Constitutive Expression of Enniatin Synthetase during Fermentative Growth of *Fusarium scirpi*

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The production of enniatins by *Fusarium scirpi* during fermentative growth in submerged cultures was measured. The fungus produced the antibiotic during mycelial growth, but not during the stationary phase of cultivation. By contrast, enniatin synthetase, the enzyme responsible for enniatin synthesis, was present during growth, during the stationary phase, and even in spores. Similarly, the enniatin synthetase mRNA was present at every stage of the cultivation of the fungus. Therefore, this multifunctional peptide synthetase is a constitutive enzyme, the expression of which is not regulated by any specific mechanism. The findings stand in contrast to the common assumption that production of secondary metabolites underlies regulatory control, leading to separation of the trophophase and the idiophase.

Enniatins are a group of cyclic hexadepsipeptides produced by various species of *Fusarium*. Many of these filamentous fungi are plant pathogens (8, 14), and the enniatins belong to a variety of low-molecular-weight phytotoxins produced by *Fusarium* species (11). Together with lycomarasmin, lysomarasminic acid, fusaric acid, fusarubin, and javanicin, they induce wilt diseases in higher plants by influencing the water economy of the host (12). Besides the effect of enniatins in the natural habitat of the fungi, their antimicrobial (11) and immunomodulatory (N. Simon-Lavoine and M. Forget, German patent 2851629, 1979) properties are noteworthy. Enniatins consist of three units each of an N-methylated branched-chain L-amino acid and a D-2-hydroxyisovaleric acid arranged in an alternating fashion (Fig. 1). The biosynthesis of these depsipeptides is well understood. A multifunctional enzyme, consisting of one 250-kilodalton polypeptide chain, synthesizes enniatins from their primary precursors, i.e., valine, leucine or isoleucine, D-2-hydroxyisovaleric acid, ATP, and S-adenosylmethionine.

This enzyme, designated enniatin synthetase, was purified from *Fusarium scirpi* and studied in our laboratory (6, 7, 20–23). Since enniatin synthetase serves as a model system for enzymes that synthesize peptidic phytotoxins, we became interested in the regulation of its expression during the life cycle of the producer organism. Here we present results of our studies on the time course of enniatin production and enniatin synthetase and its mRNA in submerged cultures of *F. scirpi*.

MATERIALS AND METHODS

Cultivation of organisms. *F. scirpi* Lamb. et Faurt. ETH 1536/9 (previously designated *F. oxysporum*) and variants thereof were maintained on FCM agar slants (3% molasses, 1% cornsteep liquor, 1.5% agar) (22). Spore suspensions (10⁷ conidia per ml) were obtained by filtration of 4-day-old submerged cultures maintained in acetate medium (2) through cotton wool.

All submerged cultures were run on a rotary shaker (115 rpm, 27°C) in 500-ml Erlenmeyer flasks containing 100 ml of medium. The cultures were inoculated with 2 × 10⁶ conidia per flask.

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Either the FCM liquid medium (22) or the chemically defined media (FDM) of Madry et al. (17) was used; the latter contained the following per liter of distilled water: 12.5 g of glucose or 25 g of lactose, 4.25 g of NaNO₃, 5 g of NaCl, 2.5 g of MgSO₄·7H₂O, 1.36 g of KH₂PO₄, 0.01 g of FeSO₄·7H₂O, and 0.0029 g of ZnSO₄·7H₂O.

* Nitrosoguanidine mutagenesis. Nitrosoguanidine mutagenesis was done by the procedure described by Madry et al. (17).

* Determination of enniatins. The content of total enniatins in the fungal cultures was assayed spectrophotometrically by the procedure described by Audhya and Russell (1).

* Determination of cell dry weight. Defined volumes (10 to 25 ml) of culture broth were suction-filtered through preweighed filter disks (MN 606; Schleicher & Schuell, Dassel, Federal Republic of Germany). The samples were dried for 16 h at 105°C.

* Preparation of crude extracts. Crude extracts of *F. scirpi* were prepared as described by Zocher and Kleinkauf (22). Lyophilized mycelia or spores were ground to a fine powder in a mortar; for homogenization of spores, sea sand was added. The powder was suspended in 50 mM phosphate buffer (pH 7.2) containing 4 mM dithiothreitol, and the suspension was stirred for 1 h at 4°C. The extract was centrifuged for 30 min at 16,000 × g and dialyzed against the same buffer. The retentate was used for measurement of enzymatic activity or for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

* Enzyme assay. Enniatin synthetase activity in crude extracts was determined as described by Zocher et al. (20) by using L-[¹⁴C]valine as the radiolabel and measuring the formation of labeled enniatin B.

* SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was done as described by Laemmli (15); gels contained 7.5% acrylamide and 0.2% bisacrylamide. Fluorography was performed by using Amplify (Amersham, Braunschweig, Federal Republic of Germany), and the instructions of the manufacturer were followed. Gels and fluorographs were scanned by using a thin-layer chromatographic scanner (CS-930; Shimadzu).

* Western blots. Immunoblotting was performed as described previously (7) by using monoclonal antibodies 21.1 and 25.91, which are directed against enniatin synthetase.
FIG. 1. Structure of enniatins. Enniatin A, R = -CH(H)3CH2; enniatin B, R = -CH2CH2(CH3)2; enniatin C, R = -CH2CH(3H)3.

Pulse-chase experiments. After 70 h of growth in submerged cultures of FCM liquid medium, 50 mCi of L-[35S]cysteine (600 Ci/mmol; Amersham) was added to 100 ml of the fungal culture. After 60 min of incubation, the mycelia were washed twice with FCM liquid medium and then transferred to 100 ml of FCM liquid medium containing 1 mM l-cysteine. Every 2 h portions were removed, from which crude extracts were prepared; samples were taken for up to 14 h.

To measure the incorporation of radioactivity into total protein, 500 μl of ice cold 50% trichloroacetic acid was added to 100 μl of crude extract. After 30 min at 4°C, the sample was filtered through filter disks (GFA; Schleicher & Schuell). After the filters were washed with 7% trichloroacetic acid, they were dried and the radioactivity was measured by liquid scintillation counting.

mRNA isolation. At different times after inoculation, mycelia were harvested by suction filtration of the culture broth through Kleenex tissue and washed with distilled water. Total RNA was isolated from 50 g of the wet mycelium by the procedure of Flurkey and Kolattukudy (10). Poly(A)+ mRNA was selected from the RNA preparation by chromatography on oligo(dT)-cellulose as described by Aviv and Leder (4), with the intermediate salt wash step omitted. mRNA was quantitated by measuring the A260 and assuming 1 A260 unit = 50 μg of RNA per ml. The A260/A280 ratio of the isolated RNA was 1.6-1.7.

In vitro translation. In vitro translation was performed in a nuclease-treated and amino acid-depleted rabbit reticulocyte lysate (Amersham). The assay contained 3.4 μl of lysate, 80 mM K+ , 0.8 mM Mg2+, 10 μCi of [35S]methionine (1,000 Ci/ mmol), 50 μM each of the 20 natural amino acids except methionine, and 0.05 to 1 μg of mRNA in a total volume of 5 μl. After 1 h at 30°C a 1-μl portion of the mixture was subjected to precipitation with trichloroacetic acid on filter disks, and acid-stable counts were detected by liquid scintillation counting. The incorporation of [35S]methionine was a linear function of the mRNA that was added to the reticulocyte lysate under the conditions used. The remainder of the assay mixture was analyzed by polyacrylamide gel electrophoresis, and the content of enniatin synthetase synthesized in vitro was determined by scanning the fluorographs of the gels (Fig. 1). The amount of enniatin synthetase formed was a linear function of the mRNA concentration (data not shown).

RESULTS

Enniatin fermentation. When the enniatin-producing fungus F. scirpi was grown in submerged cultures, production of the antibiotic occurred during mycelial growth; the enniatin content of the culture rose concomitantly with the mycelial dry weight and remained at a constant level when the stationary phase was reached (Fig. 2). This pattern of antibiotic production was observed both with the wild-type fungus, which produced a maximum of 25 mg of enniatin per liter, as well as with variants generated by nitrosoguanidine mutagenesis, which produced up to 50 times more of the antibiotic. In the studies described below, only the high producer strain J5 was used; this was because of the high level of enniatin synthetase that is present in this strain.

Growth-associated enniatin production occurred in all media tested, i.e., in a complex cornsteep molasses medium (FCM liquid medium), in FCM liquid medium supplemented with 1% glucose, and in defined medium (DFM) containing glucose or lactose as the sole carbon source. Growth of cultures on FDM medium was retarded compared with that on FCM liquid medium; the stationary growth phase and constant enniatin titer were reached after 130 and 100 h of growth, respectively (data not shown).

The final enniatin titer could be raised by feeding the precursor amino acid L-valine to the cultures on FCM liquid medium during the exponential phase of growth, as has been described for FDM cultures by Madry et al. (17) (data not shown). The enniatin content was also raised when L-valine was added at the transition from the growth to the stationary phase. The enniatin titer after 120 h on FCM liquid medium was about 1.5-fold higher when valine was included at a concentration of 10 mM after 96 h. This indicates that enniatin synthetase should still be active even in the stationary phase.

Enniatin synthetase level. F. scirpi J5 was harvested at various times during growth on FCM liquid medium, and crude extracts were prepared by homogenization of the lyophilized mycelium in phosphate buffer. The activity of enniatin synthetase in these extracts was measured by incubation of radiolabeled t-valine into enniatin B. The total activity in the mycelium rose within about 20 h, starting from zero at 50 h and reached a maximum after about 70 h (Fig. 2); at this time, mycelial dry weight and the enniatin titer were about half of the maximal amounts. The total activity of enniatin synthetase per gram of mycelium peaked after 60 h and decreased rapidly to 20% of its maximal value within 35 h (Fig. 2).

In addition to the measurement of enzymatic activity, samples of the crude extracts were separated by SDS-polyacrylamide gel electrophoresis. Enniatin synthetase, which appeared as a 250-kilodalton band, was quantitated by densitometric scanning of the Coomassie blue-stained gels. The plot of the enniatin synthetase content of the extracts versus the time of growth yielded a curve parallel to that obtained by plotting the total enzymatic activity (Fig. 2). This means that the specific activity (in units per milligram of protein) of enniatin synthetase remained constant.

Enniatin synthetase was also detected by immunoblotting the crude extracts by using monoclonal antibodies directed against the enzyme (7); with this sensitive technique enniatin synthetase was even found in very young mycelia (30 to 50 h on FCM liquid medium). Therefore, we wondered whether the enzyme might be present in spores; indeed, the protein and its activity were detectable in conidia that were freshly harvested from cultures in FCM liquid medium. The ques-
tion arose as to whether the depsipeptides produced by the enzyme were also present in the spores. A content of 2 mg of enniatin per g (dry weight) of spores was determined.

**Half-life of enniatin synthetase.** The half-life of enniatin synthetase in the mid-exponential phase of growth was determined. After 70 h of growth on FCM liquid medium, pulse-chase experiments were performed, with L-[35S]cysteine used as the radiolabel. At various times during the chase period, mycelia were harvested and crude extracts were separated by SDS-polyacrylamide gel electrophoresis. Gels were subjected to fluorography, and the amount of enniatin synthetase was quantitated by densitometry (Fig. 3); the half-life of the enzyme calculated from these data was found to be about 12 h.

For comparison, the half-life of total soluble protein of the cells was measured in similar experiments. The labeled proteins were precipitated with trichloroacetic acid on filter disks, and radioactivity was determined by liquid scintillation counting (data not shown). The half-life measured in this way was about 12 h. Thus, the rates of degradation of enniatin synthetase and total protein appeared to be similar.

**Enniatin synthetase mRNA.** Cellular RNA was isolated from *F. scirpi* grown in FCM liquid medium. From the total RNA, poly(A)+ mRNA was selected by chromatography on oligo(dT)-cellulose. The mRNA was translated in vitro, and the products were subjected to polyacrylamide gel electrophoresis followed by fluorography; the amount of enniatin synthetase formed was estimated by densitometric scanning of the fluorographs (Table 1). In each case, the same amount of enniatin synthetase was formed per microgram of added fungal mRNA; the amount was equivalent to about 3% of the total protein synthesized. If one assumes that all mRNAs translated at the same efficiency in our system, about 3% of total mRNA encoded for enniatin synthetase. The total amount of mRNA that was isolated from the cultures and, consequently, the absolute amount of enniatin synthetase mRNA varied considerably. The mRNA contents of exponentially growing mycelia were much higher than those of the cultures in the stationary phase of growth (Table 2).

**DISCUSSION**

We showed that in submerged cultures of *F. scirpi* on different media, production of enniatins occurs during mycelial growth but ceases at the beginning of the stationary phase of fermentative growth. By contrast, a study by Audhya and Russell (3) on the production of enniatins in static cultures of *F. sambucinum* led to somewhat different results. They observed that liquid surface cultures on semi-defined medium with glucose as the carbon source passed through well-defined phases corresponding to the growth phase (trophophase) and the enniatin production phase (idio-
phase); when glucose was replaced by lactose, there was no separation of the trophophase and the idiophase, but the growth rate and enniatin production were in balance throughout the growth period, as was observed in our experiments. To explain these contradictory findings, Audhya and Russell (3) have pointed out that glucose might repress or inhibit the synthesis of the depsipeptides. From results of our experiments, there was no indication that catabolite repression is exerted by glucose. We agree with Audhya and Russell (3), however, in their speculation that the accumulation of acidic metabolites might bring about inhibition of the enzymes needed for enniatin synthesis. Production of acids led to an unfavorably low pH (as low as 3.5) during the beginning of mycelial growth on their glucose-containing medium, and enniatin production was retarded. In our glucose-containing FDM medium, this drop in pH did not occur, and consequently, enniatin synthesis was not inhibited. Thus, the separation of production from the growth phase can be considered a laboratory artefact that is observed when the fungus is grown on a rapidly utilized carbon source in a weakly buffered medium.

The level of enniatin synthetase, the enzyme responsible for enniatin synthesis, cannot be deduced simply from the titer of its product in the culture, because the enzymatic activity per gram of mycelium reaches its highest level about 10 h before the enniatin content increases to its maximal level. Furthermore, as much as 50% of the maximal synthetase activity was subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. Fluorographs were scanned with a densitometer, and the relative amounts of enniatin synthetase were calculated.

**TABLE 1. In vitro translation of mRNA from F. scirpi**

<table>
<thead>
<tr>
<th>Time (h) of growth</th>
<th>Content (arbitrary units) in vitro translation assay</th>
<th>Relative amt (%) of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total protein</td>
<td>Enniatin synthetase</td>
</tr>
<tr>
<td>60</td>
<td>567,000</td>
<td>16,000</td>
</tr>
<tr>
<td>72</td>
<td>478,000</td>
<td>15,000</td>
</tr>
<tr>
<td>87</td>
<td>523,000</td>
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</tr>
<tr>
<td>110</td>
<td>532,000</td>
<td>14,000</td>
</tr>
</tbody>
</table>

* A total of 0.5 μg of mRNA was used for in vitro translation (see text). The reaction products were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. Fluorographs were scanned with a densitometer, and the relative amounts of enniatin synthetase were calculated.
sizing activity is found in the extracts of the cells after enniatin production has stopped (100 h on FCM liquid medium).

Since the time course of the levels of enzymatic activity and of enzyme protein coincide (i.e., the specific activity of enniatin synthetase remains constant), the existence of some covalent modification of the active enzyme to yield an inactive form at the end of the growth phase can be excluded. Therefore, the reason for the cessation of antibiotic production should lie in a shortage of the substrates needed for enniatin synthetase; indeed, enniatin production could be stimulated by adding valine to the culture, even at the end of the growth phase.

Many enzymes of secondary metabolism are repressed during growth of the producer organism and are induced at the end of the trophophase, e.g., penicillin acyltransferase (18) and candidicidin synthetases (16). By contrast, enniatin synthetase is present in conidia during mycelial growth and in the stationary phase of fermentative growth. Since the half-life of enniatin synthetase, like that of the total soluble protein of the cells, is only about 12 h, the high content of enniatin synthetase in the stationary phase can only be explained by a continuous biosynthesis of the enzyme. This conclusion is substantiated by the observation that enniatin synthetase mRNA is still present in the stationary phase; indeed, the relative amount of this message in the total pool of mRNA is constant. So there is no indication for a specific regulation of the enniatin synthetase gene. However, the total amount of mRNA present in the mycelium is lower in the stationary phase than in the growth phase. The reduction of the enniatin synthetase level only reflects the decreasing amount of mRNA in the fungal cell. A drop in the rate of protein synthesis, which is preceded by a drop in the rate of RNA and DNA biosynthesis, is generally observed at the transition from the growth to the stationary phase of the cultivation of microorganisms (5); this drop is correlated with a decrease in the activities of the enzymes of primary metabolism. Thus, enniatin synthetase, while producing a peptide that is conventionally regarded as a secondary metabolite, behaves like a constitutive enzyme of primary metabolism and in no way seems to be regulated by a specific mechanism either on a transcriptional or on a translational level.

A major difference between the peptide antibiotics produced by Bacillus spp. and Streptomyces spp. and the structurally related phytotoxins made by phytopathogenic fungi is that the latter are synthesized constitutively (J. D. Walton, Michigan State University, East Lansing, Mich., personal communication). The reason for constitutive biosynthesis of the fungal toxins, e.g., HC toxin (9), victorin (19) or AM toxin (13), should lie in the importance of their role in the infection mechanism and in the progress of host impairment. Seen in the context of the biological role of enniatins, it makes sense that enniatin synthetase as an enzyme that is responsible for phytotoxin production is expressed continuously.

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LITERATURE CITED


