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Effects of amino acid and trace element supplementation on pneumocandin production by *Glarea lozoyensis*: impact on titer, analogue levels, and the identification of new analogues of pneumocandin B_0

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Addition of the amino acids threonine, serine, proline, and arginine to fermentations of the fungus *Glarea lozoyensis* influenced both the pneumocandin titer and the spectrum of analogues produced. Addition of threonine or serine altered the levels of the ''serine analogues'' of pneumocandins B_0 and B_5 and allowed for their isolation and identification. Proline supplementation resulted in a dose-dependent increase in the levels of pneumocandins B_0 and E_0, whereas pneumocandins C_0 and D_0 decreased as a function of proline level. Moreover, proline supplementation resulted in an overall increase in the synthesis of both *trans*-3- and *trans*-4-hydroxyproline while maintaining a low *trans*-4-hydroxyproline to *trans*-3-hydroxyproline ratio compared to the unsupplemented culture. Pneumocandin production and the synthesis of hydroxyprolines was also affected by addition of the proline-related amino acid arginine but not by the addition of glutamine or ornithine. Zinc, cobalt, copper, and nickel, trace elements that are known to inhibit α-ketoglutarate-dependent dioxygenases, affected the pneumocandin B_0 titer and altered the levels of pneumocandins B_1, B_2, B_5, B_6, and E_0, analogues that possess altered proline, ornithine, and tyrosine hydroxylation patterns. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 216–221.

Keywords: pneumocandin; antifungal; *Glarea lozoyensis*; fermentation; hydroxyproline; nonribosomal peptide synthetase

Introduction

The fungus *Glarea lozoyensis* (previously identified as *Zalerion arboricola* [6]) produces a family of structurally related acylated cyclic hexapeptides known as the pneumocandins [11,16]. The major fermentation product of interest, pneumocandin B_0, possesses the amino acids threonine, *trans*-4-hydroxyproline, 3,4-dihydroxyhomotyrosine, 3-hydroxyglutamine, *trans*-3-hydroxyproline, and 4,5-dihydroxyornithine in addition to a 10,12-dimethylmyristate side chain (Figure 1) [11]. CANCIDAS™ (caspofungin acetate), a semisynthetic derivative of pneumocandin B_0, is a potent inhibitor of glucan synthesis and has fungicidal activity against pathogens of clinical significance [4,10].

Labeling studies on pneumocandin biosynthesis identified proline, tyrosine, and glutamic acid as precursor amino acids [1,2]. It was also noted that the medium composition influenced the amount and spectrum of pneumocandins produced throughout a fermentation [16,17]. The pneumocandins are thought to be synthesized *via* a nonribosomal peptide synthetase (NRPS) similar to the mechanism described for the peptide antibiotics cyclosporin and gramicidin S [12].

In the present study, we demonstrate how supplementation of specific amino acids and trace elements impact the pneumocandin B_0 titer and the analogue spectrum. Although achieving a high titer is important for a natural product production process, reducing the levels of analogues that impact the isolation process is paramount. A better understanding of the physiological parameters that influence pneumocandin production will allow analogue levels to be controlled during the fermentation with less reliance on downstream processing methods.

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Materials and methods

Pneumocandin fermentation

The culture and growth conditions used in this study are essentially those described previously [9]. The *G. lozoyensis* strain is a descendant of ATCC 74030 generated through a classical mutation/strain improvement program. The culture was maintained as aliquots of a mycelial suspension in 5% (v/v) glycerol at −70°C. LYCP-5 medium was used for inoculum development and consisted of (per liter of distilled water): glucose, 25 g (added after sterilization); KH_2PO_4, 9 g; yeast extract (Difco, Detroit, MI, USA), 5 g; cotton seed flour (Traders Protein, Memphis, TN, USA), 10 g; lactic acid (85%), 2 ml; and a trace element mixture, 10 ml. The pH was adjusted to 6.0 before sterilization. FGY medium was developed to support pneumocandin production at the shake-flask scale and consisted of (per liter of distilled water): fructose, 125 g (added after sterilization); monosodium glutamate, 8 g; yeast extract (BioSpringer, Minneapolis, MN, USA), 8 g; proline, 15 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; trace element mixture, 10 ml; and 2-[*N*-morpholino] ethanesulfonic acid (MES) buffer 15 g. The pH was adjusted to 5.3 before sterilization. The trace element mixture consisted of (per liter): $FeSO_4 \cdot 7H_2O$, 1.0 g; $MnSO_4 \cdot H_2O$, 1.0 g, $ZnSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g;

Pneumocandin	Position Affected	R_1	R_2	R_3	R_4	R_5	R_6
B_0		H	OH	OH	OH	CH_3	OH
C_0	*trans* 3-Hydroxyproline	OH	H	OH	OH	CH_3	OH
D_0	″	OH	OH	OH	OH	CH_3	OH
E_0	″	H	H	OH	OH	CH_3	OH
A_0	″	CH_3	OH	OH	OH	CH_3	OH
B_5	Ornithine	H	OH	OH	H	CH_3	OH
B_6	″	H	OH	H	OH	CH_3	OH
B_2	″	H	OH	H	H	CH_3	OH
B_0 Serine Analogue	Threonine	H	OH	OH	OH	H	OH
B_5 Serine Analogue	″	H	OH	OH	H	H	OH
B_1	Homotyrosine	H	OH	OH	OH	CH_3	H
D_2	*trans* 3-Hydroxyproline, Ornithine	OH	OH	H	H	CH_3	OH

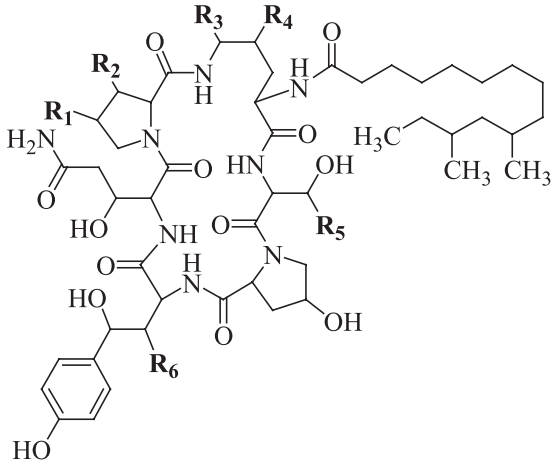

Figure 1 Structures of pneumocandins described in this study.

HBO_3, 0.056 g; $CuCl_2{\cdot}2H_2O$, 0.025 g; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.019 g; and 12 N HCl, 50 ml.

The fermentation process consisted of two stages of inoculum development before the shake-flask production stage. A first-stage inoculum was generated by inoculating 2 ml of frozen mycelia into 50 ml of LYCP-5 medium in a 250-ml Erlenmeyer flask. The culture was incubated 3 days at 25°C with 220 rpm rotary shaking. A second-stage inoculum was generated by inoculating one or more 250-ml flask(s) containing 50 ml of LYCP-5 medium with 1 ml of first-stage inoculum and incubating as above for 4 days.

For each production treatment group, 15 ml of second-stage inoculum was inoculated into 300 ml of FGY medium and subdivided at 25 ml per 250-ml Erlenmeyer flask. The cultures were incubated at 25°C with 220 rpm rotary shaking. Beginning on day 6 of the production cycle, one flask was harvested daily from each treatment group for adjusting broth pH. The amount of titrant (2 N sulfuric acid or sodium hydroxide) required to adjust the broth pH to 5.3–5.5 was determined and this volume of sterile titrant was added to the remaining flasks in the treatment group. Shake-flask fermentations were carried out for 14 days and the data presented for a given treatment group represent duplicate flasks harvested on this day. Alterations or additions to the base medium are described in the text.

Pneumocandin extraction and HPLC analysis

Extraction of pneumocandins for analysis by reverse-phase and normal-phase chromatography was carried out by adding 25 ml of water to each flask followed by homogenizing the culture with a tissue homogenizer. For reverse-phase analysis, a 10-ml aliquot of the diluted broth was added to 20 ml of 100% methanol. Extraction of the product was carried out by shaking the 6× diluted mixture for 30–60 min followed by centrifugation at 1500×*g* for 10 min to remove cellular debris. Clarified methanol extracts were filtered through a 0.45-μm pore size PVDF syringe filter before HPLC analysis.

For normal-phase analysis, a 15-ml aliquot of the 2× water-diluted broth was added to 15 ml of isobutanol. Complete extraction of the product was carried out by shaking followed by centrifugation to remove cell debris and separate the aqueous and organic phases. Ten milliliters of the organic phase were evaporated to dryness in a vial. The residue was dissolved in 1.5 ml of mobile phase (ethyl acetate:methanol:water, 84:9:7) and filtered before HPLC analysis.

For the reverse-phase determination of the pneumocandin B_0 titer (i.e., B_0 plus a small amount of co-eluting C_0) and other analogue levels, 15 μl of methanol extract were chromatographed on a YMC J'Sphere ODS-M80 reverse-phase column (YMC, Wilmington, NC, USA) using a gradient method. At an initial flow rate of 1.5 ml/min, the acetonitrile:0.1% (v/v) phosphoric acid in water ratio was maintained at 40:60 for the first 20 min (postinjection) and then a linear gradient was run between 20 and 40 min to 50:50 (acetonitrile: 0.1% phosphoric acid). Ultraviolet absorption was monitored at 210 nm.

The pneumocandin B_0 titer, expressed as arbitrary units, was determined based on calibration of the instrument with a standard of known purity. Other pneumocandin analogues were identified based on their retention times relative to the main pneumocandin peak (B_0) and quantitated as an area percent relative to pneumocandin B_0.

The pneumocandin C_0 percentage was determined by normal-phase HPLC. Twenty microliters of the extract were chromatographed on an Inertsil 5-μm SI column (MetaChem Technologies, Torrance, CA, USA) at ambient temperature employing a mobile phase of ethyl acetate:methanol:water (84:9:7) at a flow rate of 1.2 ml/min. Ultraviolet absorption of the eluant at 278 nm was monitored and pneumocandins B_0 and C_0 were identified by comparison to pure standards. The level of pneumocandin C_0 was expressed as a percentage of the total amount of pneumocandin B_0+C_0 based on area counts.

Hydroxyproline analysis

To determine the levels of *trans*-3- and *trans*-4-hydroxyproline produced during the fermentation, an aliquot of whole broth was boiled for 5 min followed by centrifugation to remove biomass and insolubles. Quantitation was carried out on the supernatant using the Hewlett Packard AminoQuant system (Hewlett Packard, Palo Alto, CA, USA) employing *o*-phthalaldehyde (OPA) and 9-fluorenylmethylchloroformate (FMOC) derivatization with fluorescence detection. Hydroxyprolines were identified and quantitated by comparison to pure standards.

Pneumocandin analogue isolation and structure determination

The structural analogues of pneumocandin B_0 (Figure 1) were isolated by a combination of normal-phase and reverse-phase chromatography. After cell removal and a series of extractions, a crude solid containing primarily pneumocandin B_0 (~50% by weight) was isolated by precipitation. This solid was the starting point for isolation of the analogues. Initially, a normal-phase, low-pressure chromatography on silica gel using an eluant consisting of 84:9:7 ethyl acetate:methanol:water was carried out on 500 g of crude solid to produce purified pneumocandin B_0, and the analogues were then isolated from the side cuts by further normal-phase and reverse-phase chromatographies. The normal-phase chromatographies were carried out using silica gel G60 (mesh 60–200) in a low-pressure column with an eluant of 84:16:2 chloroform:MeOH:water. The reverse-phase chromatographies were carried out using a Water Prep 4000 HPLC and a 1-in.-ID Inertsil C18 column with a 5-μm particle size. The eluant consisted of a gradient of acetonitrile/water containing 0.05% acetic acid. The products from the chromatographies were isolated by concentration under vacuum (for normal-phase chromatography) or by lyophilization (for reverse-phase chromatography). The structures of the analogues were determined by mass spectrometry and NMR. Key data for the newly identified analogues are provided in Table 1.

The serine analogue contains the amino acid serine instead of threonine in the cyclic hexapeptide. As an analogue of pneumocandin B_0, it has been characterized by mass (M-14); purification to a level that permits spectral characterization has proved difficult. The structure of the serine analogue was confirmed by isolation and characterization after conversion of pneumocandin B_0 containing the serine analog to the antifungal drug caspofungin acetate [5].

Results

Identification of pneumocandin B_0 analogues

During the course of this work, we isolated six new pneumocandins by combinations of normal- and reverse-phase chromatographies (Table 1). The structures of these analogues were determined by mass spectroscopy and NMR. These pneumocandins show altered hydroxylation patterns (pneumocandins B_5, B_6, D_2, and E_0) or alternative amino acid incorporations (serine analogues of pneumocandins B_0 and B_5). The absence or incorporation of hydroxyl groups versus pneumocandin B_0 were readily determined by the corresponding shifts in the ^{13}C NMR resonances. For example, in pneumocandin B_0, the two hydroxylated C4 and C5 positions of the ornithine amino acid have resonances of 76.9 and 75.8 ppm. In pneumocandin B_5 which is missing the C4 hydroxyl group, the C4 resonance shifts from 76.9 to 30.7 ppm. Likewise, in pneumocandin B_6, which is missing the C5 hydroxyl group, the C5 resonance moves upfield from 75.8 to 44.0 ppm. ^{13}C NMR is also diagnostic for replacement of the threonine amino acid with serine. In pneumocandin B_0, the threonine methyl group has a resonance of 19.7 ppm, and this resonance is absent in the serine analogues of B_0 and B_5.

Midcycle additions of serine and threonine

The serine analogues of pneumocandins B_0 and B_5 are marked by incorporation of a serine residue in place of threonine and the levels

Table 1 New pneumocandins isolated during this work

Pneumocandin	Molecular weight	Key ^{13}C NMR resonances (CD_3OD), ppm
E_0	1048	C2–5 positions of the proline amino acid: 62.0, 30.4, 26.3, 49.6
B_5	1048	C3–5 positions of ornithine amino acid: 34.7, 67.6, 44.0
B_6	1048	C3–5 positions of ornithine amino acid: 26.8, 30.7, 71.8
Serine Analog of B_0	1048	As caspofungin acetate analogue, C2,3 positions of the serine amino acid: 56.1, 63.5
Serine Analog of B_5	1032	C3–5 position of ornithine amino acid: 27.4, 30.8, 71.8; C2,3 positions of the serine amino acid: 55.9, 63.8
D_2	1048	C3–5 positions of ornithine amino acid: 27.3, 24.1, 37.5 ppm; C2–5 positions proline amino acid: 66.2, 75.9, 71.8, and 54.3 ppm.

Table 2 Effect of exogenous serine and threonine on pneumocandin B_0 titer and analogues

	Control[a]	Threonine (g/l)[b]		Serine (g/l)	
		1	5	1	5
Pneumocandin B_0 titer (arbitrary units)	472	453	344	446	364
Serine analogue of B_0 (%)	2.2	1.3	0.3	3.7	22.3
Serine analogue of B_5 (%)	0.5	0.3	ND	1.0	6.3

[a]Results are an average of duplicate flasks harvested on day 14 of the fermentation.
[b]Serine and threonine were added on day 6 of the fermentation as described in the Materials and Methods section.
ND, not detected.

of these two analogues can be manipulated by midcycle addition of serine or threonine (Table 2). Addition of 1 g/l threonine to the culture at day 6 resulted in 40% reductions in the two serine analogues. Alternatively, addition of 1 g/l serine resulted in a 70% increase in the B_0 serine analogue and doubling of the B_5 serine analogue. When threonine or serine was added at 5 g/l, further alterations in the levels of the serine analogues were observed but the pneumocandin B_0 titer was reduced by 25%.

Effects of initial proline concentration on pneumocandin and hydroxyproline production

The initial proline concentration in the medium affected both the pneumocandin titer and the levels of the analogues which are marked by alternate hydroxylation patterns at the *trans*-3-hydroxyproline position (Figure 1; Table 3). The pneumocandin B_0 titer and the level of the E_0 analogue increased as a function of initial proline concentration. Conversely, increases in the initial proline concentration resulted in dose-dependent decreases in the levels of pneumocandins C_0 and D_0.

Figure 2 reveals how proline supplementation affects the synthesis of *trans*-3- and *trans*-4-hydroxyproline. An initial proline concentration of 15 g/l results in a 50% reduction in the amount of *trans*-4-hydroxyproline produced at day 8 of the fermentation compared to cultures without proline in the medium. However, proline supplementation enhances the overall synthesis of *trans*-4-hydroxyproline at 14 days. In contrast, the amount of *trans*-3-hydroxyproline produced at day 8 is not influenced by proline supplementation but the overall synthesis at day 14 is increased by approximately 50%. In addition to the absolute

Table 3 Effect of initial proline concentration on pneumocandin B_0 titer and analogue profile

	Proline (g/l)[a]				
	0	5	10	15	20
Pneumocandin B_0 titer (arbitrary units)	274	428	494	476	535
C_0 (%)	14	5.6	3.1	2.6	2.7
D_0 (%)	4	1.6	0.7	0.5	0.4
E_0 (%)	0.9	1.5	2.0	2.1	2.2

[a]Results are an average of duplicate flasks harvested on day 14 of the fermentation.

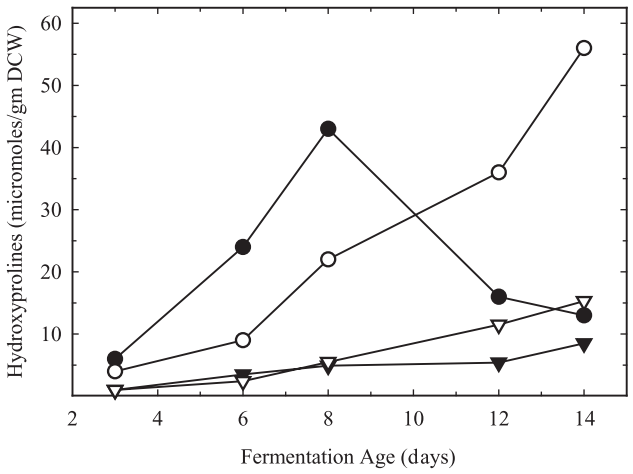

Figure 2 Production of *trans*-4-hydroxyproline (●,○) and *trans*-3-hydroxyproline (▼,▽) as a function of 0 g/l (filled symbols) or 15 g/l (open symbols) initial proline concentration.

amounts of each hydroxyproline formed, supplementation with proline impacts the *trans*-4-hydroxyproline:*trans*-3-hydroxyproline ratio (Figure 3). When 15 g/l proline is added to the medium, the hydroxyproline ratio is approximately 4:1 during the course of the fermentation. In the absence of proline in the medium, this ratio of hydroxyprolines is approximately 7.5:1 during the first 8 days of the fermentation and then drops to less than 2:1 during the balance of the cycle.

Effects of arginine supplementation on pneumocandin and hydroxyproline production

Based on the metabolism of *Saccharomyces cerevisiae*, the amino acids glutamate, arginine, ornithine, and proline share Δ^1-pyrroline-5-carboxylate as a metabolic intermediate [8]. Therefore, the effects of these proline-related amino acids were investigated to determine what, if any, effects they may have on the fermentation. Of these proline-related amino acids, the addition of 15 g/l arginine to the fermentation resulted in a 42% increase in the level of pneumocandin C_0 with no impact on the pneumocandin B_0 titer (Table 4). Neither glutamine nor ornithine, added to the

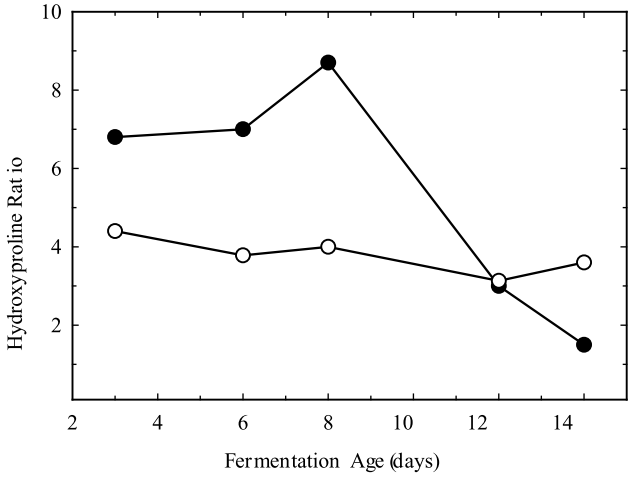

Figure 3 *trans*-4-Hydroxyproline:*trans*-3-hydroxyproline ratio for medium with an initial proline concentration of 0 g/l (●) or 15 g/l (○).

Table 4 Effect of an arginine addition on pneumocandin B_0 titer and analogues

	No additions	Arginine[a]
Pneumocandin B_0 titer (arbitrary units)	636	616
C_0 (%)	3.4	6.2
D_0 (%)	0.6	1.0
E_0 (%)	1.9	1.8

[a]Arginine was added to 15 g/l on day 6 of the fermentation as described in the Materials and Methods section.

fermentation at 15 g/l, had any impact on the pneumocandin titer or the levels of the analogues produced (data not shown). Moreover, the addition of arginine resulted in a doubling of the amount of *trans*-4-hydroxyproline produced whereas the amount of *trans*-3-hydroxyproline produced was unaffected by the arginine addition (Figure 4).

Effects of trace elements addition

The divalent metal ions, zinc, cobalt, copper, and nickel were added to the fermentation medium at 36 μM (equal to the concentration of Fe^{2+} in the medium) to evaluate their effects on titer and analogue levels (Table 5). These divalent metal ions were chosen because of their *in vitro* effects on α-ketoglutarate-dependent dioxygenases that are thought to be responsible for the hydroxylations of some of the pneumocandin constituent amino acids (Petersen, unpublished data).

Addition of zinc resulted in a 50% reduction of the pneumocandin B_0 titer and a doubling of pneumocandin E_0. Zinc addition also resulted in a minor increase in the level of pneumocandin B_1 and minor decreases in the levels of pneumocandins B_2, B_5, and B_6.

A 33% reduction in the pneumocandin B_0 titer was observed with the addition of cobalt. Increases of 30–50% were noted for the pneumocandins with altered ornithine hydroxylation patterns (B_2, B_5, and B_6). However, cobalt addition resulted in nearly an order of magnitude increase in the level of pneumocandin B_1.

Whereas the addition of copper resulted in only subtle changes in pneumocandin titer and analogue levels, the addition of nickel

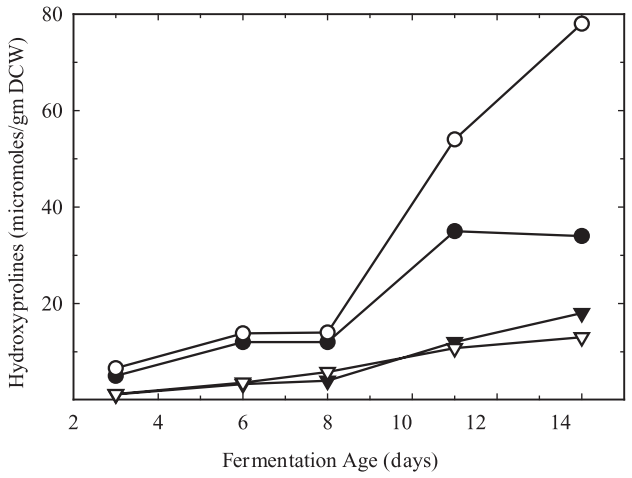

Figure 4 Production of *trans*-4-hydroxyproline (●,○) and *trans*-3-hydroxyproline (▼,▽) with (open symbols) or without (filled symbols) 15 g/l arginine added on day 6 of the fermentation.

Table 5 Effect of trace metals on pneumocandin B_0 titer and analogue profile

	Control[a]	Zn^{2+}	Co^{2+}	Cu^{2+}	Ni^{2+}
Pneumocandin B_0 titer (arbitrary units)	629	312	421	605	546
E_0 (%)	2.2	4.3	1.8	2.6	1.7
B_5 (%)	4.8	3.9	7.2	5.2	4.4
B_6 (%)	1.9	0.8	2.5	0.5	0.7
B_2 (%)	2.8	1.9	6.0	2.7	3.1
B_1 (%)	1.6	2.8	14.1	2.3	7.5

[a]The base medium contains 36 μM Fe^{2+}. Zinc, cobalt, copper, and nickel were added at 36 μM (equimolar to the iron in the base medium).

resulted in a 15% reduction in the pneumocandin B_0 titer and a 5-fold increase in the level of pneumocandin B_1.

Discussion

It has been reported that NRPSs can have broad amino acid substrate specificities resulting in the production of various natural product structures. Numerous reports describe how peptide antibiotic titer and analogue spectrum can be affected by amino acid supplementation [3,7,14,15,18]. For example, the cyclosporin peptide synthetase possesses broad substrate specificity at most of its active sites which results in the production of at least 25 cyclosporins [13].

Although we have identified six new pneumocandins specifically during this study, over two dozen different pneumocandins have been identified during the entire course of pneumocandin fermentation development. In fact, the major pneumocandin produced by wild-type *G. lozoyensis* (previously identified as *Z. arboricola*) was pneumocandin A_0, which possesses a unique 3-hydroxy 4-methyl proline residue at the position occupied by *trans*-3-hydroxyproline in pneumocandin B_0 (Figure 1). Classical mutagenesis and medium development have clearly impacted the spectrum of pneumocandins produced [16]. Currently, pneumocandin B_0 is the predominant pneumocandin produced with pneumocandin A_0 barely detectable.

Although an NRPS mechanism for the synthesis of the pneumocandins has not been conclusively proven, it would be consistent with the results generated in the amino acid supplementation experiments. In addition to the amino acid composition of the pneumocandins being dictated by the general substrate specificities of the NRPS, the metabolic pool size of the amino acids that could occupy a given position also plays a role. The serine analogues of pneumocandins B_0 and B_5 were directly and predictably affected by the levels of exogenously added serine or threonine (Table 2). Even though threonine is likely to be the amino acid preferably incorporated into the hexapeptide, the levels of serine (relative to threonine) will dictate the extent of misincorporation resulting in the formation of the two serine analogues.

The supplementation of proline to the medium affected the extent of hydroxyproline synthesis (Figure 2) and the *trans*-4-hydroxyproline:*trans*-3-hydroxyproline (hydroxyproline) ratio (Figure 3). Increasing the overall amount of hydroxyproline synthesis during the course of the fermentation was important for obtaining a good pneumocandin B_0 titer while the hydroxyproline ratio appeared to be critical in determining the levels of pneumocandins C_0 and D_0. When proline was supplemented at 15 or 20 g/l,

nonhydroxylated proline was incorporated directly into the hexapeptide forming pneumocandin E_0.

The addition of arginine at 15 g/l increased the amount of *trans*-4-hydroxyproline produced without impacting the level of *trans*-3-hydroxyproline produced (Figure 4). Hence the pneumocandin C_0 level increased with an increase in the hydroxyproline ratio (Table 4). Although both arginine and proline share Δ^1-pyrroline-5-carboxylate as a metabolic intermediate [8], it is unclear why they impact hydroxyproline and pneumocandin synthesis differently.

The divalent metal ions zinc, cobalt, copper, and nickel had different effects on pneumocandin production and analogue formation. For example, cobalt addition substantially reduced the pneumocandin B_0 titer and increased the levels of the pneumocandins B_1, B_2, B_5, and B_6 whereas the addition of nickel increased only the level of pneumocandin B_1 with only a modest reduction in pneumocandin B_0 titer. The divalent metal ions chosen for this study are known to inhibit α-ketoglutarate-dependent dioxygenases and preliminary results suggest the involvement of at least one α-ketoglutarate-dependent dioxygenase that is responsible for hydroxylation of proline (Petersen, unpublished). Therefore, it is plausible that other α-ketoglutarate-dependent dioxygenases are responsible for hydroxylations of the homotyrosine and ornithine residues. However, it is unclear if these enzymes utilize the free amino acids as substrates or if the homotyrosine and ornithine are hydroxylated after incorporation into the hexapeptide or during its synthesis.

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