THE RELATION BETWEEN SPORULATION AND THE INDUCTION OF ANTIBIOTIC SYNTHESIS AND OF AMINO ACID UPTAKE IN *BACILLUS BREVIS*

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ABSTRACT

The induction and localization of tyrocidine-synthesizing enzymes is shown to be parallel, during growth of *Bacillus brevis* (ATCC 8185, American Type Culture Collection, Rockville, Md.), with the induction of uptake of constitutive amino acids and of components of pantetheine, a coenzyme of tyrocidine synthesis. Antibiotic synthesis appears at the end of logarithmic growth when the first soluble enzymes may be obtained from homogenates. During this period, binding proteins for metabolite uptake were isolated by intensive sonication which, when studied by chromatography, were identified by the appearance of low molecular weight fractions binding the radioactively marked metabolites; their induction was prevented by addition of rifampicin.

The major purpose of this study was a comparison of antibiotic production and sporulation, the progress of which was followed by electron microscopy. The onset of tyrocidine synthesis and metabolite uptake coincided with the appearance of septum formation indicating that sporulation had progressed to stage II. With the progress of spore encapsulation, the tyrocidine production migrated from the soluble fraction into the forespore, terminating with the separation of forespores from the sporangium membrane. The resulting concentration of antibiotic in the forespore may indicate its function in sporulation, the nature of which, however, was not explored.

It has been recognized for some time that the spore-forming bacteria are apt to make antibiotic polypeptides and that this ability is related to sporulation (6, 7, 18, 22, 24). In the case of gramicidin S (4, 11), tyrocidine (10, 13, 20), the linear gramicidins (3), and bacitracin (6, 7), it was shown that the onset of the formation of antibiotic occurs at the time when logarithmic growth turns into the stationary phase just before spore formation begins. It also has been known (11, 18, 24) that there are genetic links between the formation of spores and the synthesis of antibiotics, and it has been speculated whether antibiotics have a role in the formation of spores. This was discussed by Bernlohr and Novelli (7) in the case of *Bacillus licheniformis* in relation to formation of bacitracin, and more recently also for gramicidin S (11) and tyrocidine (12, 23) formed by strains of *Bacillus brevis*. A function of antibiotics in spore formation has been proposed by Sarkar and Paulus (23) who reported that tyrothricin, the mixture of tyrocidine and linear gramicidins, in-

hibits RNA polymerase. They also tested the single antibiotics and could find a similar activity in each of them.

In our work on the biosynthesis of tyrocidine and the linear gramicidins (3, 12, 13, 20), we became aware of the relevance of this correlation when it appeared that, in order to obtain extracts suitable for studying their in vitro synthesis, it was essential to follow closely the appearance of antibiotic formation during growth of the organism. Only during a short period at the onset of the stationary phase of *B. brevis* (ATCC 8185, American Type Culture Collection, Rockville, Md.) was it possible to extract the tyrocidine-synthesizing enzymes in soluble fractions suitable for purification; farther into the stationary phase, insoluble enzyme preparations were obtained that sedimented with the particulate fraction and were unusable for purification, although antibiotic production was often enhanced in this later phase. This seemed to indicate that with the start of spore formation there might be an association of the antibiotic-forming enzymes with forespores. Therefore, it was decided to localize more closely the production of antibiotic in relation to formation of forespores and spores.

In the course of this work it was discovered that, roughly coincident with the onset of the formation of the antibiotic-forming enzymes, a very sudden increase occurred in the uptake of some of the antibiotic-constituent amino acids and of components of pantetheine. This uptake was paralleled by the appearance in intensive sonicates of proteins that bound radioactively marked antibiotic-constituent amino acids and β-alanine. The induction of tyrocidine synthesis, and of proteins presumably specifically binding metabolites related to the formation of tyrocidine, will be described in greater detail. The relationship of these events to spore formation was followed by biochemical tests and electron microscopy in order to align antibiotic formation with the progress of spore formation that causes a change of solubility in the antibiotic-synthesizing enzymes.

Before beginning the description of the experiments, it will be necessary to summarize the mechanism of tyrocidine synthesis (14, 15). As shown in Fig. 1, tyrocidine is synthesized on three enzymes, beginning with phenylalanine. The molecular weight of the enzymes, as noted in the legend, is approximately proportional to the number of activated amino acids indicated by the brackets, and

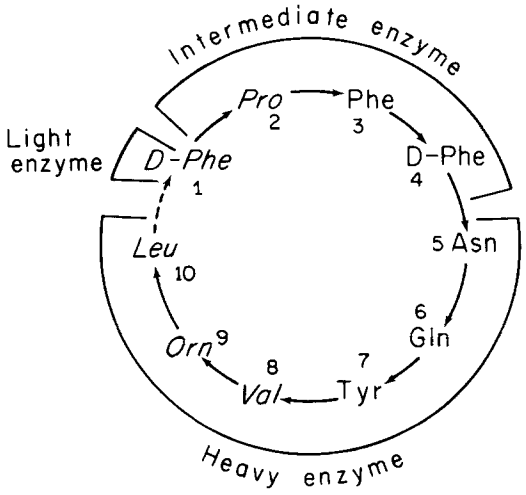

FIGURE 1 Amino acid sequence in tyrocidine. The brackets embrace the amino acids activated by the three complementary enzymes: light, 100,000 mol wt; intermediate, 230,000 mol wt; and heavy, 440,000 mol wt. Solid arrows indicate the direction of addition, dotted arrow the direction of cyclization.

amounts to approximately 75×10^3 daltons for each amino acid. Thus, the possibility was suggested that the polyenzymes represented an association of subunits, and this was confirmed (14) when three and six fractions of $70–75 \times 10^3$ daltons were separated from the intermediate and heavy enzymes, respectively. In addition, from each of the polyenzymes, one subfraction of 17×10^3 daltons was isolated containing 4′-phosphopantetheine, which acts as coenzyme in polymerization.

MATERIALS AND METHODS

Growth of Tyrocidine-Producing Organisms

B. brevis cells (ATCC 8185) were grown at 37°C in a rich medium (1% Difco-bactopeptone, 1% Difco-beef extract (Dfico Laboratories, Detroit Mich.), 0.25% NaCl, pH adjusted to 7.0 with KOH) in a New Brunswick fermenter (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) with a stirring speed of 600 rpm and aeration of 13 liters of air per min.

Uptake of Amino Acids and Pantetheine Precursors

Beginning at the middle of the exponential phase of growth (Fig. 2), cell samples were taken at 30-min intervals and incubated for 5–10 min at 37°C with radioactive amino acids and pantetheine precursors in a

New Brunswick shaker with a shaking speed of 300 rpm. The cells were then collected and their radioactivity was determined as described in the figure legends.

Tyrocidine Synthesis

At various times during growth, 10-ml samples of culture were withdrawn and incubated with 1 μCi of [^{14}C]ornithine as described for the uptake experiments. The cells were collected by centrifugation for 5 min at 20,000 *g* through 20 ml of sucrose containing 1% NaCl, and the resulting pellets were suspended in 2 ml of triethanolamine buffer, pH 7.4; the cells were then lysed with lysozyme, and the radioactive tyrocidine produced was extracted with two 2-ml portions of *n*-butanol-chloroform (4:1, vol/vol). The tyrocidine was purified by thin-layer chromatography as described previously (21) and its radioactivity was measured.

Electron Microscopy

Cell pellets obtained by centrifugation were prefixed overnight with 1% glutaraldehyde in 10 mM phosphate buffer, pH 7.0, and then postfixed with 1% osmium tetroxide in the same buffer for 2 h. After dehydration and embedding in Epon resin, the thin sections were stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop A.

RESULTS

Induction of Tyrocidine Synthesis and Amino Acid Uptake

As an example of the change of metabolite intake during growth, β-alanine and pantothenic acid uptake is compared with cell growth and tyrocidine production in Fig. 2. After the middle of the exponential phase, uptake capacity often declines to a minimum. It then increases quite abruptly, parallel with the sudden development of tyrocidine-synthesizing activity, and declines with decline of tyrocidine synthesis. The pattern of the development of β-alanine uptake, however, varies a great deal with the history of the cell culture. Generally, the use of a large amount of inoculum, which permits the cells to grow for only a small number of generations before sporulation, results in a slight increase. This is particularly true when sporulating cells are used as inoculum (Fig. 3 *a*).

On the other hand, when the cells are grown for a large number of generations in the exponential phase, either by use of a small initial inoculum or by transfer of an exponential culture to fresh medium, the increase in uptake is far greater; once

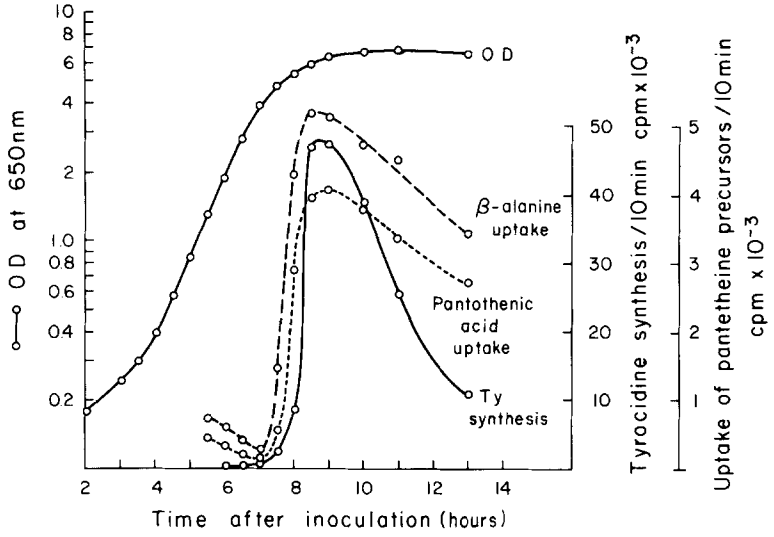

FIGURE 2 Tyrocidine (Ty) synthesis and uptake of pantetheine precursors. Culture samples of 10 ml taken at 30-min intervals were incubated in 50-ml flasks in a New Brunswick shaker at 300 rpm for 10 min at 37°C with 1 μCi each of [^{14}C]ornithine and [^{14}C]pantothenic acid, and 5 μCi of [^{3}H]β-alanine. Cells were collected by centrifugation at 20,000 *g* for 5 min through a 10% sucrose layer containing 1% NaCl. To determine uptake of β-alanine and pantothenic acid, the pelleted cells were suspended in 2 ml of triethanolamine buffer, pH 7.4, and the radioactivity of a 0.2-ml portion of the suspension was counted. Tyrocidine synthesis was determined with the cells incubated with [^{14}C]ornithine as described in Materials and Methods.

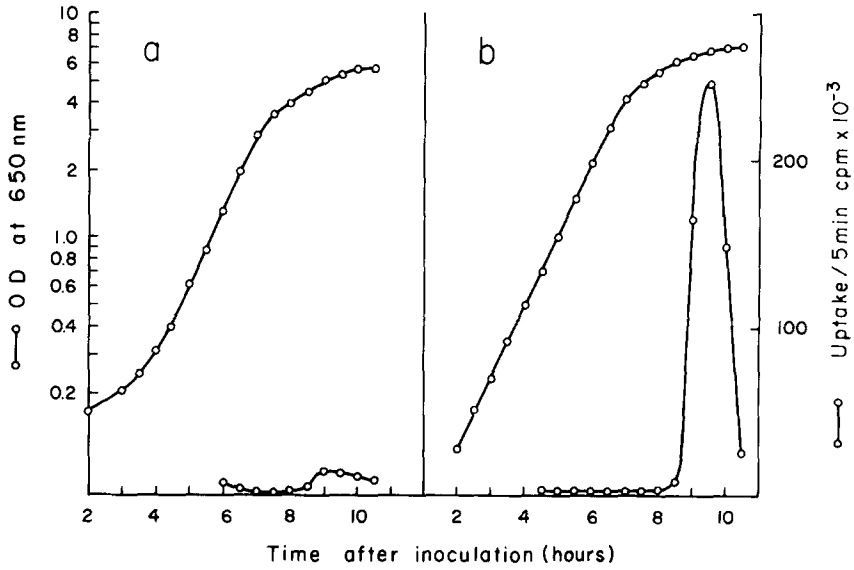

FIGURE 3 *a* β-alanine uptake by cells grown for a limited number of generations in the exponential phase. A 5-liter culture medium was inoculated with 30 ml of sporulating cell (T5) (9) culture, and the cells were grown as described in Materials and Methods. Samples of 1 ml, withdrawn at 30-min intervals, were incubated for 5 min at 37°C in 10-ml flasks with 5 μCi of [^{3}H]β-alanine in a New Brunswick shaker at 300 rpm. Cells from 0.5 ml of the incubation mixture were collected on Millipore filters (0.45-μm pore size) (Millipore Corp. Bedford, Mass.), and after washing the filters with five 2-ml portions of culture medium, they were dried and the radioactivity was counted.

FIGURE 3 *b* β-alanine uptake by cells grown for a large number of generations in the exponential phase. When the OD of the culture of Fig. 3 *a* reached 0.6, a 50-ml sample was transferred to 5 liters of fresh medium and the cells were grown until the OD reached 0.6, at which time the 50-ml culture was again transferred to 5 liters of fresh medium and growth was continued. Using this last culture, β-alanine uptake was determined as described in Fig. 3 *a*.

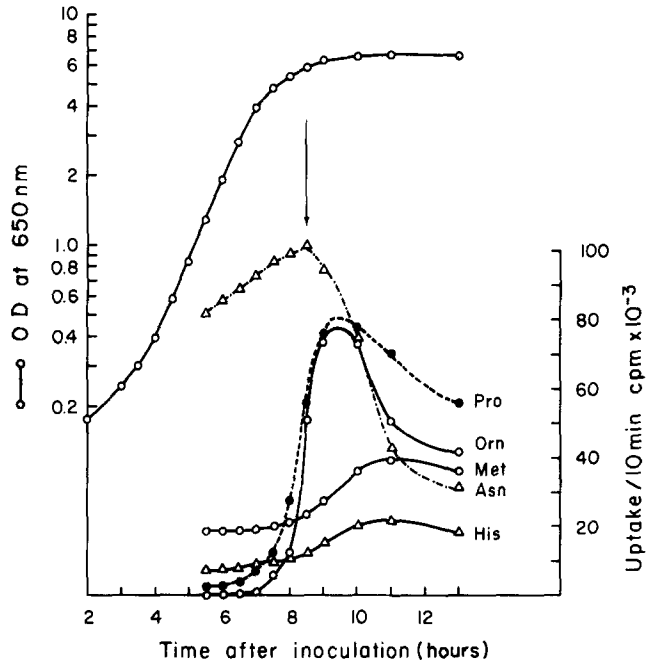

FIGURE 4 Uptake of constituent and nonconstituent amino acids of tyrocidine. Uptake of amino acids was determined using 1 μCi of ^{14}C-labeled amino acids as described in the legend of Fig. 2. The time of peak uptake of pantetheine precursors is indicated by an arrow.

the peak is reached, however, the uptake declines quite rapidly (Fig. 3 *b*). Similarly, the uptake capacity of ornithine and proline is rather low during the exponential phase, being roughly parallel with cell density, but it increases suddenly with the appearance of tyrocidine-producing enzymes (Fig. 4). Development of this uptake, however, appears to lag somewhat behind that of the precursors of pantetheine. In contrast, no abrupt development is observed for the uptake of nonconstitutive amino acids such as histidine and methionine (Fig. 4). A slight and somewhat delayed increase in uptake observed for these amino acids may be accounted for by a reduction of isotope dilution resulting from the progressive decrease in cold amino acids in the medium. The uptake of asparagine, another tyrocidine-constitutive amino acid, is high during the exponential phase with only a hump around the turn into the stationary phase; it then declines parallel with the decline of uptake of proline and ornithine and of synthesis of tyrocidine.

As shown in Fig. 5, the development in uptake of both β-alanine and proline is delayed and inhibited by rifampicin, suggesting it to be dependent on the synthesis of new mRNAs. Rifampicin inhibition is most pronounced during the early phase of development, but once uptake reaches its peak its sensitivity to the antibiotic diminishes and finally disappears in parallel with the decline of tyrocidine-synthesizing activity. Fig. 5 shows that the start and decline of rifampicin sensitivity to β-alanine uptake precede those of proline by about 1 h.

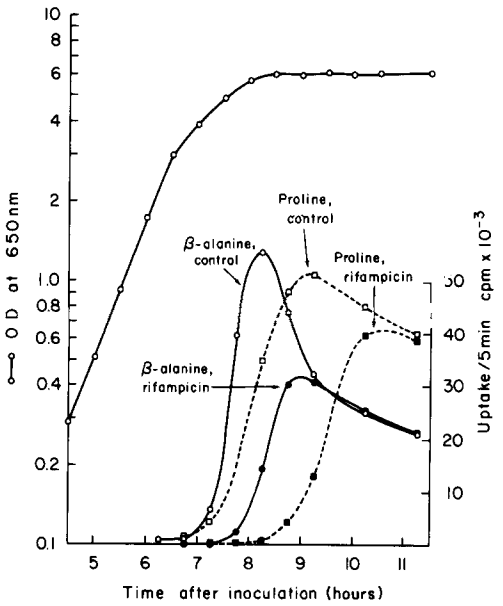

FIGURE 5 Effect of rifampicin on the uptake of β-alanine and proline. The uptake of [^{3}H]β-alanine (5 μCi) and [^{14}C]proline (1 μCi) by the control cell culture was assayed as described in the legend of Fig. 3 *a*. The rifampicin-treated cell culture was preincubated for 10 min with 6 μg/ml rifampicin, after which the radioactive compounds were added and the uptake was determined as in the control.

Isolation of Metabolite Binding Proteins

Transport of metabolites across membranes has recently been shown in various cases to involve their binding by specific proteins on the cell membrane (1, 2, 5, 8, 17, 19). Accordingly, attempts were made at the peak of uptake stimulation to test for the production of a protein that binds β-alanine. Exponentially growing cells and sporulating cells, harvested at the peak of β-alanine uptake, were subjected to intensive sonication to dissolve membrane-bound proteins; after removal of large particles by centrifugation for 20 min at 20,000 *g*, the supernatant fractions were applied to a Sephadex G-200 column (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) equilibrated with a buffer containing [^{3}H]β-alanine and the proteins were eluted with the same [^{3}H]β-alanine-containing buffer. As may be seen in Fig. 6, only sporulating cells yielded a radioactive peak at a low molecular weight region. In contrast, no clearly overlapping peak appeared when exponentially growing cells were similarly sonicated and chromatographed. The extract of the latter may be considered to present a blank in view of the very low binding shown in Fig. 6.

Relationship between Antibiotic Formation and Sporulation

Antibiotic production overlaps very nicely with the onset of sporulation and stretches into the time of spore formation (7, 13). To compare this interrelation, the progress of sporulation was followed by electron microscopy in order to correlate it more closely with the production of tyrocidine and with the concomitant metabolite uptake. The electron micrograph in Fig. 7 *a* shows spore septa which represent the late stage II of sporulation according to the scheme of Fitz-James (9). This stage corresponds to the onset of development of β-alanine uptake and tyrocidine production. When cells were harvested at the peak of

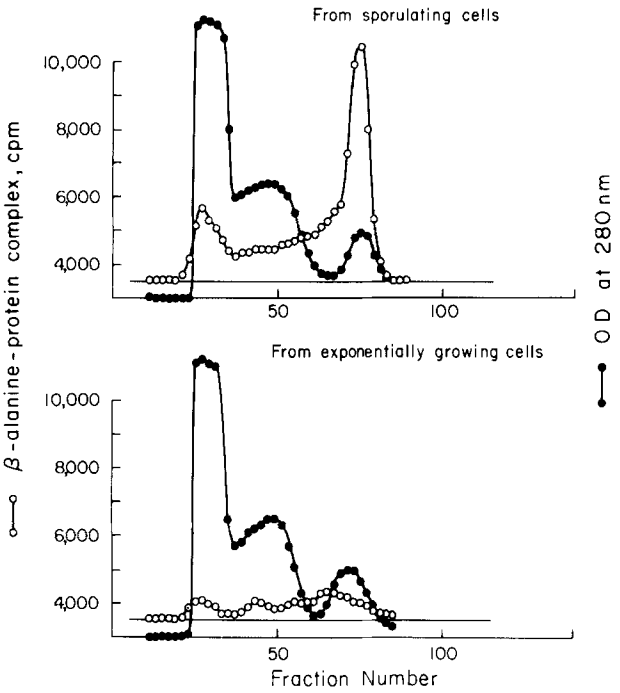

FIGURE 6 Analysis on Sephadex G-200 chromatography of β-alanine-binding protein from exponentially growing cells and sporulating cells. A 5-g batch of cells, harvested either during the exponential phase or at the peak of β-alanine uptake, was suspended in 20 ml of triethanolamine buffer, pH 7.6, containing 5 mM EDTA, 1 mM dithiothreitol, and [^{3}H]β-alanine (7,200 cpm/ml). The cell suspension was subjected to four 20-s sonications with a Branson sonifier at step 5 and 7 am, and the disrupted cell suspension was centrifuged for 20 min at 27,000 *g*. Of the resulting supernate, 6 ml was applied to a G-200 column (120 × 2 cm) equilibrated with 10 mM phosphate buffer, pH 7.0, containing [^{3}H]β-alanine (7,200 cpm/ml). The column was eluted with the same solution, and 0.5 ml from each fraction was counted for radioactivity.

β-alanine uptake, i.e. near the peak of tyrocidine production, as shown in Fig. 7 *b*, both ends of the spore septum membrane had moved towards the pole, indicating that these cells were in early stage III (9). Concomitant with the decline of β-alanine uptake and tyrocidine-synthesizing activity, as shown in Fig. 7 *c*, the spore septum membrane moved to the end of the pole, indicating that sporulation had progressed to stage IV; thus, the membrane around the forespore was completed, separating it from the sporangium membrane (9).

Since the uptake capacity for tyrocidine-constitutent amino acids and tyrocidine production paralleled the early stages of sporulation, an accumulation of amino acids and the synthesis of tyrocidine in forespores were suggested. To examine this, the cells were briefly incubated with [^{14}C]proline, after which they were disrupted by sonication and the cellular components were separated by brief low-speed centrifugation through a 20–50% sucrose gradient as described in the legend of Fig. 8. The figure shows the distribution of proline radioactivity overlayed by the OD profile at 280 nm in this gradient. Soluble proteins, ribosomes, and membranes stayed at the top. The forespores and broken cells sedimented to positions indicated by FS and BC, and the FS fraction was identified by electron microscopy as shown in Fig. 9. Whole cells sedimented to the bottom of the tubes. This separation procedure shows the forespore fraction to contain over 60% of the total radioactivity with only 16% of the total protein, indicating that proline accumulated primarily in the forespores.

The forespore and supernatant fractions were prepared as described in Fig. 8; at various times during the progress of sporulation they were tested for tyrocidine synthesis as described by Roskoski et al. (21). This is compared in Table I with β-alanine uptake (see Fig. 2). At the onset of in

vivo tyrocidine production, tyrocidine-synthesizing activity was found in the supernatant fraction because forespores had not yet developed. With the development of forespores, however, tyrocidine-synthesizing activity was almost entirely found in the forespore fraction. In spite of the lack of tyrocidine formation in the supernatant fraction, the rate of ATP-PP_i exchange activity dependent on ornithine was similar to that in the tyrocidine-forming forespore fraction. Chromatography of the supernatant fraction on Sephadex G-200 revealed that this exchange activity was found almost entirely in the region of 70,000 mol wt, which is characteristic for amino acid activating subunits formed by disaggregation of the multienzymes of tyrocidine synthesis as described (14). We did not explore whether this appearance of subunits in the supernatant fraction was due to natural decay of

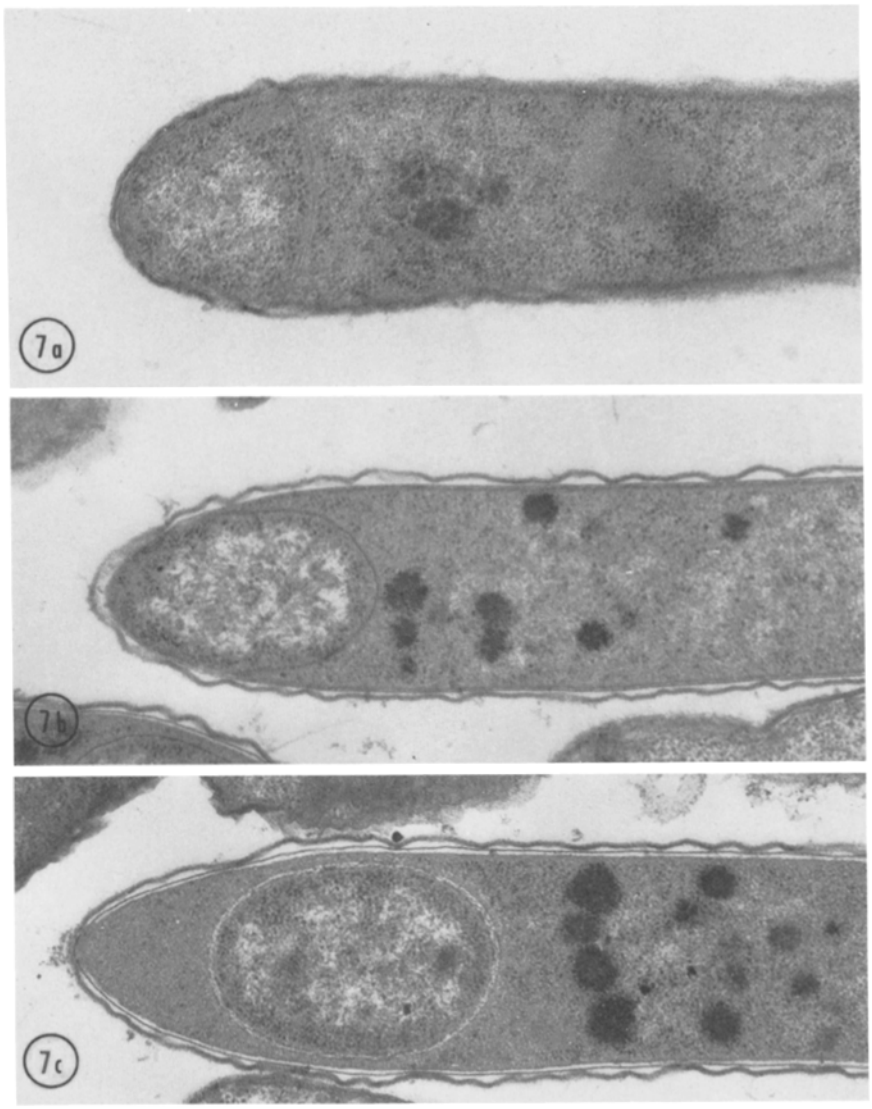

FIGURE 7 Comparison of progress of sporulation with development and decline of tyrocidine production and β-alanine uptake shown in Fig. 2. (*a*) Cells taken at the onset of tyrocidine production and β-alanine uptake. × 40,000. (*b*) Cells taken at the peak of β-alanine uptake, i.e., near the peak of tyrocidine production. × 40,000. (*c*) Cells taken during decline of tyrocidine production and β-alanine uptake, or about 2 h after peak uptake. × 40,000.

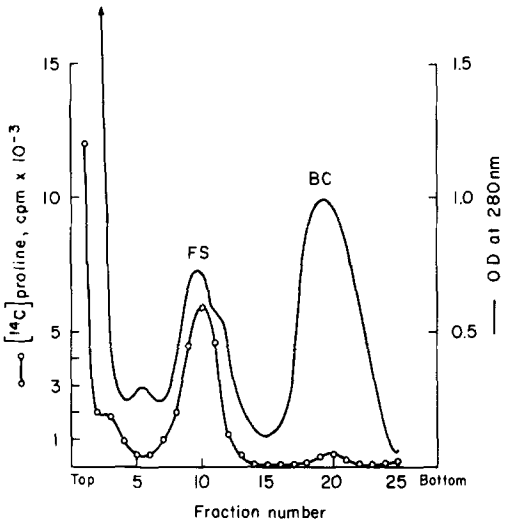

FIGURE 8 Sucrose gradient centrifugation of sonicated cells preincubated with [^{14}C]proline. A 60-ml culture taken 1 h after the peak uptake of β-alanine was incubated with 5 μCi of [^{14}C]proline for 10 min at 37°C in a rotary shaker at 300 rpm. The culture was then chilled quickly with crushed ice, and the cells were collected by centrifugation and suspended in 10 ml of 20 mM triethanolamine buffer, pH 7.6, containing 5 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, and 10% sucrose. The cells were disrupted with a Branson sonifier by four 10-s sonications at step 4 using a step horn, and 2 ml of the suspension was layered on 36 ml of a 20–50% wt/vol sucrose gradient in the same buffer and was centrifuged for 20 min at 6,000 rpm using a SW 27 rotor. The gradient was fractionated and 0.1 ml from each fraction was counted for radioactivity.

multienzymes present initially in this fraction, or to leakage from the forespores, or to secondary disruption during processing.

The observation of an appearance of low molecular weight breakdown products of the multienzymes does not obscure the fact, shown by the data of Table I, that most of the capacity to produce tyrocidine is found in the forespore fraction. The results seem to indicate a migration of antibiotic synthesis into the forespore without indicating, however, what function the antibiotic may fulfill in the process, if such is the case.

DISCUSSION

One of the most interesting observations reported here is the induction of proteins that bind specifically to metabolites connected with tyrocidine synthesis. For example, a β-alanine-binding protein appears in a Sephadex G-200 chromatograph; it is in the low molecular weight region of the supernatant fraction after intense sonication of the bacteria taken from the stationary growth phase, and it is nearly absent in similar extracts of the exponentially growing bacteria; therefore, the latter serve as a good blank. A further confirmation of induced formation of binding proteins is their isolation not only for β-alanine but also for ornithine and proline, both antibiotic-constituent amino acids. Furthermore, such proteins were obtained from the supernatant fraction after shocking the *B. brevis* by dilution, a procedure that has been used extensively for such isolation with gram-negative bacteria (1, 5, 8, 16, 20). Thus, when the growth curve turns from logarithmic to stationary, a number of new proteins appear, including the antibiotic-synthesizing enzymes and metabolite binding proteins.

Furthermore, as shown in Fig. 5, rifampicin suppresses the uptake of constituent amino acids and β-alanine. On the other hand, the change of solubility of the antibiotic-producing enzymes with the appearance of forespores attests to the interrelation between antibiotic and spore formation. Apparently, spore membrane-bound enzymes produce antibiotic which could be shown to be enclosed in forespores (Table I and Fig. 7). Such data would be consistent with the assumption of an

TABLE I

Tyrocidine Synthesis in Supernatant and Forespore Fractions during Development and Decline of β-Alanine Uptake

Time of harvest	Tyrocidine produced	
	Supernate	Forespore
	cpm	
Onset of β-alanine uptake	1,620	—
Peak of β-alanine uptake	440	3,420
1 h after the peak	230	2,300
2 h after the peak	130	1,460

Supernatant fractions (3.2–4.1 mg protein) and forespore fractions (0.9–1.1 mg protein) were incubated for 40 min in a 0.3-ml reaction volume with 1 μCi of [^{14}C]proline, 0.2 mM each of the other tyrocidine constituent amino acids, 2 mM ATP, 10 mM $MgCl_2$, 20 mM triethanolamine buffer, pH 7.6, 1 mM dithiothreitol, and 0.5 mM EDTA. The radioactive tyrocidine produced was extracted and separated as described in Materials and Methods. β-Alanine uptake was determined as described in the legend to Fig. 3.

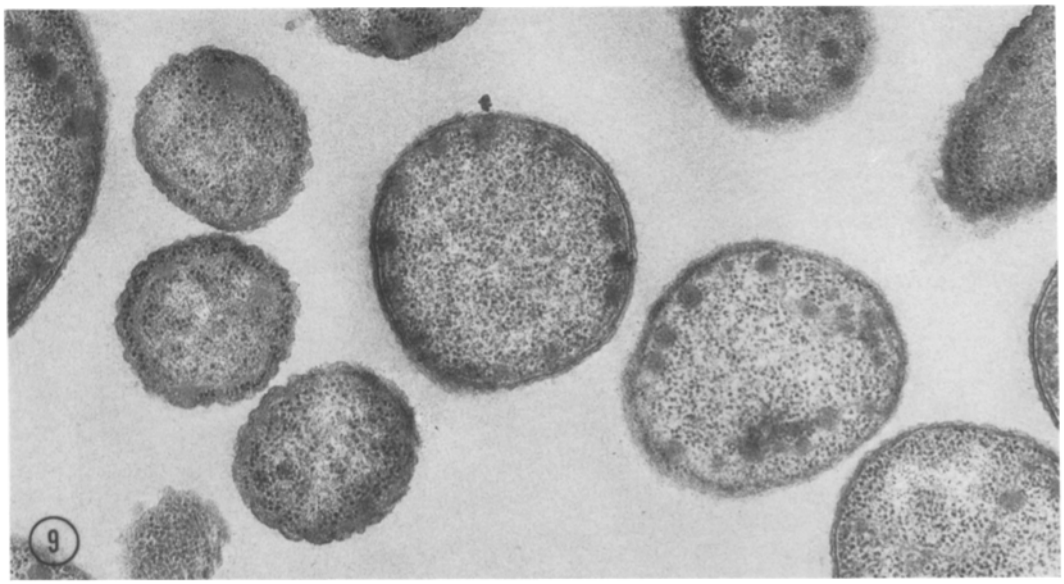

FIGURE 9 Electron micrograph of forespore fraction. The fraction designated FS in Fig. 8 was fixed and embedded in Epon resin, and thin sections were stained as described in Materials and Methods for whole cells. × 40,000.

effect of the antibiotic facilitating the transition from the vegetative phase into sporulation. However, this laboratory has not yet probed into the causative connection between antibiotic and spore formation.

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