesis and to repress the synthesis of citrate synthase and aconitase [the effective compound is in fact 2-oxoglutarate (Hanson & Cox, 1967; Ohne, 1974)]. Therefore, glutamate addition probably reduces the drain of oxaloacetate in the direction of 2-oxoglutarate synthesis, making more oxaloacetate available for aspartate synthesis. The decrease of pyruvate carboxylase activity in the *spdA* strain (61964) also explains why the concentration of alanine and valine, whose synthesis requires pyruvate, was elevated in the *spdA* mutant (61964).

We do not know whether the decreased activity of pyruvate carboxylase is the only enzymic alteration caused by the *spdA1* mutation. The *spdA1* gene is located close to the *thr* marker and is also linked to the *citG* marker. It is therefore genetically different from the *pyc* gene whose mutation causes complete deficiency of pyruvate carboxylase activity (Diesterhaft & Freese, 1973). Presumably, one of the two genes (*pyc* or *spdA1*) is the structural gene for pyruvate carboxylase, whereas the other gene controls the synthesis of this enzyme. A fumarase (*citG*) mutant (61423) behaved similarly to the *spdA* mutant. It grew slowly in glucose medium because it lacked aspartate. In addition it sporulated at a high frequency in glucose medium, a property that was prevented by the addition of aspartate or malate but not by glutamate. The sporulation of the *citG* strain was also prevented by the introduction of a *relA* mutation. The *spdA1* gene is not identical to the *citG* gene, although it is linked to it, because the *spdA1* mutant (61964) did not

have a decreased specific activity of fumarase (results not shown) and, unlike the *citG* mutant, did not excrete acid (fumaric acid).

The continual sporulation of all strains containing the *spdA1* or *citG* mutations was effectively prevented by aspartate, whereas other amino acids, including those derived from aspartate (isoleucine, lysine, methionine, threonine) were much less effective (Table 2). Therefore, it appears likely that a partial deficiency of aspartyl-tRNA (or asparaginyl-tRNA) was responsible for the partial stringent response that caused the continual sporulation.

Although the involvement of a mild stringent response in the continual sporulation of *spdA* strains was clearly established by genetic means, we have not found a significantly increased pool concentration of ppGpp or pppGpp, or a significantly decreased concentration of GTP in the *spdA1* strain (61964) grown in glucose medium as compared with the concentrations of these compounds in cells grown in the presence of 20 mM-glutamate, which prevented sporulation (results not shown). Therefore, we cannot claim that the change in the concentration of any nucleotide (in particular GTP) was responsible for the increased sporulation frequency. However, the frequency at which cells of an *spdA1* strain entered sporulation per unit time was much lower than that at which cells enter massive sporulation after their sudden transfer to a poor medium, where a drastic (though transient) stringent response is produced (Lopez *et al.*, 1981*a*; Lopez *et al.*, 1981*b*; Ochi *et al.*, 1981). Presumably, every cell experiences a decrease in certain metabolites at a certain time in the cell cycle. At that time it may be particularly sensitive to an additional reduction in the synthesis of an aminoacyl-tRNA. Therefore, a slight reduction in the rate of aspartyl-tRNA synthesis in the *spdA1* and *citG* strains may have a pronounced effect for the few cells at a critical stage of their cell cycle. To examine these biochemical details of continual sporulation, one would have to use cells that are highly synchronized with respect to the cell cycle.

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