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Improvement in the titer of echinocandin-type antibiotics: a magnesium-limited medium supporting the biphasic production of pneumocandins A_0 and B_0

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SUMMARY

We have developed a liquid fermentation medium for the submerged culture of the fungus, *Zalerion arboricola*, which supports the rapid production of an echinocandin-type antibiotic, pneumocandin A_0 (formerly L-671,329), in yields increased at least 4-fold over those reported previously. The improvements were achieved through medium simplification, substitution of high levels of mannitol for glycerol as the major source of carbon, and restriction of available magnesium. Antibiotic formation in batch cultures with this mannitol-based medium is not confined to the idiophase; rather production appears to be biphasic, with synthesis beginning during growth (i.e., at day 3) and increasing in rate at day 11, well after rapid growth has ended. Accumulation of antibiotic continues beyond 14 days, and by 21 days titers greater than 500 μg/ml are attained. For the synthesis of a related compound, pneumocandin B_0, by a mutant strain of *Z. arboricola*, the medium gives similar production kinetics and a titer of 800 μg/ml. Although supplementation of the medium with magnesium ions stimulates growth, it decreases titer by preferentially affecting the second phase of antibiotic synthesis. This decline in synthesis in the magnesium-supplemented medium is explained by the depletion of mannitol before the second phase of synthesis can begin. In contrast, mannitol in the magnesium-limited medium is used more slowly with approximately half still available at day 11 to support continued antibiotic formation.

INTRODUCTION

Pneumocandin A_0 (formerly L-671,329) is an acylated cyclic hexapeptide antibiotic related to echinocandin [27] and produced by the helicosporous hyphomycete fungus, *Zalerion arboricola* [6,10,21,22]. Recently, it has also been found in a *Cryptosporiopsis* sp. (teleomorph *Pezicula*) [18]. Interest has arisen in the clinical potential of this and similar compounds for the treatment of systemic *Candida* infections as well as pneumonia caused by *Pneumocystis carinii* [9,20], and consequently there is need to develop fermentation procedures for the production of these compounds in significant titers. Further information concerning this group of antibiotics can be found in a recent review [23].

The initial report of pneumocandin A_0 from *Z. arboricola* described a complicated, glycerol-based, liquid fermentation medium in which production commenced at about the fifth day and proceded for 19 days thereafter, supporting titers of approx. 130 μg/ml (reported as 5.8 mg/flask) [21]. Although it was recognized that certain components of this medium exerted a negative influence upon production, medium improvement (for example, by deletion of these components and adjustment of the ratios of the remaining constituents) was not explored. The work described in this paper was undertaken to simplify the fermentation medium and to improve production with respect to both time of onset and titer. Our results lead to the conclusion that the production of this and a structurally related antibiotic, pneumocandin B_0, is favored in a medium that contains a high level of mannitol as the major carbon source and a growth-limiting quantity of magnesium ions.

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MATERIALS AND METHODS

Microorganisms and growth conditions

Two strains of *Zalerion arboricola* were used: MF5402 which is identical to MF5171 (ATCC 20868), the original wild-type isolate that produces pneumocandin A_0 as its major antibiotic product [21], and MF5533 (ATCC

74030), a mutant that makes pneumocandin B_0 as its main product. The derivation of MF5533 from the wild-type strain is described in a separate communication [16]. The strains were grown in the seed medium described previously [21] and in various production media specified in Table 1 and in the Results. Production experiments were performed in 250-ml flasks that contained 50-ml portions of media and that were plugged with cotton. Media were steam sterilized at 121 °C and 15 psi for 20 min. Standardized inocula were prepared by mixing a vegetative culture grown in seed medium with an equal volume of 20% glycerol and freezing portions of the mixture at −70 °C. Transfers were made by 25-fold dilution with fresh medium when the standardized inoculum was passed into seed medium and also when the seed culture was used to inoculate the production media. Cultures were incubated at 25 °C with shaking at 220 rpm for 3–4 days in the case of the seed medium or for the periods specified in the text for the production media.

Media components

The following nutrients were obtained from the sources indicated: corn steep liquor, Grain Processing Corp.; oat flour, Quaker Oat Co.; lard water, Inland Molasses Co.; Ardamine PH, Champlain Industries Inc.; tomato paste, Beatrice/Hunt-Wesson, Inc.; citrus pectin and peanut meal, Bio-Serv Inc.; Peptonized milk nutrient, NZ-Amine (type E), HY-Soy, and HY-Case SF, Sheffield Products; Bacto Peptone, Bacto Yeast Nitrogen Base (without amino acids), Bacto Yeast Nitrogen Base (without ammonium sulfate and amino acids), Bacto Yeast Carbon Base, Bacto Vitamin-Free Yeast Base, Casamino Acids (technical and certified grade), and Bacto Yeast Extract, Difco Laboratories. Yeast Extract obtained from the Fidco division of Nestlé Co., Inc., was also used. Salts, vitamins, glutamic acid, glycine, sugars, and polyols were reagent- or research-grade commercial products.

Determination of pneumocandins A_0 and B_0 by HPLC

To extract the antibiotics, each culture was mixed with an equal volume of methanol, and the mixture was shaken at 220 rpm for 30 min at 25 °C. Following the removal of the large particulates by low-speed centrifugation, the extracts were filtered through 0.45-μm nylon syringe filters. HPLC separations were performed on a 5 μ Ultracarb ODS-20 column (4.6 mm × 25 cm; Phenomenex) eluted isocratically at 37 °C with acetonitrile/water (50:50) at a flow rate of 0.75 ml/min. The column effluent was monitored at 210 nm. Under these conditions, pneumocandin B_0 and pneumocandin A_0 had retention times of 13.8 and 15.5 min, respectively. Titers were calculated from the peak areas obtained by integration of the elution profiles;

TABLE 1

Production media employed[a]

Components	Media								
	PB9	A	B	C	D	E	F	G	H
Glycerol	85								
Mannitol		40	40	40	40	40	40	40	100
Bacto Peptone			20		20	20	33		
NZ-Amine (type E)								33	33
Bacto Yeast Extract			10		10	10	10		
Fidco Yeast Extract								10	10
Bacto Yeast Nitrogen Base[b]				6.7	6.7				
$(NH_4)_2SO_4$						5	5	5	5
KH_2PO_4	2	2				1	9	9	9
Corn steep liquor	4	4							
Lard water	4	4							
Citrus pectin	10	10							
Tomato paste	4	4							
Glycine	2	2							
Peptonized milk nutrient	4	4							
Peanut meal	4	4							

[a] All components are given in g/l. Media B–H were used without pH adjustment; PB9 and medium A were adjusted to pH 7.0 before sterilization.

[b] Without amino acids.

pure compounds used to determine the response factors were supplied by Dr. R. Schwartz, Merck & Co. Each titer value given is presented either as the average of three replicate cultures ± the standard deviation (tables) or as the average of two replicates (figures).

Assay of residual mannitol

Mannitol in the culture broth was measured by HPLC employing a BioRad Aminex HPX-87H column (7.8 mm × 30 cm) at 60 °C eluted isocratically with 4.5 mM H_2SO_4 at a flow rate of 0.6 ml/min. The effluent was monitored with a refractive index detector. Under these conditions mannitol was well separated from other medium components and exhibited a retention time of 10.4 min.

Other methods

The packed cell volume used as an index of cell yield was estimated after subjecting a culture to centrifugation in a 50-ml graduated conical polypropylene tube (Falcon) at $1200 \times g_{ave}$ for 15 min. Actual cell yield was determined by collecting the cells from a measured volume of culture on a weighed 4.25-cm Whatman GF-B filter, washing the cells with two 50-ml portions of water, and drying the loaded filter to constant weight over silica gel desiccant in vacuo. Yields are expressed as mg dry weight per ml of culture. Harvest pH was measured to the nearest half pH unit with commercially available indicator paper (Schleicher & Schuell). The magnesium content of culture media was determined by flame atomic absorption spectrophotometry.

RESULTS

An understanding of the physiological characteristics of the microorganism for which a fermentation medium is desired should be a helpful guide in the development of that medium. Although morphological descriptions of *Zalerion arboricola*, which is isolated from tree wounds and cankers, have appeared [6,19], there is almost no published information regarding its physiology. Consequently we began by determining the basic growth requirements of our wild-type strain (MF5402) in chemically defined agar media (Bacto Yeast Nitrogen Base without amino acids, Bacto Yeast Carbon Base, or Bacto Vitamin-Free Yeast Base). The following could be utilized as the sole carbon source: cellobiose, sucrose, lactose, glucose, mannitol, sorbitol, xylitol, and glycerol plus amino acids (Casamino acids). Sorbose, glutamic acid, or acetate plus amino acids did not support growth. Ammonium ions were suitable as a sole source of nitrogen. Biotin but apparently no other vitamin was required for growth.

Medium simplification

In the preceding report, the medium specified as PBM supported the highest titers of pneumocandin A_0 (see Fig. 1 of Ref. 21). Despite this, another medium from that earlier study, namely PB9, was used as the point of departure for the experiments described here because chromatograms of PB9 extracts were less complicated in the region of pneumocandin A_0 allowing more reliable analytical measurement. We first examined the influence of the major carbon source upon production by strain MF5402. Medium PB9 was prepared with either lactose, sucrose, glucose, sorbitol, or mannitol (each at 4%) in place of 8.5% glycerol. The antibiotic production after 14 days of culture in these media is shown in Table 2. The disaccharides and glucose performed more poorly than glycerol, whereas the titer obtained with sorbitol equaled that with glycerol. However, an encouraging improvement of production was observed in the medium containing mannitol. To evaluate the influence of mannitol further, the time-course for the appearance of the antibiotic in the mannitol medium (designated medium A) was directly compared with that in the glycerol medium, and the results are presented in Fig. 1. In medium PB9, synthesis began at day 9 and continued through day 21. In contrast, production of the antibiotic in medium A started before day 6 with the titer reaching a maximum by day 9 (69 μg/ml) which approached the best titer in medium PB9 (83 μg/ml at day 21). Thereafter, the titer slowly decreased. In this experiment, mannitol did not influence the maximal titer greatly but appeared to allow synthesis to be initiated during rather than after the growth of the culture.

With the identification of this favorable characteristic of mannitol, a survey was conducted of fermentation media containing this polyol at a concentration of 4%. All the media tested were less complicated than medium A.

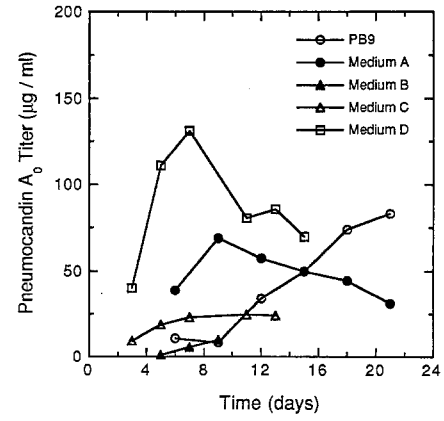

Fig. 1. Pneumocandin A_0 production by *Zalerion arboricola* MF5402 in various media.

TABLE 2

Influence of carbon source upon pneumocandin A_0 production by strain MF5402 in PB9-based media

Medium	Titer of pneumocandin A_0 at 14 days (μg/ml)
PB9 minus glycerol plus:	
4% lactose	29.9 ± 1.03
4% sucrose	29.8 ± 1.84
4% glucose	31.6 ± 3.72
4% sorbitol	37.3 ± 1.20
4% mannitol	58.1 ± 6.98
PB9 (8.5% glycerol)	36.0 ± 0.59

Among them were medium B, a yeast extract-peptone medium, and medium C, a chemically defined medium. Surprisingly, medium C sustained a low level of synthesis extending from day 3 to day 7 whereas the more complex medium B did not (Fig. 1). Since the pH values at harvest were different in these two media (ranging from 3.0 to 4.0 for medium C and from 8.0 to 8.5 for medium B), one explanation for the failure of medium B was that conditions detrimental to the stability of pneumocandin A_0 are generated during growth in this broth. Alternatively, medium B might contain an inhibitor of production or might lack a necessary factor that is present in medium C. To aid in choosing among these possibilities, the effect of combining the components of medium B and medium C was examined. In this new medium (medium D, Fig. 1), the initial kinetics of antibiotic synthesis were similar to those in the chemically defined medium (medium C): pneumocandin A_0 began to appear on day 3 and was maximal by day 7. However, the peak titer achieved in medium D was significantly improved with respect to medium C (131 μg/ml vs. 25 μg/ml). The performance of medium D in comparison to medium B indicated that at least one factor necessary for either the stability or the production of pneumocandin A_0 was provided to medium B by the chemically defined base of medium C. An unanticipated benefit of this experiment was the finding that medium D was also markedly superior to medium A in terms of both pneumocandin A_0 titer and kinetics (Fig. 1). The decline in pneumocandin A_0 levels seen in medium D after day 7 coincided with a rise in pH of the culture from 6.0 on day 7 to 8.0 on day 9.

Bacto Yeast Nitrogen Base (without amino acids) which was used as the base of medium C contains ammonium sulfate, vitamins, trace elements, and salts. For the identification of the component(s) responsible for the positive characteristics of medium D, a set of media, each a variation of medium D lacking one or a group of the components in Bacto Yeast Nitrogen Base, was tested for the ability to support production. The results (data not shown) indicated that $(NH_4)_2SO_4$ and KH_2PO_4 contributed to the stimulation, whereas the other salts, trace elements and vitamins did not. To test whether the ammonium and phosphate salts were sufficient to produce the effect, they were added individually or in combination to medium B at concentrations equal to those they had when added as part of Bacto Yeast Nitrogen Base in medium D (i.e., 0.5% and 0.1%, respectively). The pneumocandin A_0 titers attained in 7 days with these supplements are presented in Table 3. Individually each salt was stimulatory, but the effect of KH_2PO_4 was far greater than that of $(NH_4)_2SO_4$. Each salt had the potential to lower the pH of the culture, but at the concentration tested, neither salt alone resulted in a harvest pH as acidic as that of medium D. When both salts were added, a harvest pH like that of

TABLE 3

Components of Bacto Yeast Nitrogen Base required to support production of pneumocandin A_0 in medium B

Medium	Titer of pneumocandin A_0 at 7 days (μg/ml)	Harvest pH[a]
Medium B plus:		
Bacto Yeast Nitrogen Base[b,c]	123 ± 4.18	4.0, 4.0, 4.5
$(NH_4)_2SO_4$	34.1 ± 4.19	7.5, 8.0, 7.5
KH_2PO_4	130 ± 3.13	8.0, 8.0, 8.0
$(NH_4)_2SO_4$ and KH_2PO_4[d]	141 ± 3.37	4.0, 5.0, 5.0
Nothing	5.54 ± 7.17	8.0, 8.0, 8.0

[a] Values from three replicates in this table and Table 4.
[b] Without amino acids.
[c] i.e., medium D.
[d] i.e., medium E.

medium D was obtained along with a titer slightly better than that of medium D. Medium D with 0.5% $(NH_4)_2SO_4$ and 0.1% KH_2PO_4 substituted for the Bacto Yeast Nitrogen Base was designated medium E.

Medium optimization

In the hope of further improving the production medium for pneumocandin A_0, we determined the optimal ratios of peptone, yeast extract, $(NH_4)_2SO_4$, and KH_2PO_4 in medium E through an experiment with these four components as simultaneous variables. The concentration ranges were these: peptone, 1% to 4%; yeast extract, 0.5% to 2%; $(NH_4)_2SO_4$, 0.5% to 2%; and KH_2PO_4, 0.1% to 1%. The matrix of variables was based on the Box-Behnken design [5,11] and was generated with the aid of RS/Discover software (BBN Software Products Corporation) which was also used for data analysis (response-surface methodology). Since the earliest onset of synthesis was desired, production data from 5-day cultures were used in the calculation of optimal levels of medium components. The results are not presented in detail, but increases in the peptone and phosphate levels to 3.3% and 0.9%, respectively, were indicated with no change in the concentrations of ammonium sulfate or yeast extract. Based upon the results of this experiment, a new medium (medium F) was formulated, and its superior performance was verified in a direct comparison with medium D and medium E (Table 4).

To improve the feasibility of transferring pneumocandin A_0 production from shake flasks to large stirred fermentors, substitutes for two of the costly components of medium F (viz., Bacto Peptone and Bacto Yeast Extract) were sought. We evaluated Peptonized milk, Casamino acids (technical and certified grade), HY-Soy, HY-Case SF, and NZ-Amine type E (each at 3.3%) as replacements for Bacto Peptone and Fidco Yeast Extract and Ardamine PH (each at 1%) as substitutes for the Bacto Yeast Extract. NZ-Amine (type E) and Fidco Extract appeared to be satisfactory when tested individually. A medium prepared with both of these alternate components (medium G) supported the production of pneumocandin A_0 with a titer at 7 days of 138 ± 3.30 μg/ml, which compared favorably with production of medium D and medium E in the same time period (Table 3). Another relatively expensive component of medium F and medium G is mannitol. Since our initial experiments indicating the superiority of mannitol as the major carbon source were performed in a PB9-based medium which differs substantially from medium G, it was appropriate to re-examine the effect of carbon sources in this new medium. Production of pneumocandin A_0 was checked at both 7 and 14 days in medium G unmodified or prepared with lactose, sucrose, glucose, or sorbitol (each at 4%), or glycerol (at 8.5%) in place of mannitol. The results (not shown) confirmed our previous conclusion that mannitol is superior to other polyols which are, in turn, superior to mono- and disaccharides.

This carbon source experiment provided an additional observation, namely, that the titers in medium G measured at 7 and 14 days were nearly equivalent. In contrast, a loss of antibiotic had been noted in medium D after day 7 (Fig. 1). Thus, the improvements that had been made to the peptone-yeast extract fermentation medium resulted in a stabilization of the product; presumably the acidic culture pH was the primary factor responsible for this stabilization (see Discussion). On the other hand, the improvements had not extended the production phase beyond the seventh day. Since high carbon to nitrogen ratios can prolong the synthesis of a secondary metabolite in a fungal fermentation (see for example [7,25]), we examined the effect of increasing the mannitol content of medium G over the range of 4% to 10% (Fig. 2). In this experiment, as before, the titer with 4% mannitol was the same at 7 and 14 days. As the mannitol concentration was raised, production was initially retarded so that titers at day 7 were slightly lower with 6% to 10% mannitol than they were with 4% mannitol. However, at 14 days, titers with 6% and 8% mannitol were increased over the corresponding

TABLE 4

Optimization of components in medium E

	Titer of pneumocandin A_0 (μg/ml) at day 5	Harvest pH
Medium D	73.5 ± 4.24	3.5, 3.5, 3.5
Medium E	74.8 ± 7.21	4.0, 4.0, 4.0
Medium F	127 ± 4.56	4.5, 4.5, 4.5

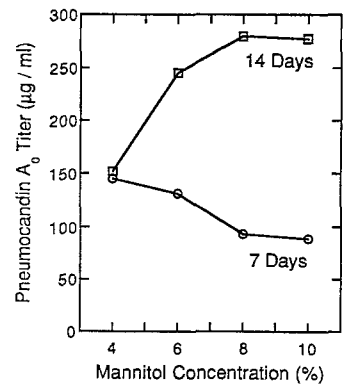

Fig. 2. Effect of increasing the mannitol content of medium G upon the synthesis of pneumocandin A_0.

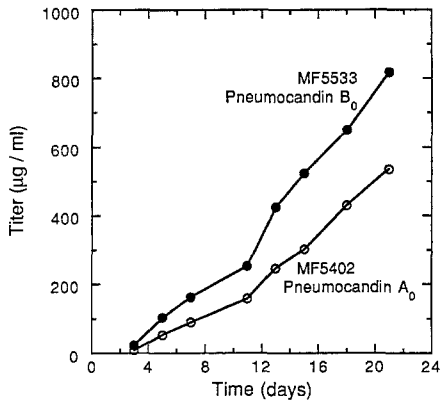

Fig. 3. Performance of fermentation medium H for the production of pneumocandin A_0 by *Zalerion arboricola* MF5402 and pneumocandin B_0 by *Z. arboricola* MF5533.

7-day values and were nearly double the values obtained with 4% mannitol. These increases verified our presumption that 7 to 14 days was sufficient time for the organism to utilize all or most of the mannitol normally present in medium G. The fact that the 14-day values for the media with 8% and 10% mannitol did not differ raised the possibility that production in these media might extend well beyond 14 days. The improved medium G modified to contain 10% mannitol was named medium H.

Production of pneumocandin B_0 in medium H

The wild-type *Z. arboricola* culture (MF5402) which was used throughout the development of medium H produces pneumocandin A_0 together with smaller quantities of several structurally related bioactive compounds. The most prominent of these minor products is pneumocandin B_0; it differs from the parent compound by having a residue of 3-hydroxyproline in the cyclic peptide core of the antibiotic at the position normally occupied by 3-hydroxy-4-methylproline [12,22]. In mutant MF5533, synthesis is shifted in favor of pneumocandin B_0 with pneumocandin A_0 the principal minor product. We found that medium H supported not only the production of pneumocandin A_0 by strain MF5402 but also the formation of pneumocandin B_0 by mutant MF5533; this is shown in Fig. 3. Antibiotic production with both strains began at day 3, and in each case, synthesis appeared to be biphasic with an increase

TABLE 5

Determination of the factor limiting growth of MF5402 in medium H[a]

	Concentration (mg/l)	Packed cell volume[b]
Medium H (with usual 1% yeast extract) plus:		
Nothing	–	8.6
Bacto Yeast Nitrogen Base[c]	1700	17.1
Vitamins	[d]	9.0
Trace elements	[e]	8.0
NaCl	100	8.6
$CaCl_2 \cdot 2H_2O$	100	8.8
$MgSO_4 \cdot 7H_2O$	500[f]	17.0
$MgSO_4 \cdot 7H_2O$	100	15.2
$MgSO_4 \cdot 7H_2O$	50	13.7
Medium H (with 0.5% yeast extract) and $MgSO_4 \cdot 7H_2O$	50	14.5
Medium H (with 0.25% yeast extract) and $MgSO_4 \cdot 7H_2O$	50	16.0
Medium H (with no yeast extract) and $MgSO_4 \cdot 7H_2O$	50	8.0

[a] Measured after 14 days of growth.
[b] Average of duplicates expressed as ml per 50 ml of culture.
[c] Without ammonium sulfate and amino acids.
[d] Vitamins and trace elements were used at the concentrations they had when added as part of Bacto Yeast Nitrogen Base. Vitamins (per liter): biotin, 2 µg; calcium pantothenate, 400 µg; folic acid, 2 µg; inositol, 2 mg; niacin, 400 µg; *p*-aminobenzoic acid, 200 µg; pyridoxine hydrochloride, 400 µg; riboflavin, 200 µg; thiamine hydrochloride, 400 µg.
[e] Trace elements (per liter): boric acid, 500 µg; copper sulfate, 40 µg; potassium iodide, 100 µg; ferric chloride, 200 µg; manganese sulfate, 400 µg; sodium molybdate, 200 µg; zinc sulfate, 400 µg.
[f] The concentration equivalent to the level achieved as part of Bacto Yeast Nitrogen Base.

in the rate occurring at day 11. Accumulation of the antibiotics continued beyond 14 days, and by 21 days, the last point assayed in this experiment, titers greater than 500 and 800 μg/ml had been achieved for pneumocandin A_0 and pneumocandin B_0, respectively.

Growth-limiting factor in medium H

Medium H is rich in carbon, nitrogen, and phosphorus, and consequently, it seemed reasonable that growth of MF5402 in this medium was limited by some other factor, perhaps an inorganic ion or vitamin (biotin). Indeed, supplementation of medium H with a mixture of salts, trace elements, and vitamins (commercially available as Bacto Yeast Nitrogen Base without ammonium sulfate or amino acids) resulted in a growth yield (measured as packed cell volume) at 14 days which was double that obtained with unmodified medium H (Table 5). Testing of the individual components of Bacto Yeast Nitrogen Base revealed that magnesium sulfate was sufficient to produce this growth enhancement (Table 5) and that cell yield increased in proportion to the magnesium sulfate added over the range of 50 to 500 mg/l (i.e., 0.203 to 2.03 mM). In a separate experiment (data not shown), the stimulatory effect of magnesium ions was observed with glucose in place of mannitol and with the mutant MF5533 in place of the parental strain MF5402. Therefore, the effect was neither carbon source- nor strain-specific. These results indicated that cells of *Z. arboricola* growing in medium H experience magnesium limitation.

By flame atomic absorption spectrophotometry, the magnesium ion content of medium H was 0.206 mM. With the addition of 50 mg of magnesium sulfate to a liter of medium H, the total magnesium ion content should have nearly doubled to 0.409 mM. As noted in Table 5, this level of magnesium supplementation supported a 1.6-fold increase in cell yield. Withholding the yeast extract from medium H reduced its magnesium ion content to 0.148 mM, indicating that only about 28% of the magnesium in medium H is provided by the yeast extract. Nevertheless, lowering the yeast extract content while maintaining the added magnesium sulfate level at 50 mg/l (0.203 mM) resulted in an increase in cell yield; data for the cases in which the yeast extract was reduced to a half and a quarter of its usual concentration are shown in Table 5. This observation suggested that the yeast extract contains a factor that antagonizes the stimulatory effect of magnesium ions or that suppresses growth. No attempt to identify this factor was made, but calcium ions are known to antagonize the biological effects of magnesium [17]. Eliminating the yeast extract altogether while maintaining the level of added magnesium sulfate at 50 mg/l resulted in a reduction of the growth yield to the level obtained in unmodified medium H.

Effect of magnesium ion upon antibiotic production

The stimulation of MF5402 growth that resulted from the addition of magnesium sulfate was accompanied by a reduction in pneumocandin A_0 synthesis. At 14 days maximal reduction was observed when the level of added magnesium sulfate was as low as 60 mg/l (i.e., 0.45 mM total magnesium ion content; data not shown). The nature of this suppressive effect became clear when the time-courses for growth, mannitol utilization, and antibiotic production were examined in supplemented and unsupplemented medium H. As seen in Fig. 4, production began at 3 days in each medium; this is after rapid growth was about 50%

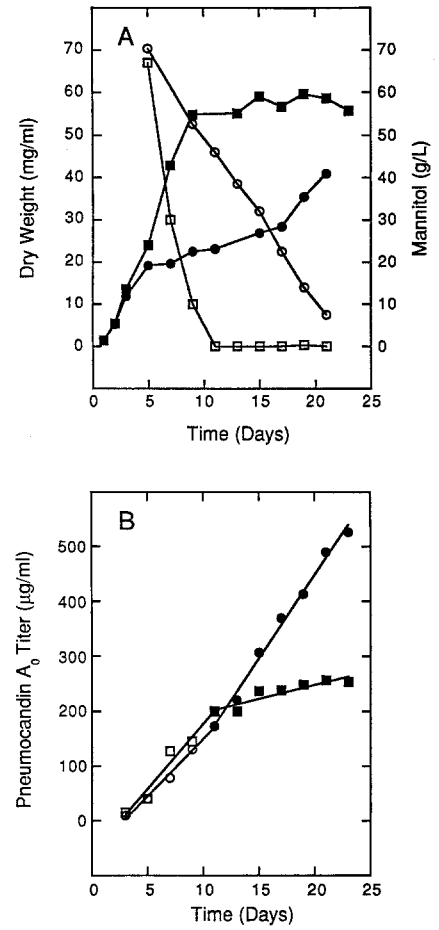

Fig. 4. Influence of magnesium ions in medium H on growth of and mannitol utilization by *Z. arboricola* MF5402 (A) and production of pneumocandin A_0 (B). Circles show the data obtained with unmodified medium H, and squares give the results in medium H supplemented with magnesium sulfate (60 mg/l). In panel A, dry weight is indicated by the filled symbols and residual mannitol concentration is specified by the unfilled symbols. In panel B, the data points for each type of medium are divided into two groups to emphasize the biphasic nature of the production curves, i.e., 3 to 11 days (open symbols) and 11 to 23 days (filled symbols); linear regression lines are shown for each group.

completed in the unsupplemented medium but only 25% completed in the supplemented medium. In this experiment as before (Fig. 3), production appeared to be biphasic; to emphasize this, the data points in Fig. 4B are divided into two groups (3 to 11 days and 11 to 23 days) and linear regression lines are shown for each group. In the magnesium-fortified medium, the rate of synthesis in the first phase was virtually identical to that in unmodified medium, but the rate in the second phase declined sharply. Magnesium addition also extended the period of rapid growth (Fig. 4A) from 5 days to 9 days and slightly more than doubled the growth yield. Mannitol utilization was markedly different in the two media. With magnesium supplementation, mannitol was entirely consumed by day 11. On the other hand, approximately half of the original 100 g/l remained in the unmodified medium at day 11, and at day 21 residual mannitol was still detectable. Thus, with magnesium limitation, mannitol was available after day 11 to support antibiotic synthesis whereas none was available in the magnesium-fortified medium.

DISCUSSION

The use of medium H for the production of pneumocandins A_0 and B_0 provides several advantages that were not afforded by the fermentation media used previously [21]. Synthesis begins early in the fermentation; the products are stable; and the titers achieved are at least 4-fold higher than the values obtained earlier for pneumocandin A_0. The medium has been used successfully in large stirred fermentors, and a facile procedure for the recovery of these and other pneumocandin species has been developed [22]. Because all the components of the medium are soluble, antibiotic-producing cells may be easily washed free of exogenous nutrients for the preparation of respiring-cell suspensions that efficiently incorporate labeled precursors for biosynthetic studies [1,2].

Pneumocandin production in medium H does not follow the typical pattern of a trophophase–idiophase fermentation. Although unusual, this situation has been reported for other fungal fermentations. For example, the formation of enniatin in liquid surface cultures of *Fusarium sambucinum* was converted from a phenomenon limited to the idiophase to one occurring both during and after growth by substituting lactose for glucose as the major source of carbon in the medium [4]. Fermentations of this sort serve to emphasize the assertion of Aharonowitz and Demain that "metabolites are not 'secondary' because they are produced after growth; they are 'secondary' because they are not *essential* for growth" [3]. Among the carbon sources we tested, mannitol was advantageous in that it permitted the early onset of pneumocandin production, i.e., production during the growth of the culture. The underlying reason for this is not clear. Mannitol is a storage carbohydrate used during spore germination in many fungi [14]. Of the compounds examined, mannitol may have minimized catabolite repression most effectively. Alternatively as a substrate for mannitol dehydrogenase [13], its metabolism may have generated the levels of NADPH required for antibiotic synthesis. In any event, the pneumocandin fermentation is reminiscent of that developed for the formation of clavine alkaloids by *Claviceps* spp., which, like *Z. arboricola*, are plant-inhabiting fungi [25]. In both fermentations, the best yields were obtained by using relatively high concentrations of mannitol (i.e., 10%), although for alkaloid synthesis, a small portion of the polyol could be replaced with glucose. The substitution of glucose, even at low levels, for mannitol in the pneumocandin fermentations depressed synthesis (unpublished results).

The formation of clavine alkaloids, however, is inhibited by phosphate [25]. In the development of the *Z. arboricola* fermentation media, not only did KH_2PO_4 and $(NH_4)_2SO_4$ emerge as important factors (medium E), but the subsequent optimization experiment leading to media F and G called for an increase in the level of KH_2PO_4. The role of these components may be appreciated by recalling that the apparent degradation of pneumocandin which occurred in medium A or D (Fig. 1) was correlated with an increase in pH to neutrality and above. Chemical stability studies underway during the course of this fermentation development work showed that pneumocandin is subject to a base-catalyzed inactivation that results from splitting the hemiaminal linkage between the substituted proline and dihydroxyornithine residues [12,22]. Thus, the positive impact of KH_2PO_4 and $(NH_4)_2SO_4$ on the fermentation is likely to be due, in large part, to their capacity to counteract an increase in the pH of the culture. During the development of medium D, we attempted to buffer at pH 5.5 with various organic acids, each at a concentration of 0.1 M (unpublished data). Citrate proved satisfactory for pneumocandin synthesis, neither increasing nor decreasing production. Succinate and oxalate reduced overall titers somewhat, and acetate inhibited growth. Since citrate offered no clear advantage, we continued to utilize phosphate as the primary buffering component. Subsequently, we also found that 60 mM MES (2-(*N*-morpholino)ethanesulfonic acid) buffer could be substituted for KH_2PO_4 in medium H without affecting titer. The results with citrate and MES suggest that phosphate, per se, is neither stimulatory nor inhibitory for pneumocandin synthesis.

Several complex nitrogen sources were evaluated in this fermentation, and the most satisfactory was NZ-Amine (type E). According to information provided by the manufacturer, this peptone is rich in glutamic acid, a fact

that might account for its performance since glutamic acid is a precursor for three of the six amino acid residues in pneumocandin A_0 [2].

The production of pneumocandins in high titer is favored when growth is limited by magnesium. Magnesium has not often been encountered as an effector of secondary metabolism in fungi [8,15,26], but an example of magnesium regulation has been found [24]. In the present case, excess magnesium ions limit titer by stimulating the conversion of the available mannitol to biomass leaving no energy source to support antibiotic formation after growth has ended.

Other media supporting the production of pneumocandins are described elsewhere [16].

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