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Biosynthesis of the Pyrroindomycins by *Streptomyces rugosporus* LL-42D005; Characterization of Nutrient Requirements

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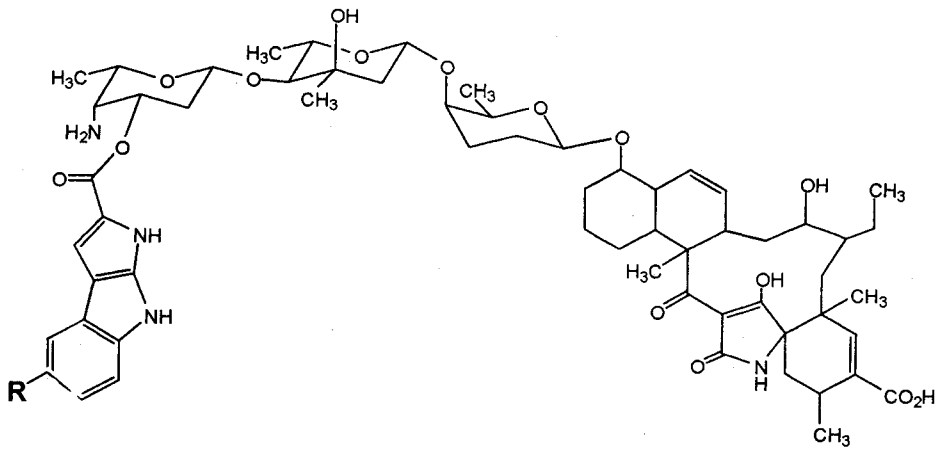
Streptomyces rugosporus LL-42D005 was shown to produce the novel pyrroindomycin antibiotics. Production of pyrroindomycin (α) and chloro-pyrroindomycin (β) was characterized in a semi-defined fermentation medium containing glucose, casein, phosphate, vitamins and minerals. Accumulation of pyrroindomycin β increased with increasing concentrations of glucose, reaching maximum titers at approximately 5g/L glucose. Glucose concentrations greater than 7.5 g/L decreased pyrroindomycin β yields. Inhibition of pyrroindomycin accumulation at higher glucose concentrations could be reversed by increasing the casein concentration. Ammonium chloride, arginine or glutamine could replace casein as the sole nitrogen source for growth and pyrroindomycin production. Glucose, fructose or mannitol were utilized as the sole carbon source, while sucrose, maltose, glycerol, corn oil and starch were poorly metabolized. Incubation of this isolate in a vitamin-deficient medium resulted in a delay in growth and pyrroindomycin production; this delay was eliminated by the addition of biotin. Addition of L-tryptophan to the medium resulted in the production of pyrroindomycin α as the major species.

In response to the development of antibiotic resistance in human pathogens, many pharmaceutical and biotechnology companies have instituted screening programs to discover new antibiotics with novel modes of action. During the course of our screening for compounds active against methicillin-resistant staphylococci and vancomycin-resistant enterococci, the pyrroindomycins were discovered.[1,2] These compounds are composed of an unusual pyrroloindole group linked to a deoxytrisaccharide and a tetramic acid-containing moiety (Figure 1).

Despite decades of research, detailed studies of the nutritional factors regulating secondary metabolite production have been characterized in a relatively small percentage of microorganisms. Control of secondary metabolism remains unpredictable for new isolates, requiring extensive experimentation to maximize the variety and yields of idiolytes produced.

Fig. 1. Structure of pyrroindomycins α (R=H) and β (R=Cl).



To understand the nutritional factors controlling production of the pyrroindomycins, a semi-defined medium able to support growth and pyrroindomycin production was developed. The resulting studies outlined in this report have provided information important for the optimization of pyrroindomycin yields and have provided a means for the selective biosynthesis of pyrroindomycin α or β. It is anticipated that knowledge of the role of nutrients in secondary metabolism of culture LL-42D005 will be useful for studies on the biosynthesis and biotransformation of the pyrroindomycin structure.

Materials and Methods

Fermentation Conditions

To prepare a seed culture, LL-42D005 (NRRL 21084) was inoculated into 50 ml medium A-1 (10 g/L dextrose, 20 g/L soluble starch, 5 g/L yeast extract, 5 g/L N-Z Amine A, 1 g/L $CaCO_3$, 0.4 g/L agar) in a 250 ml Erlenmeyer flask and incubated at 28°C, 200 rpm for 72 hours. Production fermentations containing 50 ml semi-defined, defined, or rich medium per 250 ml Erlenmeyer flask were inoculated with 1 ml seed culture and incubated at 28°C, 200 rpm. The semi-defined medium (SDM) contained glucose (5.0 g/L), vitamin-free casein (5.0 g/L, ICN Biochemicals), K_2HPO_4 (0.14 g/L), MOPS buffer (Na salt, 11.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $MnSO_4 \cdot 4H_2O$ (51 mg/L), NaCl (100 mg/L), KCl (35.2 mg/L), $FeSO_4 \cdot 7H_2O$ (10 mg/L), $Fe(NH_4)_2(SO_4)_2$ (20.7 μg/L), $CoCl_2 \cdot 6H_2O$ (10 mg/L), $ZnSO_4 \cdot 7H_2O$ (37 mg/L), $CuSO_4 \cdot 5H_2O$ (3.2 mg/L), $AlK(SO_4)_2$ (1.0 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (1.0 mg/L), $CaCl_2$ (10 mg/L), $CaPO_4$ (140 mg/L), H_3BO_3 (1 mg/L), KI (60 μg/L), $NaSeO_4$ (12.5 μg/L), $NiSO_4$ (2.5 μg/L), $SnCl_2$ (5.0 μg/L), $NaVO_3$ (5.0 μg/L), Na_2SiO_3 (5.0 μg/L) and vitamins A (2500 units/L), D3 (200 units/L), E (15.0 units/L), C (30 mg/L), B1 (0.75 mg/L), B2 (0.85 mg/L), B6 (1.0 mg/L), B12 (3.0 μg/L), K1 (12.5 μg/L), folic acid (0.2 mg/L), niacinamide (10.0 mg/L), biotin (15.0 μg/L) and calcium pantothenate (5.0 mg/L). The complex medium SO-7 contained glucose (10 g/L), molasses (20 g/L), Bacto-peptone (10 g/L), ferric ammonium citrate (1 g/L) and $CaCO_3$ (1 g/L).

For experiments characterizing the carbon, nitrogen and vitamin requirements, the cell pellet from the seed culture was washed twice with 50 ml dH_2O and re-suspended in 50 ml dH_2O prior to inoculation of SDM or defined medium (SDM minus casein). The effects of various nitrogen sources on growth and pyrroindomycin yields were determined in defined medium. The effects of different sugars as sole carbon source were performed in defined medium without glucose and casein, and with arginine (4 mM) and NH_4Cl (38 mM) as sole nitrogen sources. Duplicate fermentations of each medium variation were tested, and the data points were calculated as mean values. Growth was estimated by quantitation of cell pellet wet weight obtained from 5 ml of fermentation broth. Glucose was determined using an IBI Biolyzer Rapid Analysis System (Johnson & Johnson Clinical Diagnostics, Inc., New York, NY); the lower limit of detection was 1.1 mM glucose. Specific production of the pyrroindomycins was calculated by dividing the concentration of the pyrroindomycins (μg/ml fermentation broth) by the cell mass (g wet weight per ml broth).

Quantitation of Pyrroindomycins

Fermentation broth was lyophilized and extracted with methanol. Extracts were analyzed as 10-fold concentrates by reverse phase chromatography using a HP 1090M LC system with diode array detection.[1] Extracts were resolved on a VYDAC C_{18} column (4.6 mm × 250 mm, catalog #218TP54) with gradient elution using acetonitrile/water/0.1% TFA, increasing from 50% to 90% acetonitrile over 10 minutes. The pyrroindomycins were detected by UV absorption at 280 nm and were identified as α or β according to retention time and UV chromophore. Pyrroindomycins were quantified by comparison of fermentation extract peak areas with those obtained from the injection of known amounts of purified pyrroindomycin standards.

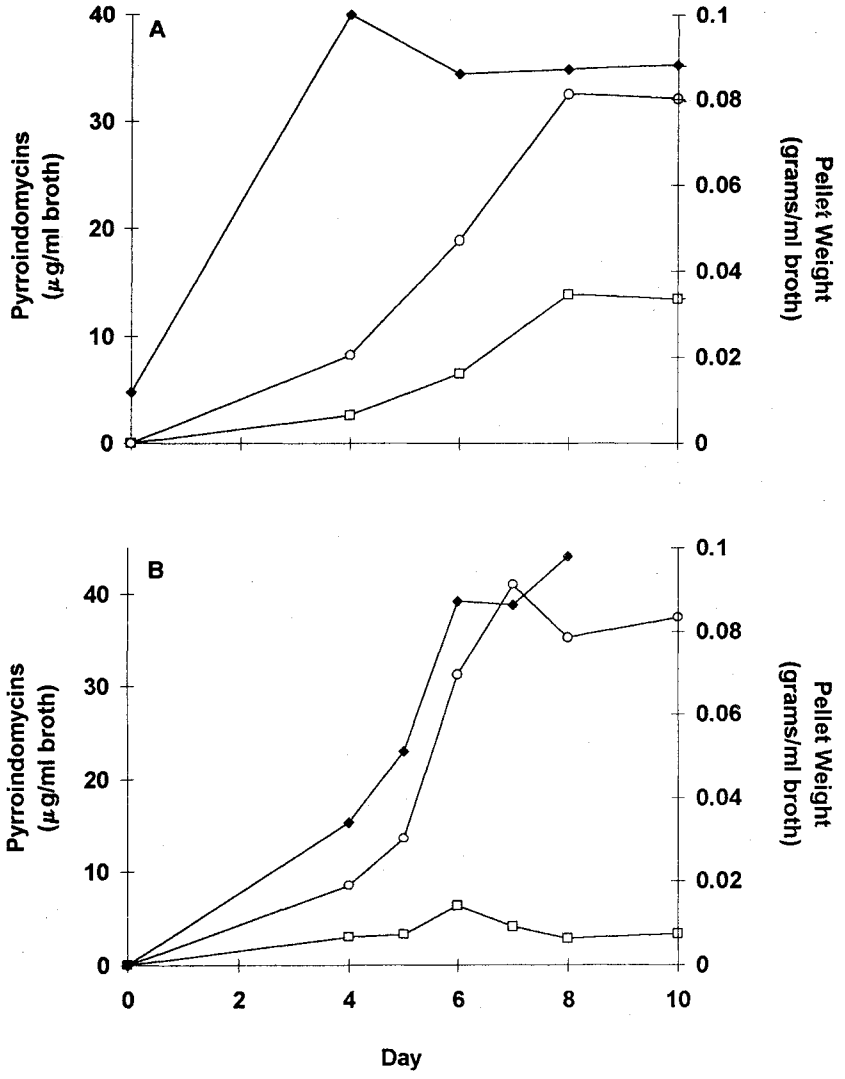
Results

Cell Growth and Pyrroindomycin Production

Growth of culture LL-42D005 in the complex medium SO-7 resulted in a distinct production phase (idiophase) for pyrroindomycins α and β following the growth phase (trophophase), reaching maximum titers after approximately 8 days (Figure 2A). Levels of the chlorinated form, pyrroindomycin β (approximately 35 μg/ml), were approximately 2-fold greater than those of the non-chlorinated α form. In contrast, growth of the culture in SDM resulted in production of pyrroindomycins in the trophophase (Figure 2B). Cell mass and pyrroindomycin titers began to increase after 3~4 days, reaching maximum levels after approximately 6~7 days. Pyrroindomycin β was the major compound produced during growth on SDM, typically reaching titers of 20 to 45 μg/ml. Because yields of pyrroindomycin α were insignificant in this medium, the experiments described

Fig. 2. Cell growth and pyrroindomycin production in (A) SO-7 and (B) SDM.

□ Pyrroindomycin α, ○ pyrroindomycin β, ● pellet weight.



below report only the accumulation of pyrroindomycin β, unless otherwise noted.

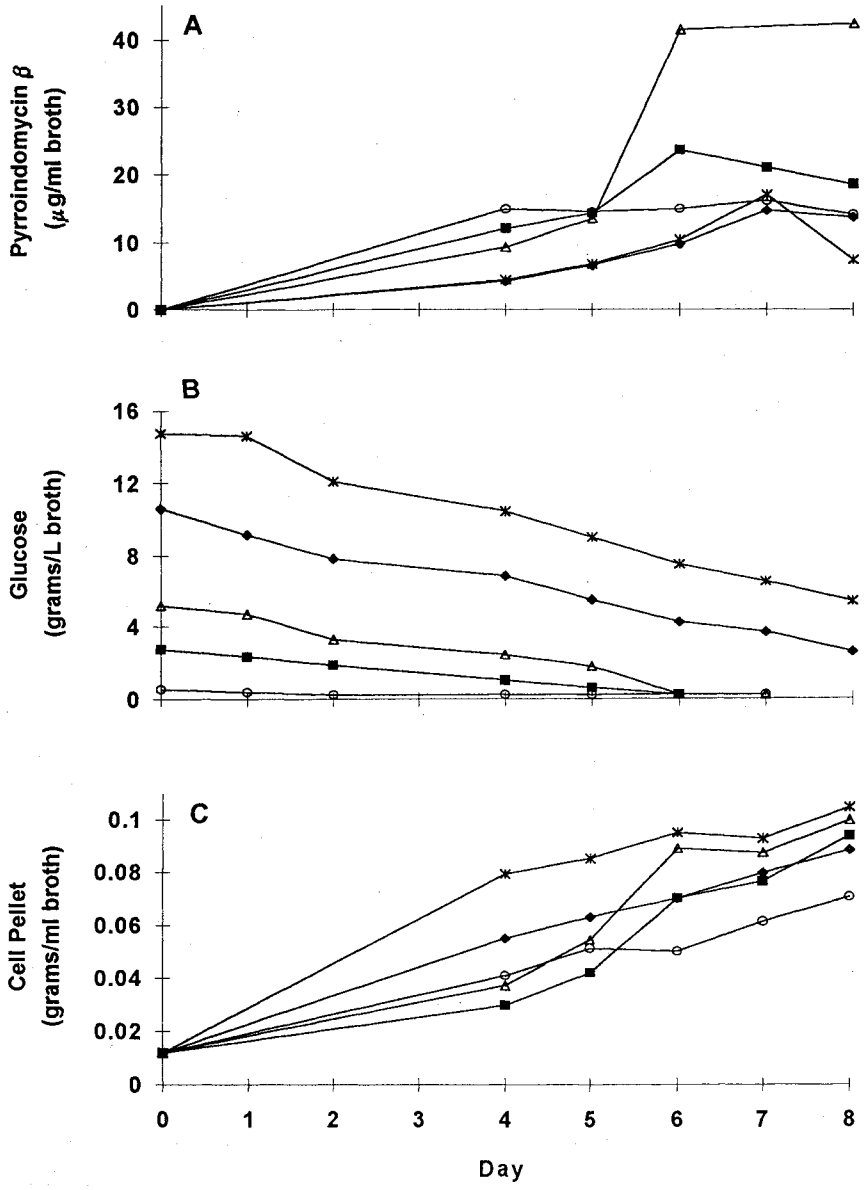
Effects of Glucose and Casein

To determine the effects of glucose, culture LL-42D005 was grown in SDM supplemented with varying glucose concentrations, and pH, growth, glucose depletion and pyrroindomycin yields were monitored (Figure 3). In the absence of glucose, cell growth decreased by approximately 25%, and pyrroindomycin β yields were 4-fold lower than peak levels observed in complete medium (data not shown). Maximum pyrroindomycin β titers were observed in SDM prepared with 5 g/L glucose (Figure 3) or 7.5 g/L glucose (data not shown). Although initial rates of pyrroindomycin β formation were slightly increased at glucose concentrations of 0.5 or 2.5 g/L, the maximum yields attained at these concentrations were approximately 2-fold lower than those observed at 5 g/L glucose. In contrast, increasing the glucose concentration to 10 g/L or more decreased both the production rate and yields of pyrroindomycin β.

The pH of fermentations in SDM remained at or near neutrality. With initial glucose concentrations of 5 g/L or more, the pH remained at 6.8 ± 0.2, while in fermentations with glucose levels of 2.5 g/L or less, the pH rose slightly to $7.3 \sim 7.6$ by day 8. Presumably, in SDM prepared with low concentrations of glucose, carbohydrate depletion during growth resulted in the increased utilization of casein and the release of excess ammonia.

Fig. 3. Effect of glucose on cell growth and pyrroindomycin β production.

Culture LL-42D005 was grown in SDM with varying amounts of glucose and measured for pyrroindomycin β (A), remaining glucose (B) and cell growth (C).
[Glucose]: ○ 0.5 g/L, ■ 2.5 g/L, △ 5 g/L, ◆ 10 g/L, ✳ 15 g/L.



Decreased pyrroindomycin production due to high glucose concentrations (10 g/L glucose) could be relieved by increasing casein levels to 10 g/L (Figure 4). In the presence of 5 g/L glucose, an increase in casein to 10 g/L appeared to stimulate pyrroindomycin accumulation above the levels observed with control glucose and casein concentrations. Under these conditions, there were no significant differences in cell growth.

Nutrient Requirements

With casein as the sole nitrogen source, nitrogen concentrations in the defined medium were estimated to be approximately 50 mM (calculated from analytical specifications, ICN Biochemicals). Growth of culture LL-42D005 in defined medium supplemented with 20 mM arginine or 30 mM glutamine as a sole nitrogen source (concentrations were calculated to provide 60 mM nitrogen from catabolism of arginine or glutamine amide groups) demonstrated that either amino acid could replace casein (Figures 5A and 5B). Glutamine supported a rate of growth similar to that observed with casein, but pyrroindomycin β levels decreased by 35%. With

Fig. 4. Effect of casein on glucose repression of pyrroindomycin β formation.

SDM was made with the indicated levels of glucose and casein.
[Glucose]: ■ 5 g/L, ◇ 10 g/L, △ 10 g/L, ○ 5 g/L.
[Casein]: ■ 5 g/L, ◇ 5 g/L, △ 10 g/L, ○ 10 g/L.

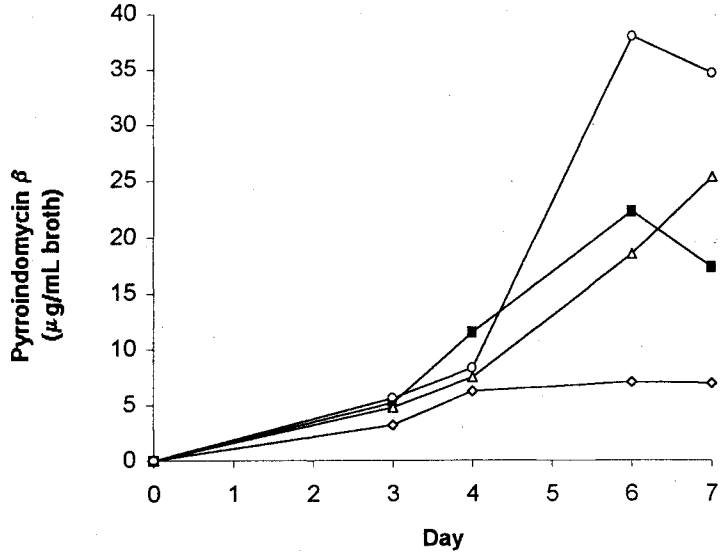


Fig. 5. Effect of nitrogen sources on pyrroindomycin β accumulation (A) and cell growth (B).

Defined media were prepared with 5 g/L glucose and the indicated nitrogen sources.
■ Casein (5 g/L), ● arginine (20 mM), ▲ glutamine (30 mM), ◆ arginine (4 mM), NH_4Cl (38 mM), × arginine (4 mM).

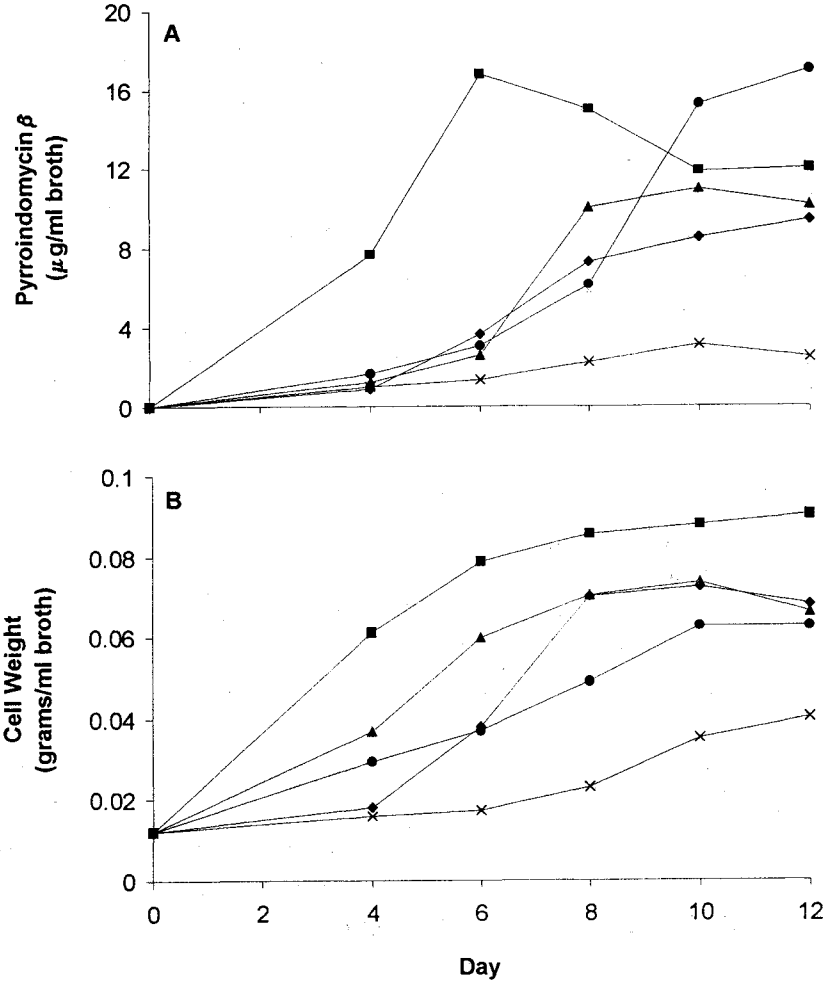
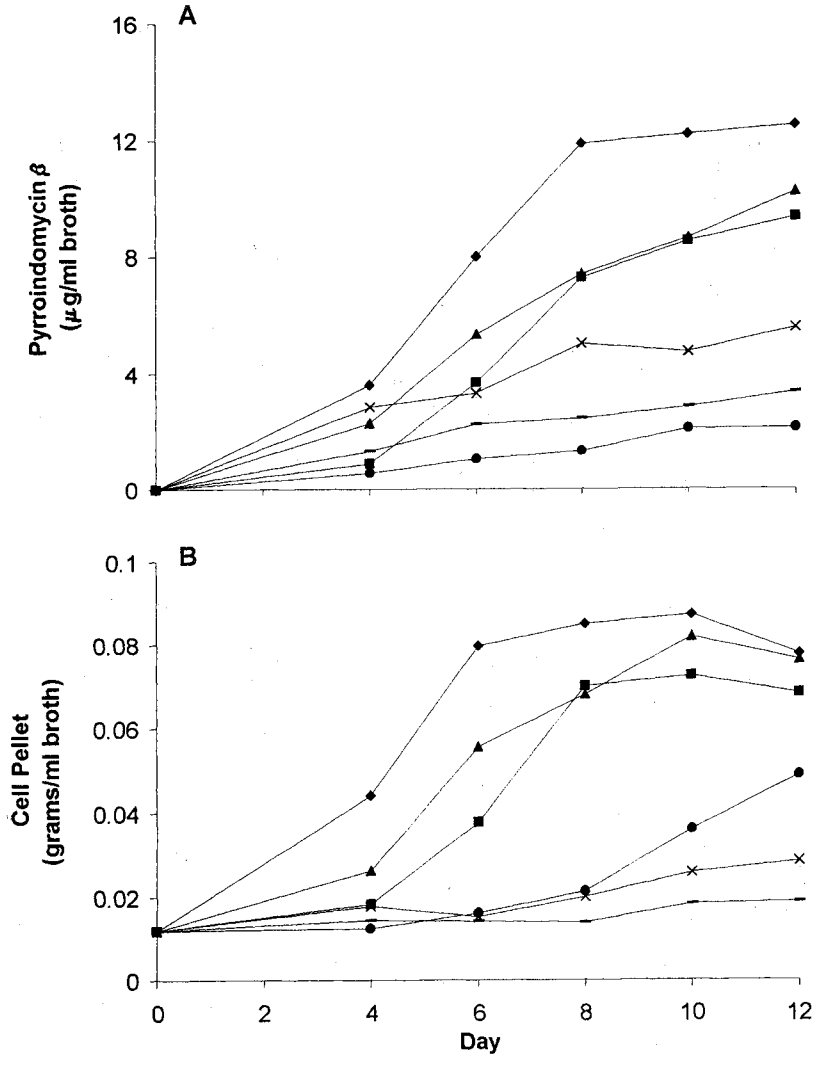


Fig. 6. Effect of carbon sources on pyrroindomycin β accumulation (A) and cell growth (B).

Culture LL-42D005 was grown in defined medium with arginine (4 mM) and ammonium chloride (36 mM) as nitrogen sources, and with the indicated sole carbon source (5 g/L).
■ Glucose, — maltose, ▲ fructose, ◆ mannitol, ● glycerol, × starch.

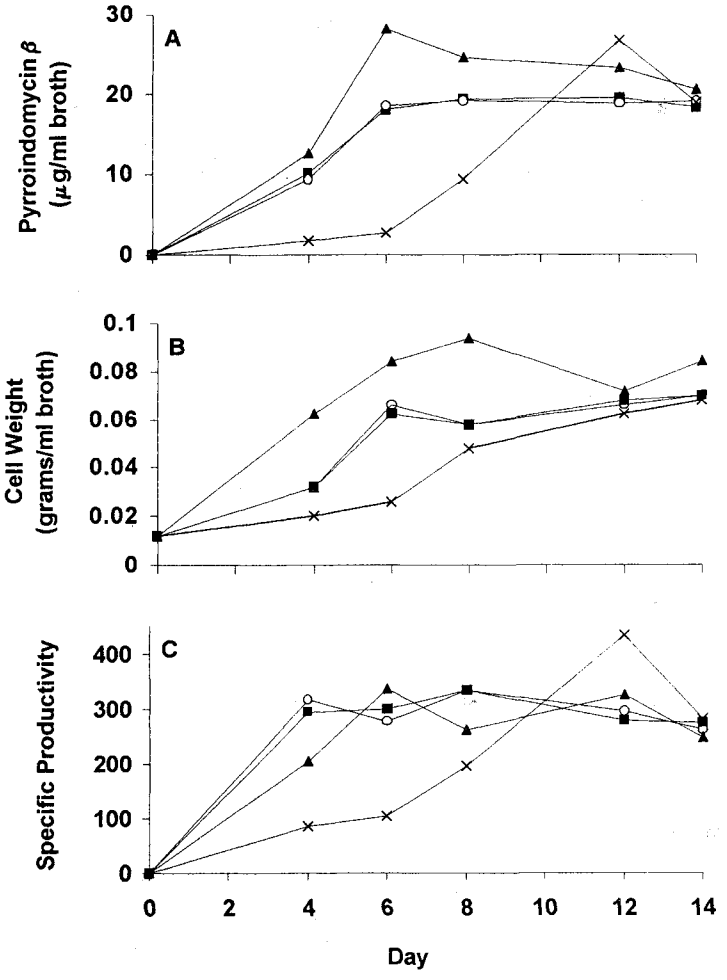


20 mM arginine as the sole nitrogen source, pyrroindomycin β production and cell growth lagged, but the yields of pyrroindomycin β ultimately reached levels observed with the casein-supplemented defined medium. Supplementation with 4 mM arginine resulted in decreased growth and pyrroindomycin production, indicating that nitrogen was limiting at this concentration. Addition of 4 mM arginine and 38 mM ammonium chloride resulted in levels of growth and pyrroindomycin β production approaching those observed with glutamine. Substitution of ammonium chloride (60 mM) for casein resulted in a 2~3 day lag in growth and a 40% decrease in the levels of growth and pyrroindomycin production; higher concentrations of ammonium chloride (200 mM) added as sole nitrogen source inhibited growth and pyrroindomycin β production but had no effect when added in the presence of casein (data not shown). These results suggest that ammonium ion does not mediate nutrient suppression of pyrroindomycin production.

The ability of corn oil, selected sugar alcohols, or mono-, di- and polysaccharides to serve as the primary carbon source was determined using defined medium with 4 mM arginine and 38 mM ammonium chloride as nitrogen sources to minimize the contribution of organic nitrogen to the carbon pool. For comparisons with results obtained from fermentations in SDM medium, refer to Figure 5. Fructose was equivalent to glucose for growth and pyrroindomycin β production, while mannitol

Fig. 7. Biotin requirements of culture LL-42D005.

Requirements were determined by measuring the effects of added biotin (15 μg/ml) on growth and pyrroindomycin β production in SDM prepared with or without the vitamins mix.
■ Vitamins, ▲ no vitamins, added biotin, ○ vitamins, added biotin, × no vitamins, no biotin.



increased growth and pyrroindomycin β production (Figures 6A and 6B). Glycerol, maltose and soluble starch supported reduced levels of growth and pyrroindomycin production. No significant growth or pyrroindomycin production was observed with sucrose or corn oil (data not shown).

Phosphate concentrations from 80 μM to 2.4 mM had no effect on growth or pyrroindomycin production (data not shown). These data indicate that pyrroindomycin biosynthesis was not regulated by phosphate at the concentrations tested.

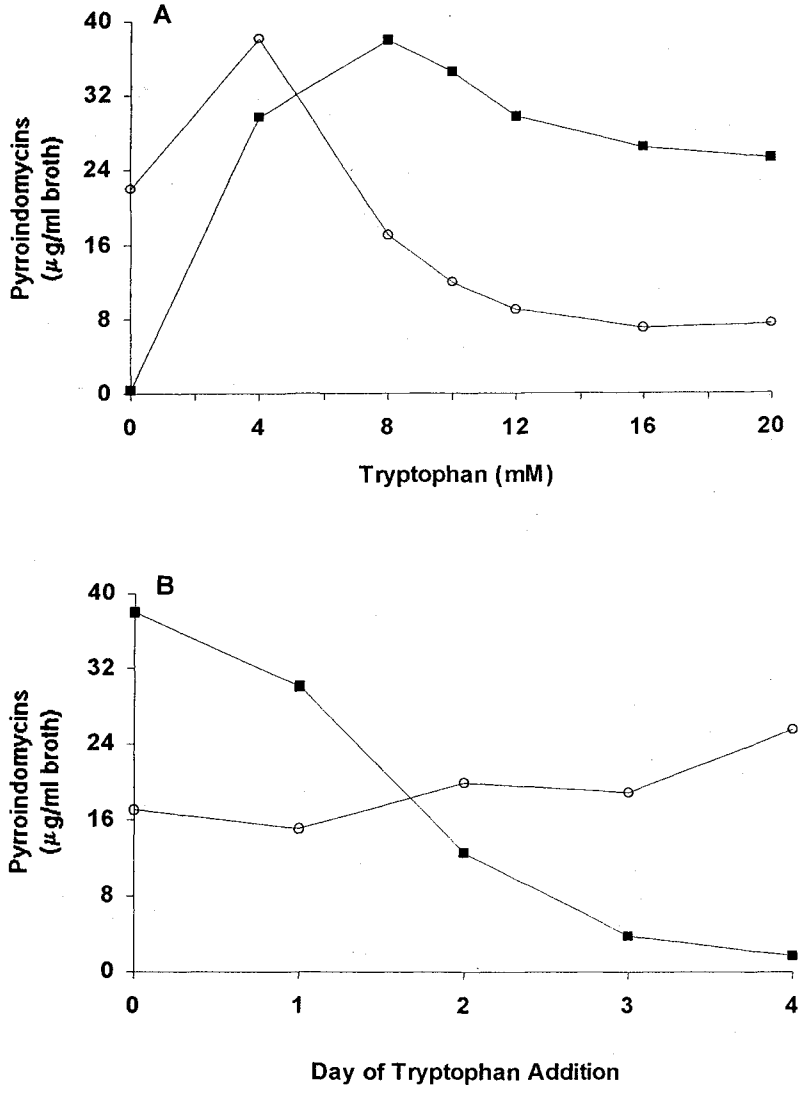
The vitamin requirements of LL-42D005 were measured using vitamin-deficient SDM supplemented with individual vitamins. Growth of culture LL-42D005 in medium prepared without vitamins resulted in a 2~4 day lag in pyrroindomycin β production and growth (Figures 7A and 7B). Addition of biotin to the vitamin-deficient medium restored growth and pyrroindomycin production. Serial transfer of inoculum (late log phase) from vitamin deficient SDM into fresh vitamin-deficient medium also resulted in a delay in growth and pyrroindomycin production (results not shown).

Effects of Tryptophan

Tryptophan is the presumed precursor to the pyrroloindole moiety of pyrroindomycin (manuscript in preparation). Supplementation of SDM with L-tryptophan resulted in a concentration-dependent decrease in the accumulation of pyrroindomycin β and an increase in pyrroindomycin α (Figure 8A). At 4 mM tryptophan, titers of the two pyrroindomycin species were similar, while at concentrations greater than 8 mM, pyrroindomycin α was the predominant form. The effect of tryptophan on pyrroindomycin production decreased as the time of

Fig. 8. Effect of L-tryptophan on the production of pyrroindomycins α and β.

(A) SDM was supplemented with the indicated concentrations of L-tryptophan prior to inoculation with culture LL-42D005. (B) L-tryptophan was added on days 0, 1, 2, 3 or 4 of the fermentation. Fermentations were analyzed on day 9 for pyrroindomycins. ■ α, ○ β.



tryptophan addition was delayed (Figure 8B); no changes in the relative concentrations of α or β were observed if tryptophan was added on the fourth day of incubation, at which point pyrroindomycin titers had reached approximately 25% of the final yields observed.

Discussion

Production of secondary metabolites by actinomycetes grown on nutritionally 'rich' media typically occurs during a distinct idiophase after growth is complete. Production often shifts to the trophophase if the organism is cultivated in a nutritionally limiting medium. Under conditions of nutritional excess, cell metabolism is directed toward the generation of cell mass rather than the production of secondary metabolites; depletion of key nutrients shifts the cell cycle to the stationary phase and signals the transition from primary to secondary metabolism. With defined or nutritionally poor media, nutritional 'limitation' is sensed from the onset, often resulting in the simultaneous initiation of secondary metabolism and growth[3]. Similar patterns of growth and secondary metabolite production were observed with culture LL-42D005. During cultivation on a nutritionally complex medium, cell mass accumulation was rapid, and the production of the pyrroindomycins occurred during the stationary phase. In the semi-defined medium, there was a lag in the onset of growth and a shift to pyr-

roindomycin production during the trophophase.

Glucose has been shown to decrease secondary metabolite production directly through catabolite repression of biosynthetic pathways or inhibition of enzymes functioning in secondary metabolite production (catabolite inhibition or inactivation), or indirectly by affecting the growth rate[3~6]. Glucose appears to regulate pyrroindomycin production in culture LL-42D005, through an as yet undetermined mechanism. Varying the glucose concentration of the semi-defined medium affected the pyrroindomycin yields. Initial rates of pyrroindomycin accumulation were more rapid at lower starting glucose concentrations (1.25 g/L or less), while maximum levels of pyrroindomycin accumulated at intermediate glucose concentrations (5 g/L). Glucose concentrations of 10 g/L or more resulted in a decrease in pyrroindomycin accumulation. These results may be explained by glucose repression. At low or intermediate initial glucose concentrations, pyrroindomycin synthesis would be derepressed as the sugar was depleted, while higher glucose concentrations would result in increased residual sugar levels and the down-regulation of pyrroindomycin production. Alternatively, as was noted for chloramphenicol production[5], the ratio of glucose to organic nitrogen (casein) in the medium may be important for regulation of pyrroindomycin production. The latter hypothesis is supported by the ability of additional casein to reverse repression of pyrroindomycin accumulation at high glucose concentrations (Figure 4) without increasing cell growth.

Idiolyte production in actinomycetes is often stimulated by fermentation with slowly assimilated complex carbohydrates or oils in the fermentation media and is decreased when more rapidly utilized monosaccharides such as glucose are present[6,7]. Rapidly utilized substrates support increased growth rates at the expense of secondary metabolite production, while slowly utilized substrates diminish growth rates, resulting in higher idiolyte accumulation. This association between the carbon source, growth, and secondary metabolite production has been documented most frequently where there is a separation of growth and idiolyte production phases. The relationship between the carbon source and idiolyte production is not as well characterized when secondary metabolite production is coincident with the trophophase. Under the latter conditions, maximum titers of secondary metabolites can be associated with carbon sources that are more readily utilized[8,9]. Incubation of culture LL-42D005 in SDM resulted in production of pyrroindomycins during the cell growth phase; under these conditions, carbon sources supporting the highest growth rates (monosaccharides) yielded the highest titers of pyrroindomycins. Glycerol, di- and polysaccharides, or corn oil were less efficiently utilized as carbon sources and supported lower levels of cell growth and pyrroindomycin production.

Glutamine or arginine replaced casein as the sole nitrogen source for growth and pyrroindomycin production. Glutamine yielded a slightly higher rate and level of growth, while arginine supported delayed, but ultimately higher production of pyrroindomycin. The often-sited inverse relationship between growth rate and idiolyte production[6] may explain the differing effects of glutamine and arginine, although carbohydrates which increased the growth rate increased rather than decreased pyrroindomycin production.

Biotin was important for the optimum growth of culture LL-42D005. In the absence of added biotin, growth and pyrroindomycin production were delayed, even after serial transfer in biotin-deficient SDM. These results suggest that the rate of biotin biosynthesis in culture LL-42D005 may be limiting, causing a delay in growth and pyrroindomycin production until the required intracellular biotin concentrations are achieved. Biotin, an essential coenzyme for carboxylation reactions involving carbon dioxide, is necessary for the catalytic activity of the acetyl-CoA carboxylase enzyme utilized in fatty acid and polyketide biosynthesis. Because the macrolide of pyrroindomycin is polyketide in origin (manuscript in preparation), the effect of biotin limitation on antibiotic production was assessed by determining specific productivity. A decrease in the specific production of pyrroindomycins was noted during early stages of growth in biotin-limited medium, suggesting that limiting biotin concentrations may have selectively delayed pyrroindomycin production. Whether this decreased productivity was due to a lack of functional acetyl-CoA carboxylase or some other effect on idiolyte production remains to be determined.

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