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Curing of a Sporulation Mutant and Antibiotic Activity of *Bacillus subtilis*

RÜDIGER SCHMITT[1] AND ERNST FREESE

Laboratory of Molecular Biology, National Institute of Neurological Diseases and Blindness, Bethesda, Maryland 20014

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Sporulation mutants of *Bacillus subtilis*, blocked either in the citric acid cycle or in another pathway necessary for uracil incorporation during the developmental period, were tested for their sporulation response to different carbon sources. All of the citric acid cycle mutants and all but one of the other mutants failed to respond. The one phenotypically curable mutant, 60764, responded to most metabolizable carboxylic acids, such as acetate and fatty acids, and to some other carbon sources. For an optimal response, it was necessary to add the compound at a certain concentration and time (0.08 M for acetate, when the extinction of the culture at 600 nm was 1). A liquid medium was devised in which an appreciable amount of antibiotic activity against *Staphylococcus aureus* was produced by our standard strain 60015. The mutant 60764 produced, even in the presence of palmitate, 20 times less antibiotic and sporulated more slowly than 60015. The antibiotic activity in both strains consisted of three major and several minor molecular species, as detected by thin-layer chromatography. When purified antibiotic was added to an exponentially growing culture of our standard strain of *B. subtilis*, lysis ensued, the extent of which increased with the concentration of the antibiotic added; later, resistant bacteria grew up. Three mutants unable to produce antibiotic activity were isolated and found to be deficient in sporulation. These findings show the close correlation between sporulation and antibiotic activity, but they do not prove that antibiotic activity is needed for sporulation.

The development of spores in bacilli is a complex metabolic process which requires the presence of many vegetative and developmental enzymes. Mutants unable to sporulate normally can be blocked at different developmental stages (11, 14). Many sporulation mutants are unable to incorporate uracil into ribonucleic acid during the developmental period (1), but they recover this ability in the presence of different carbon sources (4). Some of them respond to all carbon compounds, including acetate, whereas others do not respond to acetate. Most of the latter mutants are blocked at different steps of the citric acid cycle (2).

This study was prompted by the question as to whether sporulation mutants can be phenotypically cured (with respect to sporulation) by the addition of a missing metabolite or a metabolically related compound. Such a sporulation response is possible only when the compound can enter (or otherwise act on) the cell, but it may also depend on the time and amount of addition, because differentiation presumably requires a delicate balance of cellular components. Added too early, the compound may help repress sporulation (as some 6- or 5-carbon sugars or glycerol do) or it may have been used up when needed. To determine whether some of our sporulation mutants can be cured, we have tested their sporulation response to a number of different carbon sources. Only one mutant (60764) responded; it sporulated nearly normally when any one of several carbon sources was added at the proper time of growth.

The curable mutant produced only little antibiotic activity, even when it was grown in media in which it could sporulate. To analyze whether the antibiotic activity might be required for sporulation, we have determined some of the antibiotic properties, the growth inhibition of *Bacillus subtilis* by antibiotic, and the quantitative correlation between antibiotic concentration and the rate of sporulation in the standard strain and the curable mutant.

[1] Present address: Institut für Mikrobiologie, Universität Erlangen-Nürnberg, 852 Erlangen, Germany.

MATERIALS AND METHODS

Bacterial strains. The transformable strain 60015 of *B. subtilis*, which requires indole or tryptophan and methionine for growth, and its sporulation mutants described earlier (4) were used. For the detection of antibiotic activity, *Staphylococcus aureus* of the American Type Culture Collection number 10537 was employed.

Media. The following complete media were used: Difco Tryptose Blood-Agar Base (TBAB), 33 g/liter; Nutrient Sporulation Medium (NSM; reference 4); NSM plus 0.1 M potassium phosphate (*p*H 6.5), added after autoclaving and cooling to 50 C (NSMP). For plates, these media were solidified with 1.5% Difco agar. Two synthetic media were employed. Minimal medium (M) contained (per liter): 14 g of K_2HPO_4, 6 g of KH_2PO_4, 0.2 g of $MgCl_2 \cdot 6H_2O$, 2 g $(NH_4)_2SO_4$, and 5 g of sodium citrate, supplemented after autoclaving with 5 g of glucose per liter as carbon source (MG) and with L-methionine (10 μg/ml) and L-tryptophan (25 μg/ml). Sporulation medium SSM 13 contained 0.05 M potassium phosphate (*p*H 7.0), 8×10^{-3} M glucose, 3×10^{-2} M L-glutamate (adjusted to *p*H 7.0 with KOH), 10^{-3} M $MgSO_4$, 5×10^{-5} M $ZnCl_2$, 5×10^{-5} M $MnCl_2$, 10^{-5} M $FeCl_3$, 7×10^{-4} M $CaCl_2$, 0.5% tryptone (Difco).

Antibiotic test plates. Antibiotic activity was determined on TBAB plates overlayed with 5 ml of Nutrient Broth (Difco) plus 0.7% agar which was heated to melting, cooled to 45 C, and then supplemented with 0.0003% methylene blue, 0.01% 2,3,5-triphenyltetrazolium chloride, and 0.05 ml of *S. aureus* (grown overnight in Difco Antibiotic Medium II). Subtilin was a gift of J. C. Lewis, Western Regional Research Laboratory, Albany, Calif.; bacitracin was from General Biochemicals Corp., Chagrin Falls, Ohio.

Growth conditions. Bacteria grown overnight on TBAB plates were inoculated into NSMP or SSM 13, respectively, at an initial extinction at 600 nm (OD_{600}) of 0.1 to 0.2 and then shaken in a reciprocal water bath shaker at 37 C and 120 strokes per min. The bacterial culture occupied 10 to 15% of the volume of the culture flask. For removal of samples, the shaker was stopped for less than 10 sec. For the determination of sporulation frequencies, 5-ml samples were removed from the culture flask at the desired OD_{600} and transferred to prewarmed culture tubes with an inner diameter of 2 cm. The tubes contained 1 M solutions of the carbon source to give the desired final concentration. They were incubated for 27 hr at 37 C on a rotary shaker (160 rotations per min) and then plated for total cell and spore titers, respectively.

Sporulation. The titer (V) of all colony-forming units in a culture was determined by plating on NSM. The titer (S) of heat-resistant colony-forming units (spores) was similarly ascertained by first heating the diluted cultures for 15 min at 75 C and then plating on NSM. The frequency of spores per viable cell was measured by the ratio S/V.

Production and isolation of antibiotic activity. The ability of cells to produce antibiotic activity was determined by an overlay of *S. aureus* in soft agar over small patches of these cells on TBAB plates as described previously (4).

It was important to incubate the patches of *B. subtilis* for at least 15 hr before overlaying. When both *B. subtilis* and *S. aureus* were plated simultaneously, no inhibition of *S. aureus* growth was observed. For extraction of the antibiotic activity, cells were grown in 100 ml of SSM 13 on a rotary shaker at 37 C. When the OD_{600} was 1, the cultures were divided into two equal parts, one part being supplemented with potassium palmitate or other carbon sources to a final concentration of 10 mM. Both parts were shaken for another 68 hr and then extracted in two ways. (i) For quantitative assays (*see below*), a 5-ml sample was shaken with an equal volume of *n*-butyl alcohol, evaporated to dryness at 40 C in vacuo, and resuspended in 1 ml of sterile water. It was important to remove *n*-butyl alcohol completely by evaporation to dryness, because *n*-butyl alcohol also inhibited bacterial growth. The concentrates were assayed immediately. (ii) For application to thin-layer chromatography, the remainder of the cultures was extracted with an equal volume of *n*-butyl alcohol and then, after removal of the *n*-butyl alcohol phase, with an equal volume of a mixture of *n*-butyl alcohol and methanol (7:3). After centrifugation of the mixtures, the organic layers were pooled, concentrated in vacuo to a thick, brown syrup, redissolved in 5 ml of absolute methanol, and subjected to centrifugation for 5 min at 12,000 $\times$ *g*. The supernatant solution containing most of the antibiotic activity was concentrated to about 1 ml and stored at 4 C pending application to a thin-layer plate.

Quantitative assay of antibiotic activities. Glass filter-paper discs of 0.95 cm diameter (Carl Schleicher and Schuell, Keene, N. H.) were soaked with 0.03-ml samples of the aqueous extract, dried under a heat lamp, and placed on antibiotic test plates. The diameter of the circular halos around the filter discs was determined with a caliper after 16 hr of incubation at 37 C. One unit of antibiotic activity was defined as the smallest amount of antibiotic that produced a halo of 1-mm width (diameter of clear area, 1.15 cm). Test solutions were diluted appropriately to produce halos of this diameter.

Thin-layer chromatography and detection of antibiotic activities. The extracts were applied in 10-μliter samples onto thin-layer plates (Silica Gel G, 250 μm; Analtech, Wilmington, Del.) and developed with a solvent mixture of chloroform-methanol-water (60:35:4) or chloroform-methanol (1:1), respectively. After development, the plates were placed horizontally on a support in a Pyrex glass pan (22 $\times$ 33 $\times$ 3 cm). By employing a 25-ml serological pipette, the surface of the thin-layer chromatographic plate was carefully covered with a layer of melted TBAB agar which had been cooled to 45 C and to which had been added 0.0003% methylene blue, 0.01% 2,3,5-triphenyltetrazolium chloride, and 10^7 cells/ml of *S. aureus* 10537. The Pyrex glass pan contained 100 ml of water at the bottom, which provided sufficient humidity to prevent the agar from drying. The pan was immediately covered with aluminum foil and incubated overnight at 37 C. Clear, green zones of

antibiotic activity were visible against a purple background of bacterial growth. Zones containing growth-supporting compounds were indicated by a more intense purple coloring.

Preparative extraction of antibiotic activity and column chromatography on Sephadex LH 20. A 300-liter amount of SSM 13 was inoculated with 3 liters of strain 60709 and grown overnight in SSM 13 minus tryptone plus L-methionine (10 μg/ml) plus L-tryptophan (25 μg/ml) to OD_{600} = 2; this strain produced normal amounts of antibiotic but relatively few spores (4). Sharples centrifugation of these cultures 28 hr later yielded between 2.5 and 3 kg of wet bacteria, which was then subjected to *n*-butyl alcohol extraction; the liquid medium was discarded. A 500-g amount of wet bacteria was mixed with 500 ml of aqueous *n*-butyl alcohol, agitated in a 2-liter Waring Blendor for 3 min, and centrifuged. The brown, *n*-butyl alcohol supernatant fluid was collected, and the bacterial pellet was subjected to two more extractions with fresh water-saturated *n*-butyl alcohol. The combined extracts were concentrated under reduced pressure to a dark syrup.

Sephadex LH 20 (Pharmacia, Uppsala, Sweden) was allowed to swell in water-saturated *n*-butyl alcohol. To prepare the column (2 × 100 cm), it was filled with water-saturated *n*-butyl alcohol and the Sephadex slurry was allowed to drip into it from a separatory funnel at a slow rate that was determined by the outflow at the bottom of the column. In the separatory funnel, the slurry was kept in suspension by stirring. After the column was prepared, a 2-ml sample of the concentrated antibiotic extract was carefully layered on top and eluted with water-saturated *n*-butyl alcohol at the rate of 0.2 ml/min. Fractions of 3.8 ml were collected and their absorbancy at 250 nm was determined in a Zeiss spectrophotometer.

For the inhibitory studies, fractions 86 to 98 from the Sephadex LH 20 column were collected, flash evaporated, redissolved in methanol, filtered through a VF 6 filter (Gelman Instrument Co.; pore size 0.45 μm), and dried in high vacuum. The residue was redissolved in water, filtered through a Metricel Alpha 6 filter (Gelman Instrument Co.; pore size 0.45 μm), lyophilized, and redissolved in sterile distilled water.

Assay of pyruvate dehydrogenase. Pyruvate dehydrogenase was assayed by the method of Reed et al. (9) in crude or dialyzed (0.05 M potassium phosphate plus 0.005 M L-cysteine, *p*H 7) extracts prepared by lysozyme lysis of a bacterial suspension (at an OD_{600} of 5.0) and removal of debris (4). The total reaction mixture of 1 ml contained 0.2 ml of extract, corresponding to an OD_{600} of about 50, and was incubated for 15 min at 37 C. The amount of acetyl phosphate produced in this reaction was determined by the method of Lipmann and Tuttle (8). Protein was measured by the biuret reaction (5).

Gas chromatography. Acetate was identified and quantitated on a 12-ft glass column (¼ inch outside diameter) filled with 20% Reoplex 400 on Chromasorb W (Analabs, Inc., Hamden, Conn.). The column temperature was 140 C. A 3-μliter amount of bacterial supernatant fluid was directly injected into the column. A flame photometer was used for detection.

RESULTS

Sporulation response of several mutants to different carbon sources. Since succinic dehydrogenase plays an important role in oxidative phosphorylation, mutants blocked in the citric acid cycle before succinate might recover their sporulation when succinate is added to the medium. Seven sporulation mutants, six deficient in α-ketoglutarate dehydrogenase and one in aconitase (4), were grown in NSMP with and without succinate, which was added when the OD_{600} was 0.2. The ratio of spores to vegetative cells was determined 27 hr later. The addition of succinate did not adversely affect the sporulation of our standard sporulating strain 60015 (Table 1), but it also did not increase the frequency of heat-resistant colony-forming units (spores per viable cells, S/V) for the mutant strains.

To increase the scope of testing, 21 different sporulation mutants were examined for their response to four different carbon sources: acetate, L-glutamate, succinate, and ribose (all at 50 mM concentration). Three of these mutants were blocked in three enzymatic steps of the citric acid cycle, whereas 16 of the remaining 18 mutants had shown a uracil incorporation response to all carbon sources including acetate (4). The four compounds were added at two different stages of growth: at an OD_{600} of 1, representing the exponential growth period, and at T_1 (1 hr after

TABLE 1. *Absence of a sporulation response of citric acid cycle mutants to succinate in NSMP*[a]

Strain[b]	Maximal (OD_{600})	S/V upon addition of		
		None	10 mM Succinate	50 mM Succinate
60015	2.5	5×10^{-1}	5×10^{-1}	3×10^{-1}
60705	1.2	2×10^{-4}	7×10^{-5}	5×10^{-4}
60790	1.35	3×10^{-2}	1×10^{-2}	3×10^{-2}
60813	1.0	2×10^{-3}	8×10^{-4}	1×10^{-3}
60818	1.0	8×10^{-3}	2×10^{-4}	8×10^{-5}
60871	1.5	2×10^{-3}	1×10^{-2}	3×10^{-3}
60998	0.8	2×10^{-4}	2×10^{-4}	8×10^{-5}
61105	0.3	1×10^{-4}	1×10^{-4}	1×10^{-3}

[a] Succinate was added 30 min after the onset of logarithmic growth. The total cell titer (V) and the spore titer (S) were determined after 27 hr of shaking at 37 C.

[b] Strain 60015 was the standard sporulating strain. All mutants except 60871 were deficient in α-ketoglutarate dehydrogenase. Strain 60871 lacked aconitase (2).

the end of exponential growth), representing the early developmental phase. The three citric acid cycle mutants, as well as all but one of the other strains, did not show any significant increase in the frequency of sporulation upon addition of any carbon source (Table 2). One strain, 60764, displayed a significant increase of S/V, by a factor of 10^2 to 10^3, when acetate, succinate, or ribose was added.

General properties of strain 60764. When grown in liquid NSM or NSMP, strain 60764 sporulated with low frequency, giving an S/V value between 10^{-5} and 10^{-4}, as compared to a value of 0.7 to 1 for the parental strain 60015.

On NSMP or TBAB plates, strain 60764 produced pale colonies with a smooth surface. They could be easily distinguished from the thicker, brown colonies with a rough, wrinkled surface that were produced by both strain 60015 and revertants of 60764. Such revertants could be isolated from a lawn of strain 60764 on NSM plates incubated for several days. Strain 60764 exhibited neither protease activity on NSM plates containing a top layer of 3% bovine serum albumin, nor antibiotic activity when tested by the standard procedure on TBAB plates (4). Its revertants, however, recovered all properties of strain 60015: colony appearance, normal sporulation frequency, protease production, and antibiotic production. The pleiotropic changes of phenotype observed for strain 60764 seem to result, therefore, from a single revertible mutation.

Sporulation response of strain 60764 to acetate. We assumed that optimal curing of sporulation by different carbon sources would depend on at least two variables—the stage of growth and the concentration at which the compound was added. To obtain the optimal conditions for at least one compound, acetate was used which had caused a strong sporulation response (Table 2). In a preliminary experiment, we had determined that maximal sporulation was obtained when the concentration of acetate, added at an OD_{600} of 1, was between 50 to 100 mM. The optimal time of acetate addition to a culture growing in NSMP was, therefore, determined at an acetate concentration of 80 mM. Figure 1 shows that the optimal sporulation response was obtained when acetate was added at an OD_{600} of 1. When it was added later than T_0 (the time at which the growth rate declined), much smaller S/V values were observed. In subsequent experiments, the time of addition of carbon sources was always chosen at an OD_{600} of 1. This does not necessarily represent the ideal condition for other compounds.

The dependence of the sporulation frequency of strain 60764 on the concentration of acetate is shown in the doubly logarithmic plot of Fig. 2. An increase in the acetate concentration from 2 to 80 mM raised the sporulation frequency by a factor of 5×10^4. Concentrations of acetate higher than 80 mM inhibited growth and again produced lower S/V values after 27 hr of incubation. Under all of the above conditions, the vegetative titers were about the same (2 to 5×10^8/ml), so that the S/V values in Fig. 2 reflect the titer of spores.

The spores produced in the presence of acetate or other carbon sources appeared under a microscope as refractile bodies, and they were heat-resistant. When heated spores were plated on

TABLE 2. *Sporulation response of different sporulation mutants to acetate, glutamate, succinate, and ribose*[a]

Strain[b]	S/V upon addition of									
	None		50 mM Acetate		50 mM Glutamate		50 mM Succinate		50 mM Ribose	
	OD = 1	T_1	OD = 1	T_1	OD = 1	T_1	OD = 1	T_1	OD = 1	T_1
60015	5×10^{-1}	6×10^{-1}	1×10^{-1}	5×10^{-1}	1×10^{-1}	2×10^{-1}	3×10^{-1}	8×10^{-1}	1×10^{-1}	3×10^{-1}
60644	5×10^{-5}	1×10^{-5}	1×10^{-4}	6×10^{-5}	1×10^{-5}	1×10^{-6}	5×10^{-5}	8×10^{-5}	1×10^{-5}	3×10^{-5}
60661	3×10^{-4}	2×10^{-4}	3×10^{-4}	2×10^{-3}	3×10^{-3}	7×10^{-5}	2×10^{-4}	1×10^{-4}	4×10^{-4}	4×10^{-3}
60764	2×10^{-5}	2×10^{-4}	8×10^{-2}	2×10^{-2}	4×10^{-4}	2×10^{-4}	4×10^{-2}	2×10^{-1}	1×10^{-2}	2×10^{-2}
60871	1×10^{-4}	2×10^{-4}	2×10^{-4}	1×10^{-4}	2×10^{-3}	2×10^{-3}	3×10^{-3}	5×10^{-3}	3×10^{-4}	3×10^{-3}
60763	1×10^{-4}	1×10^{-5}	1×10^{-4}	2×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-4}	9×10^{-4}	2×10^{-4}	4×10^{-5}
60998	2×10^{-4}	2×10^{-3}	2×10^{-3}	5×10^{-4}	5×10^{-4}	3×10^{-6}	8×10^{-5}	6×10^{-5}	3×10^{-3}	1×10^{-3}

[a] Cultures were titered for V and S after 27 hr of incubation at 37 C.

[b] Strain 60871 was blocked in aconitase, 60763 in succinic dehydrogenase, and 60998 in α-ketoglutarate dehydrogenase (2, 4). The mutant blocks of the other sporulation mutants are not known. The following sporulation mutants, blocked outside the Krebs cycle, were tested in the same way and found not to respond: 60645, 60651, 60662, 60693, 60703, 60710, 60722, 60725, 60732, 60733, 60735, 60737, 60755, 60759, 60793.

NSM or TBAB plates, they produced smooth pale colonies which contained no spores detectable under a microscope. These spores carried, therefore, the nonsporulating mutant character of strain 60764 and were not revertants. The latter appeared under our conditions with a frequency lower than 10^{-5} of the vegetative titer and could be easily distinguished by their different colony morphology.

Sporulation response of strain 60764 to other carbon sources. A large number of other carbon sources were tested for their ability to restore sporulation in strain 60764; they were added at a concentration of 50 mM when the OD_{600} was 1. Acids were neutralized by KOH. The results (Table 3) show that the best effects were obtained by carboxylic acids for which the S/V values increased slightly with the number of carbon atoms. The highest sporulation frequencies were observed in experiments with fatty acids such as pentadecanoate and palmitate. Of the members of the citric acid cycle, succinate and malate were most effective. Ribose showed some effect, but glucose and fructose scarcely increased sporulation, possibly owing to their repression of sporulation.

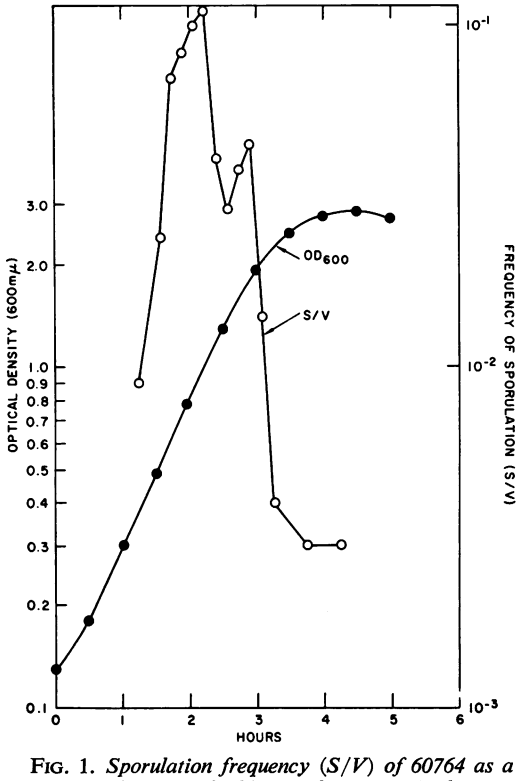

FIG. 1. *Sporulation frequency (S/V) of 60764 as a function of the time of addition of acetate. At the time points measured on the abscissa, samples were withdrawn from a culture of 60764 growing in NSMP; they were added to prewarmed tubes containing potassium acetate to give a final concentration of 80 mM. Incubation at 37 C was continued for 27 hr with aeration. Sporulation frequencies were determined as described in Materials and Methods.*

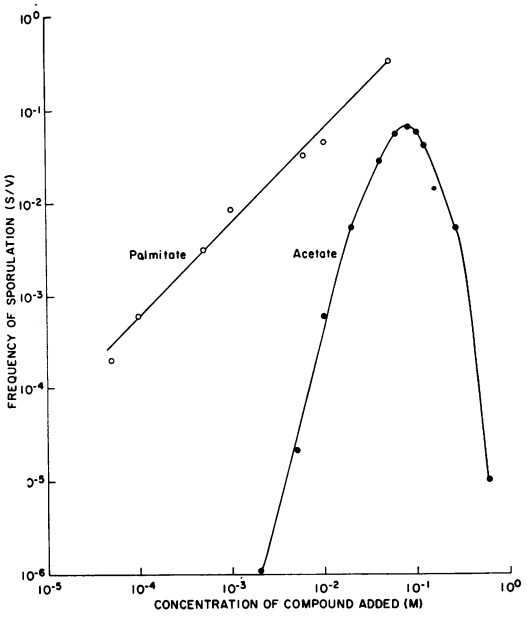

FIG. 2. *Sporulation frequency (S/V) as a function of the concentration of added acetate and palmitate, respectively. Samples of a culture of 60764 logarithmically growing in NSMP were withdrawn at an OD_{600} of 1. They were added to prewarmed tubes containing the potassium salts of acetate and palmitate, respectively, to give the final concentrations shown. Sporulation frequencies were determined after 27 hr of incubation.*

The dependence of S/V on the concentration of palmitate is illustrated in Fig. 2. The frequency of sporulation increased linearly with the concentration of the substrate but, contrary to acetate, there was no decrease at high concentrations of palmitate. However, the solubility of palmitate at 37 C was very low, the compound producing a turbid (colloidal) suspension at concentrations $\geq$ 5 mM.

Pyruvate dehydrogenase activity and acetate production in strain 60764. Since strain 60764 could be cured by acetate or palmitate, it was possibly deficient in acetyl coenzyme A (CoA). This appeared unlikely, because this strain grew at the normal rate on MG and its sporulation could well be cured by compounds such as malate or pyruvate, which are converted into acetyl CoA. The specific activities of pyruvate dehydrogenase, assayed at different times of growth in cell-free extracts, were, in fact, about the same

as those found in the standard strain 60015 and another sporulation mutant, 60661 (Table 4). All of these strains produced about the same amounts of acetate in NSMP as detected by gas chromatography. The maximal amount of acetate was always observed at T_0.

Correlation between sporulation and antibiotic activity. When a patch of our sporulating standard strain 60015 was plated on TBAB and later overlayed with *S. aureus*, strong antibiotic activity was routinely observed. A similar response was also observed for all mutants in which acetate did not restore uracil incorporation (e.g., the three mutants blocked in the citric acid cycle). In contrast, all other mutants examined here showed no (or very weak) antibiotic activity (2, 4). In particular, strain 60764 exhibited no antibiotic activity on TBAB. However, when this strain was plated on NSM, tiny halos of antibiotic activity were observed which slightly increased in size when the medium was supplemented with 1% acetate. When examined microscopically, the patches on the supplemented plates contained refractile spores.

The small amount of antibiotic activity observed in strain 60764 could be necessary for sporulation or it could be merely a by-product of sporulation. One approach to distinguish between these alternatives would be to isolate mutants unable to produce antibiotic activity and to determine whether they could sporulate. This was done by plating mutagenized (by ^{60}Co) spores of the standard strain 60015 on TBAB plates (to give 50 colonies per plate) and overlaying the plates, after 16 hr of incubation, with soft nutrient agar containing *S. aureus*. Among 10,000 colonies inspected, 3 displayed no antibiotic activity. After purification, they were examined for sporulation, which they were able to perform only at a low rate (S/V values were 2×10^{-5}, 6×10^{-5}, and 6×10^{-5} for strains 60457, 60458, and 60806, respectively). These results emphasize the close correlation between antibiotic formation and sporulation.

Another approach to examine the correlation between antibiotic activity and sporulation would be to compare quantitatively the amount of antibiotic produced and the rate of sporulation. This was hampered by the fact that, even for the standard strain 60015, no (or very little) antibiotic activity was detectable in the cultures grown in liquid NSM or NSMP to different stages, although, on NSM plates, antibiotic activity was clearly displayed. Therefore, another medium producing more antibiotic activity had to be employed, and the antibiotic synthesis had to be examined more closely in strain 60015.

Isolation and properties of antibiotic activity. A large amount of antibiotic production was observed when strain 60015 was grown in a synthetic medium containing limiting amounts of glucose and excess glutamate. On medium SSM 13, which contained only small amounts of free ammonia, growth was enhanced, and there

TABLE 3. *Response of strain 60764 to several carbon sources*

Compound added (50 mM, pH 7-7.5)	S/V[a]
None	1×10^{-5}
Formate	4×10^{-3}
Acetate	8×10^{-2}
Propionate	1×10^{-1}
Butyrate	1×10^{-1}
β-Hydroxybutyrate	1×10^{-1}
Crotonate	1×10^{-1}
Valerate	1×10^{-1}
2-Hydroxyvalerate	3×10^{-2}
Oxalate	2×10^{-4}
Malonate	2×10^{-4}
Glucose	5×10^{-4}
Fructose	5×10^{-4}
Ribose	3×10^{-2}
Lactate	1×10^{-2}
Pyruvate	5×10^{-2}
Citrate	8×10^{-3}
Glutamate	4×10^{-4}
2-Ketoglutarate	2×10^{-3}
Succinate	4×10^{-2}
Malate	2×10^{-1}
Oxaloacetate	1×10^{-2}
Pentadecanoate	4×10^{-1}
Palmitate	3×10^{-1}
Heptadecanoate	5×10^{-2}
Stearate	2×10^{-2}

[a] Average values of several experiments.

TABLE 4. *Pyruvate dehydrogenase activity in three strains*[a]

Strain	Harvested at OD_{600}	Specific activity
60015	0.45	153
	0.92	142
60661	0.60	202
	1.05	265
	1.55	259
60764	0.51	219
	1.15	182
	1.70	189

[a] Assayed in crude extracts. After dialysis for 19 hr, the activities decreased by about a factor of 3. Specific activity = nanomoles of acetylphosphate produced per minute and per milligram of protein at 37 C.

was a slight increase in antibiotic production per cell. When 60015 was inoculated into this medium at a titer of 10^8 cells/ml, maximal sporulation and antibiotic formation were obtained after 20 to 30 hr of shaking at 37 C (Table 5), and were maintained at this level up to at least 70 hr. Extracts of strain 60015 cultured in this medium for 28 hr (timing from an OD_{600} of 1) exhibited strong antibiotic activity. About one-half of this activity was attached to the bacteria themselves, since *n*-butyl alcohol extracts of the bacterial pellet showed about the same total activity as extracts of the supernatant fluid.

In the paper chromatographic procedure of Snell et al. (12), most of our antibiotic activity chromatographed differently than either subtilin or bacitracin. For the separation of different antibiotic components, we found thin-layer chromatography more discriminating than paper chromatography: antibiotic was chromatographed on Silica Gel G plates with a mixture of chloroform-methanol-water (60:35:4).

The zones of antibiotic activity were detected by a novel method employing a TBAB agar overlay which contained both the antibiotic-sensitive *S. aureus* and dyes indicating the production of reducing compounds. Three major and two minor zones of antibiotic activity were observed for extracts of strain 60015 (Fig. 3). Close behind the fastest-running antibiotic species, some growth factor was evident by red staining (formazan formation) of the bacterial overlay.

Growth factor and antibiotic activity, isolated from the standard strain 60015, could be separated by a Sephadex LH 20 column prepared and eluted with water-saturated *n*-butyl alcohol (Fig. 4, fraction numbers 41–55). The isolated growth factor produced excellent sporulation of strain 60764, whereas the separated antibiotic fractions did not. The ultraviolet-absorbing

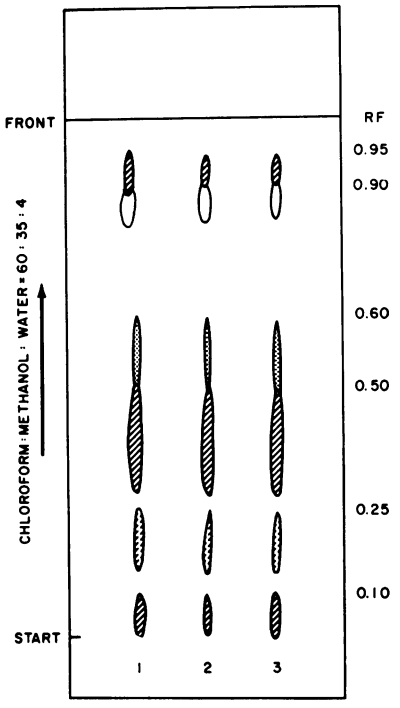

FIG. 3. *Thin-layer chromatogram of antibiotic activities extracted from strains 60015 and 60764, respectively. Cultures of 60015 and 60764 grown in SSM 13 for 68 hr in the presence of 10 mM palmitate were extracted with n-butyl alcohol and a mixture of n-butyl alcohol and methanol (7:3). Approximately 10 μliters of the concentrated extracts of 60015 (1), 60764 (3), and a 1:1 mixture of both these extracts (2) were applied on a plate of Silica Gel G and developed with a solvent mixture of chloroform-methanol-water (60:35:4). The intensities of antibiotic activities as detected by the overlay method are indicated by three different shades: strong, medium, weak. Zones without shading indicate increased growth.*

TABLE 5. *Sporulation and antibiotic activity determined after growth in SSM 13 with and without palmitate*[a]

Strain	Palmitate	V	S	Antibiotic activity
	(10 mM)			*units/ml*
60015	−	1.3×10^9	1×10^9	1,050
60015	+	1.6×10^9	1.6×10^9	1,200
60661	−	$<10^5$	$<10^3$	<0.5
60661	+	$<10^5$	$<10^3$	<0.5
60764[b]	−	1×10^7	1×10^7	20
60764	+	1.1×10^9	5.4×10^8	60

[a] Cultures were grown on a rotary shaker at 37 C for 68 hr.

[b] Contained approximately 10% revertants after 68 hr.

(at 250 mn) peaks eluted from the LH 20 column were rechromatographed on a thin-layer plate. Each peak produced several zones of either antibiotic or growth-supporting activity or both (Fig. 5).

The crude antibiotic activity could not be extracted from bacteria by water but only by less polar solutions, such as 70% ethyl alcohol or (not quite as efficiently) by shaking of the aqueous solution with *n*-butyl alcohol. In contrast, the activity found in fractions 86 to 98 of the Sephadex LH 20 column was sufficiently soluble in water to pass through a membrane filter excluding bacteria (pore size, 45 nm). The antibiotic activity was stable to heating for 10 min to 120 C.

The purified fractions 86 to 98 (freed of organic

solvents and sterilized by membrane filtration) were used to determine the effect of the antibiotic activity on the growth of *B. subtilis* itself and to compare it to that on *S. aureus*. After the cells had grown in NSMP to an OD_{600} of 0.5, the antibiotics, dissolved in water, were added at different concentrations. Under these conditions, *B. subtilis* was more sensitive to the antibiotic than was *S. aureus* (Fig. 6). Whereas low antibiotic concentrations inhibited growth, high concentrations (≥2.5 units/ml) produced lysis of *B. subtilis*. Eventually, the turbidity of the *B. subtilis* cultures increased again (not shown in Fig. 6).

The antibiotics also inhibited growth of strain 60015 on TBAB plates. For this test, 0.1 ml of

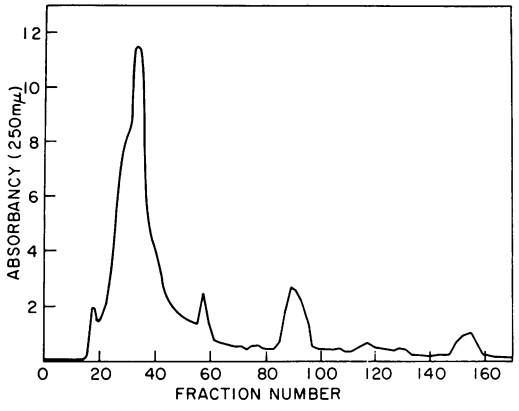

FIG. 4. *Column chromatography on Sephadex LH20 of an n-butyl alcohol extract of antibiotic-producing strain 60709. n-Butyl alcohol extracts from 500 g (wet weight) of bacteria were concentrated to about 2 ml and applied to a column (2 × 100 cm) of Sephadex LH20. The column was prepared and eluted with water-saturated n-butyl alcohol. Fractions of 3.8 ml were collected and their absorbancy at 250 nm was determined.*

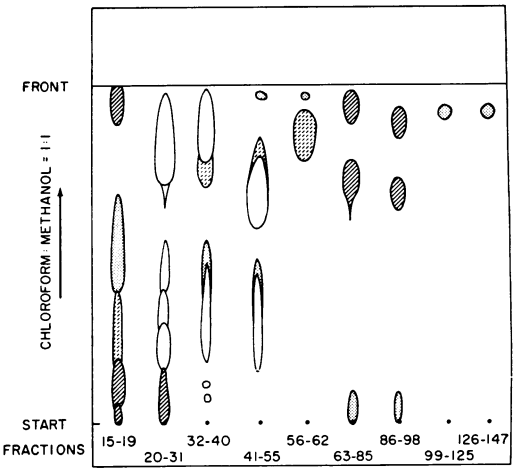

FIG. 5. *Thin-layer chromatographic analysis of the 60709 extract after fractionation on Sephadex LH20. Fractions from the Sephadex LH20 chromatography were pooled as indicated by the fraction numbers and concentration in vacuo. Samples of 10 μliters were applied on a plate of Silica Gel G and developed with a solvent mixture of chloroform-methanol (1:1). Zones of antibiotic activity and increased growth were detected by the overlay method; different intensities are shaded as in Fig. 3.*

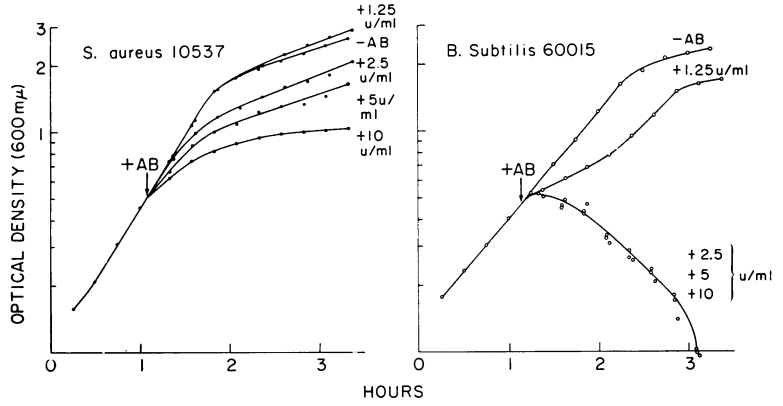

FIG. 6. *Growth inhibition of S. aureus and B. subtilis by increasing concentrations of antibiotic activity from B. subtilis. S. aureus 10537 and B. subtilis 60015 were grown in NSMP in a shaker water bath at 37 C. At OD_{600} = 0.5, 10-ml samples of the two cultures were distributed into small flasks containing increasing amounts of pre-purified antibiotic activity from B. subtilis 60709 (Fig. 4, fractions 86–98) and were further incubated. Growth was followed spectrophotometrically at OD_{600} at 15-min intervals. Units of antibiotic activity given in this figure (AB) refer to final concentration and are defined in Materials and Methods.*

B. subtilis and *S. aureus* cultures, grown in NSMP to an OD_{600} of 0.5, were spread on TBAB plates. The above antibiotics were serially diluted in water by factors of two, and a drop of each dilution was placed, with a 0.2-ml serological pipette, on the seeded plates. After 8 to 10 hr at 37 C, inhibited areas were observed on a lawn of bacterial growth. Estimating from the smallest antibiotic concentration that still produced visible inhibition, *B. subtilis* was at least eight times more sensitive than *S. aureus*. Nevertheless, *S. aureus* was more suitable for the assay of antibiotic activity, because its inhibited areas remained clear after extended incubation, whereas, in the inhibited areas of *B. subtilis*, many colonies grew up eventually.

Comparison of sporulation and antibiotic activity of strains 60015 and 60764. Strain 60764 grew in SSM 13 at the same rate as 60015 (doubling time: 50 min) and both cultures reached a viable cell titer of 2 to 3 $\times$ 10^9/ml after 27 hr; subsequently the titer decreased, leaving only sporulating cells viable. Strain 60764 sporulated to some extent in SSM 13, but the number of spores increased at a much slower rate than for 60015; sporulation was complete only after 68 to 70 hr. Refractile endospores could not be detected microscopically up to 40 hr after the culture had reached an OD_{600} of 1, whereas, in strain 60015, endospores were first seen after 11 hr and observed in more than 50% of the cells after 13 hr. The addition of 10 mM pentadecanoic or palmitic acid (at an OD_{600} of 1) greatly increased the titer of spores for strain 60764, whereas acetate at various concentrations had no effect. Another sporulation mutant, 60661, showed no response to any agent (Table 5). For example, in the presence of palmitate (10 mM, added at an OD_{600} of 1), 60764 produced 5 $\times$ 10^8 spores/ml (containing less than 1% revertants), 50 times more than without palmitate and only 3 times less than the standard strain 60015. The absolute titer of revertant spores of 60764, discerned by their different colony appearance on NSM plates, was the same for cells grown with and without palmitate; the revertants were apparently introduced with the inoculum, since their titer was 10 times lower in other experiments with SSM 13.

In the medium SSM 13, strain 60764 also produced antibiotic activity; its concentration was increased by the presence of palmitate (10 mM), but still remained 20 times lower than that of the standard strain (Table 5). In contrast, strain 60661 did not produce antibiotic activity in the presence or absence of palmitate.

The low antibiotic activity in 60764 could result either from the absence of one or more of the antibiotic species or from the reduction in the amount of all species. To decide between these alternatives, the crude antibiotic extracts of cells grown on SSM 13 were analyzed by thin-layer chromatography on silica gel. For this purpose, the extracted antibiotic activity of strain 60764, grown in SSM 13 plus palmitate, was adjusted to the strength of the standard strain. The same antibiotic bands were observed in thin-layer chromatography for both strains (Fig. 3); a mixture of extracts from 60015 and 60764 gave the same pattern (Fig. 3). Apparently all classes of antibiotic activity, characterized by their mobility in a semipolar solvent, were produced by both strains. In extracts of strain 60764, however, the antibiotic band with the highest mobility was less active, relative to the other two bands, than in 60015 extracts, indicating a quantitative difference. Nevertheless, the 20-fold or higher reduction of antibiotic activity in strain 60764, compared to 60015, cannot result merely from the lack of one particular antibiotic species, but must be the consequence of an over all reduction in the production of all species.

DISCUSSION

Most of our sporulation mutants did not recover the ability to sporulate when a number of carbon sources were added to the medium. This result is understandable for mutants blocked in the citric acid cycle, which is needed for the continued production of energy (TTP). Citric acid-cycle mutants accumulate intermediates which eventually may inhibit the whole cycle by direct or indirect feedback inhibition. Less obvious are the results for mutants which are not blocked in the citric acid cycle and which are unable to incorporate uracil during the developmental period but regain this capacity in the presence of many carbon sources, including acetate. The latter response had indicated that acetyl CoA, which supplies the carbon for adenosine triphosphate production in the citric acid cycle, is not available in these mutants during the developmental period (2, 4). The inability to sporulate in the presence of acetate shows that sporulation requires not only acetyl CoA but also some other compound(s) which cannot be made in sufficient amounts by these sporulation mutants.

One of our sporulation mutants, strain 60764, whose uracil incorporation responds to acetate, could recover the ability to sporulate upon the addition of different carbon sources, such as acetate, malate, palmitate, and several others. The high concentration of these compounds required for optimal sporulation indicates their use as energy sources. Palmitate and other fatty acids apparently can still be catabolized to acetyl CoA in this mutant. The high efficiency of long

chain fatty acids in curing sporulation may be due to their tendency to attach to lipophilic membrane, facilitating their entry into the cell. Strain 60764 displays no new growth requirement on minimal glucose medium and grows at the same rate and to the same final optical density as the standard strain on three different media. The absence of a growth requirement and the presence of normal amounts of pyruvate dehydrogenase activity indicate, furthermore, that strain 60764 produces normal amounts of acetyl CoA. This is also verified by the normal production of acetate in media in which this strain is grown.

The above results suggest that strain 60764 is unable to store some acetyl CoA precursor which is used as energy source during sporulation. The strain may either lack an enzyme specifically needed for this purpose or have a faulty control mechanism which regulates the production of this storage compound.

Acetate or other carbon sources were most effective in recovering sporulation of strain 60764 when they were added during the vegetative phase of growth at an OD_{600} of 1. When added later than T_0, they were not effective. Their addition may have to prevent the complete utilization of all potential acetyl CoA precursors in the medium to ensure that not only energy, but also metabolites needed for sporulation, can be produced in sufficient amounts.

The curing of a developmental mutant has also been observed for *Streptomyces griseus* by Szabo et al. (13). They partially purified a factor C (presumably containing a polypeptide) from the fermentation liquid of *S. griseus*, which induced conidia formation of a filamentous mutant.

The standard sporulating strains of *B. subtilis* produce an antibiotic activity which can be detected on plates by the growth inhibition of *S. aureus* (11). In liquid nutrient sporulation medium, however, almost no activity can be detected. Another liquid sporulation medium, SSM 13, was therefore designed which contains excess glutamate but little free ammonia. In this medium, both excellent sporulation and appreciable antibiotic activity have been observed. There was no activity during exponential growth, but it greatly increased during the developmental period. A thin-layer chromatographic analysis of crude antibiotic extracts revealed that the activity could be separated into at least three major and two minor components. The existence of even more components was indicated when crude extracts were chromatographed on Sephadex LH 20. As is known from other antibiotic-producing organisms, the different components of activity usually represent biochemically related compounds.

A purified antibiotic fraction of *B. subtilis* was tested for its effect on the producer strain itself. During the vegetative growth phase, *B. subtilis* was even more sensitive to the antibiotic activity than was *S. aureus*. In fact, many *B. subtilis* cells lysed after antibiotic addition. The lytic response is restricted to vegetatively growing cells, because cells in the developmental period, during which they produce the antibiotics, do not lyse but sporulate. (They lyse only eventually when the spores have ripened completely.) Consequently, the antibiotic activity is produced in large quantity only after the cells have entered the developmental period. A few cells of *B. subtilis* enter the developmental phase during the exponential growth period, during which a few spores are continuously produced. These sporulating cells presumably produce their own antibiotics, which apparently do not affect the majority of duplicating cells because they stick to bacteria and therefore tend to stay with the bacteria by which they are produced. During the developmental period, however, at least 100 times more antibiotic activity is produced in SSM 13 than is needed to lyse vegetative cells, and about 50% of it eventually appears in the medium. At this time, the activity would affect any vegetatively growing cells that might be present, unless they are (or have become) antibiotic-resistant. The antibiotic activity may therefore be (at least partially) responsible for the commitment of sporulating cells; i.e. the inability of cells in the developmental period to resume growth when glucose is added to the medium (3, 6; H. M. Nakata, Bacteriol. Proc., p. 47, 1962) or the lag in growth response when they are transferred to a medium containing both glucose and an amino acid mixture (Freese, *unpublished observations*).

The growth of antibiotic-resistant *B. subtilis* was observed both in liquid cultures and on plates in which resistant colonies grew up within the clear patches caused by the presence of the antibiotics. Resistance may result from the presence of an enzyme inactivating the antibiotics, similar to the nisin-inactivating enzyme described by Jarvis (7).

The curable strain 60764, grown in SSM 13, produced very little antibiotic activity and only three times more when the strain was grown in the presence of palmitate; the activity was always at least 20 times less than that of the standard strain. Thin-layer chromatography showed that this reduction in activity was not caused by the lack of one particular antibiotic species but rather by the general reduction of all antibiotic synthesis.

In medium SSM 13 supplemented with pal-

mitate, strain 60764 sporulated nearly as well as the standard strain, but the spore titer increased much more slowly, although both strains grew vegetatively at the same rate. The slow rate of sporulation and the small amount of antibiotic activity produced by strain 60764 may be causally related.

Several findings now show the close correlation between sporulation and antibiotic activity and suggest that an antibiotic or its metabolic precursor or product may be required in the early stages of sporulation. (i) Sporulation mutants that are blocked at the first stage of development produce no antibiotic activity, whereas mutants blocked at a later stage still produce it (11). This shows that some biochemical reaction which occurs early in sporulation is also required for antibiotic formation. (ii) Schaeffer (10) isolated one mutant and we isolated three mutants that are unable to produce antibiotic activity. All four mutants are also unable to sporulate normally. These mutants do not prove the need of the antibiotic activity for sporulation, because they could all be blocked in a biochemical reaction or in a regulatory step controlling both sporulation and antibiotic synthesis. (iii) The antibiotic activity produced by *B. subtilis* during the developmental period inhibits growth and causes lysis of *B. subtilis* when added during the vegetative period. Since the *B. subtilis* antibiotics tend to stick to cells, they mainly affect the cells in which they are produced, preventing their resumption of growth and perhaps maintaining their developmental state. (iv) In SSM 13, strain 60764 produces less antibiotic activity than 60015 and its spores appear at a slower rate. However, in NSMP, strain 60015 produces even less antibiotic and yet sporulates rapidly (sporulation complete 8 hr after T_0). This finding cannot be regarded as proof that antibiotic activity is not needed for sporulation, because its required amount may depend on the composition of the medium.

Whether the antibiotic activity is really necessary for sporulation can be decided only by finding a sporulation mutant that can sporulate only in the presence of a highly purified antibiotic.

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