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Biosynthesis of Candicidin by Phosphate-Limited Resting Cells of *Streptomyces griseus*

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Summary. A phosphate-limited resting cell system of *Streptomyces griseus* in a synthetic medium has been developed in which biosynthesis of the polyene macrolide, candicidin, is linear for at least 36 h without cell growth. Glucose and to a lesser degree sucrose, but not lactose, support antibiotic synthesis. Glucose is utilized at a constant rate for antibiotic synthesis without affecting mycelial dry weight. Acetate and propionate, the building units of the macrolide aglycone, stimulate candicidin biosynthesis in cultures supplemented with glucose but do not support its synthesis in the absence of glucose. Maximal stimulation of candicidin biosynthesis was produced by 40 mM propionate or 250 mM acetate. The biosynthetic intermediate, methyl malonate, and the analog, 1-propanol, were more stimulatory than propionate at the same concentration.

Candicidin is a polyene macrolide antifungal antibiotic produced by *Streptomyces griseus,* having an aromatic moiety (p-aminoacetophenone) and the rare aminosugar, mycosamine (3-amino-3,6 dideoxy D-mannopyranose),attached to the macrolide ring (Waksman et al., 1965). The macrolide aglycone appears to be synthesized by condensation of acetate and propionate units through the polyketide pathway. The condensation of acetate and propionate units as their activated derivatives, acetyl-CoA and propionyl-CoA, is a process similar to but different from fatty acid biosynthesis (Martin & McDaniel, 1975c).

Metabolic factors controlling the biosynthesis of several polyene macrolide antibiotics have been studied (Martin & McDaniel, 1974; Liu et al., 1975). Previous studies were carried out in long term batch experiments in which factors affecting cell growth (RNA or protein synthesis) also indirectly

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influence antibiotic synthesis. We have developed a phosphate-limited resting cell system to study the factors which differentially influence mycelial growth and antibiotic production. This system is advantageous in that the effect on growth can be selectively eliminated.

Biogenetically, the macrolides have been classified as homogeneous (simple), which are synthesized from one single building block, either acetate or propionate, or heterogeneous (compound) derived from both acetate and propionate (Corcoran & Chick, 1966). Incorporation of acetate into candicidin was previously reported (Liu et al., 1972). Propionate is also effectively incorporated into candicidin (Martin & Liras,submitted).Rafalsky & Raczynska-Bojanowska (1972) suggested that the formation of propionate by the action of the malonyl-CoA oxalacetate transcarboxylase is a rate limiting step in the biosynthesis of macrolides. However, stimulation of candicidin biosynthesis by acetate and propionate has not been reported. Using a phosphate-limited resting cell system we have studied the precursor effect of acetate, propionate and their analogs as well as the effect of the presumed intermediates, malonate and methyl malonate, on the biosynthesis of candicidin.

MATERIALS AND METHODS

Organism and Growth Conditions. *Streptomyces griseus* IMRU 3570 was grown in a glucose-soya peptone medium as described previously (Martin & McDaniel, 1975b). In this medium cells start producing candicidin after about 18 h of growth.

Resting Cell System. Candicidin-producing cells grown in glucose-soya peptone medium for 20 h were collected, washed twice with sterile saline and resuspended at 5 mg of dry cell weight/ml in a synthetic medium containing glucose 50 mg/ml, L-asparagine 10 mg/ml, $ZnSO_4 \cdot 7H_2O$ 0.02 mg/ml, $MgSO_4 \cdot 7H_2O$ 0.2 mg/ml, $FeSO_4 \cdot 7H_2O$ 0.02 mg/ml, and $CuSO_4 \cdot 5H_2O$ 0.02 mg/ml. Unless otherwise indicated, no phosphate was added to the medium. In phosphate-supplemented media, a growth-limiting 5×10^{-4} M (0.087 mg/ml) concentration of K_2HPO_4 was used.

Five ml of the cell suspension were incubated in 50 ml triple deep-baffled flasks (Bellco Glass, Inc.) at 250 rpm on a rotary Psycrotherm incubator-shaker (New Brunswick Scientific Co.). For candicidin determination, 250 µl samples were removed in duplicate and extracted with 5 ml of chloroform-methanol. Candicidin was determined spectrophotometrically as described before (Martin & McDaniel, 1975b). Candicidin was identified by thin-layer chromatography on Silicagel G plates using the solvent system chloroform-methanol-20 % ammonium hydroxide 2:2:1 (lower phase) and detected by spectrodensitometry (Martin & McDaniel, 1975a).

Study of Possible Precursors for Effect on Candicidin Synthesis. Glucose in the medium was substituted for by sucrose, lactose, acetate, propionate, malonate or methyl malonate at concentrations of 10 to 50 mg/ml. In supplementation studies, citrate, acetate, propionate, malonate or their analogs were added to the glucose medium at final concentrations of 0.1 to 20 mg/ml as sodium salts at pH 7.0.

Materials. (2,2) Dimethyl malonic acid, methyl malonic acid, malonate dimethyl ester and 1,3-propanediol were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Sodium propionate, sodium acetate, 1-propanol, isopropanol, sodium butyrate and 1-butanol were all of Reagent quality.

RESULTS

Candicidin Biosynthesis by Resting Cells of Streptomyces Griseus. S.griseus cells collected at the onset of antibiotic formation in batch culture produced candicidin linearly for at least 36 h after resuspension in the synthetic medium. A constant rate of approximately 16 µg/ml/h (3.2 µg of candicidin/mg of mycelial dry weight/h) was maintained during the period (Fig.1). The specific candicidin production rate was dependent upon the cell concentration in the resting cell system. A dry weight of 5 mg/ml was found to be optimal for antibiotic formation. Occasionally a lag period (up to 4 h) was observed before the cells started to produce antibiotic. The pH of the culture increased from 6.5 at the time of suspension to 7.8 during the linear pro-

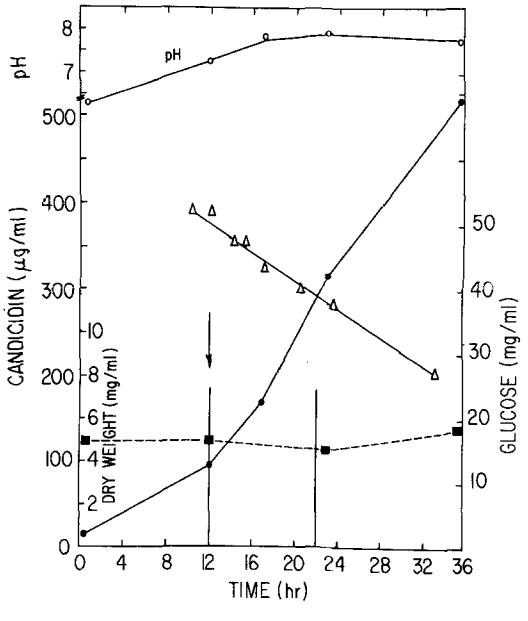

Fig.1
Production of candicidin by phosphate-limited resting cells of *S. griseus* in a synthetic medium. • candicidin; o pH; ■ dry weight; Δ glucose. Additions of precursors were made at the time indicated by the arrow

duction period. Glucose was utilized linearly at a rate of 1.2 mg/ml/h. The dry weight of the cell suspension increased slightly following cell resuspension if supplemented with 5×10^{-4} M phosphate but remained constant during the phase of linear production of the antibiotic. In synthetic medium without phosphate, there was no change in cell dry weight (Fig.1).

Utilization of Glucose and Disaccharides for Candicidin Synthesis. We have described previously (Martin & McDaniel, 1974) that slow-feeding of glucose results in increased synthesis of the polyene macrolides candidin and candihexin, presumably due to bypassing of catabolite repression (Magasanik, 1961). Candicidin synthesis by phosphate-limited cells requires glucose in the medium (Fig.2) as source of intermediates and of reduced cofactor NADPH for antibiotic synthesis. The small amount of candicidin produced in the absence of glucose is due to the intracellular pool of sugar carried over from the growth medium. When a low glucose concentration was used, the initial rate of antibiotic synthesis was similar to that in a medium with high glucose levels but antibiotic synthesis ceased at 24 h. A similar result had

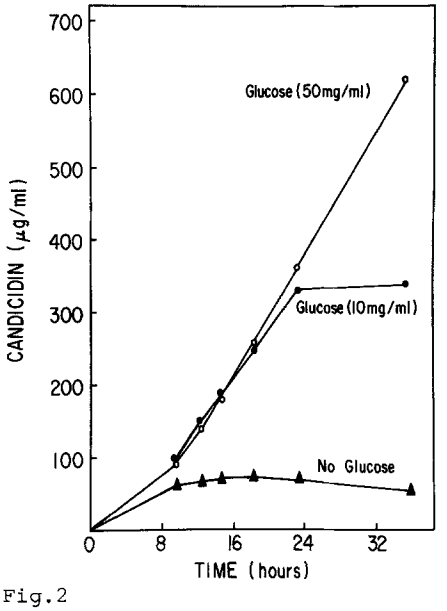

Fig.2

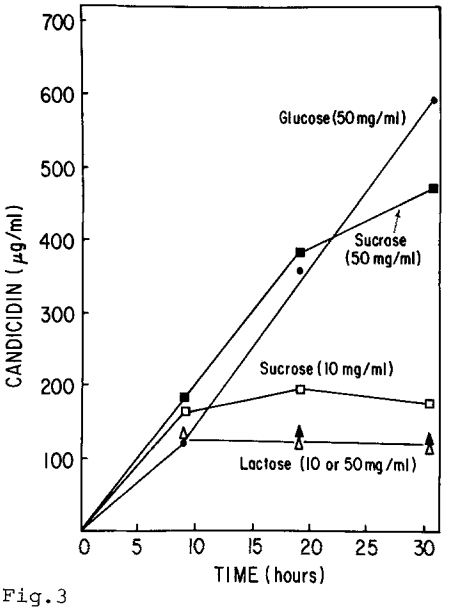

Fig.3

Fig.2
Production of candicidin by phosphate-limited resting cells of *S.griseus* at different levels of glucose

Fig.3
Production of candicidin by phosphate-limited resting cells of *S.griseus* using glucose and disaccharides as carbon sources

been found previously using a high-producing complex medium (Martin & McDaniel, 1975b).

Slowly-utilizable disaccharides have been used as a way of avoiding catabolite repression in the production of several antibiotics (Demain, 1972). The disaccharide lactose was a very poor substrate for candicidin synthesis (Fig.3) while antibiotic production with sucrose was almost as good as with glucose.

Short Chain Fatty Acids as Substrates for Candicidin Biosynthesis. Acetate and propionate as their biologically active units, acetyl-CoA and propionyl-CoA, and their carboxylated derivatives, malonyl-CoA and methylmalonyl-CoA, are the building blocks used in the head to tail condensation leading to the formation of the macrolide antibiotics. Using the phosphate-limited cell system we found that acetate, propionate or malonate do not support candicidin synthesis in the absence of glucose even though they are biogenetically closer to the antibiotic (Table 1). However, when resting cells producing antibiotic in glucose were supplemented with acetate, propionate or malonate, there was a stimulation of the rate of candicidin synthesis by propionate and 250 mM acetate but not by malonate (Table 2). Sodium mevalonate failed to support or stimulate candicidin synthesis in the resting cell system.

Citrate is a positive effector of the acetyl-CoA carboxylase of several organisms (see review by Volpe & Vagelos, 1973). Since acetyl-CoA carboxylase in the first enzyme involved in the

Table 1
Candicidin production by phosphate-limited cells of *S.griseus* from glucose, acetate, propionate and malonate

Candicidin (µg/ml)[a]			
Additions	Time		
	9 h	19 h	31 h
None	123	124	104
Glucose (50 mg/ml)	118	362	596
Acetate (10 mg/ml or 50 mg/ml)	110	146	110
Propionate (10 mg/ml or 50 mg/ml)	86	86	78
Malonate (10 mg/ml or 50 mg/ml)	110	116	98

[a]Pre-grown candicidin-producing cells of *S.griseus* were collected, washed and suspended in the synthetic medium without phosphate as indicated in Materials and Methods, with acetate, propionate, malonate or glucose as carbon source. Values of candicidin concentrations are averages of four determinations.

Table 2
Effect of acetate, propionate and malonate on candicidin biosynthesis by phosphate-limited resting cells of *S.griseus*

Amount			Candicidin Production[a] µg/ml	
Addition	mg/ml	(mM)	15 h[b]	48 h
None	-		279	553
Acetate	4	(48)	283	553
Acetate	20	(244)	283	612
Propionate	4	(41)	283	671
Propionate	20	(208)	283	585
Malonate	4	(27)	279	549
Malonate	20	(135)	283	553

[a]Average of four determinations. [b]Samples before additions were made. A phosphate-limited resting cell system was preincubated overnight and acetate, propionate and malonate were added at 15 h after starting incubation.

branching of acetyl-CoA for polyketide biosynthesis, a regulatory role of citrate on candicidin biosynthesis seemed possible. Results (not shown) indicate the citrate in the presence of glucose is neither stimulatory nor inhibitory between 0 and 1 mg/ml. Higher concentrations of citrate were clearly inhibitory, apparently due to its metal-chelating properties.

Stimulation of Candicidin Biosynthesis by Propionate and Acetate. The effect of acetate and propionate on candicidin biosynthesis was

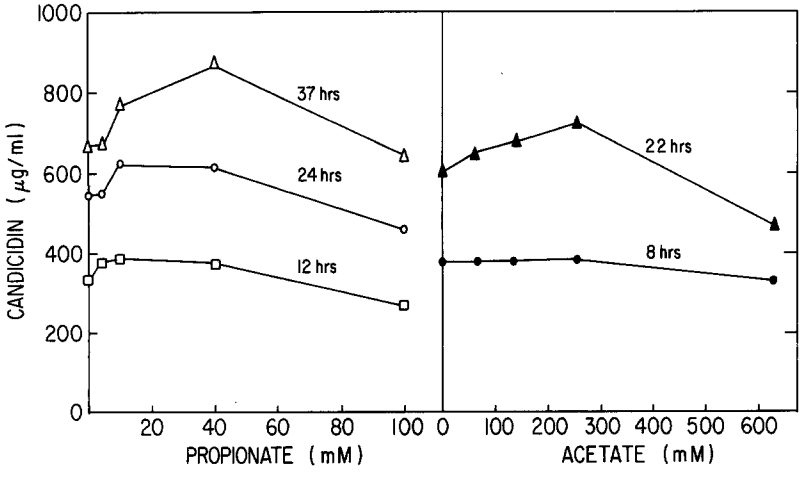

Fig.4
Stimulation by acetate and propionate of the biosynthesis of candicidin by phosphate-limited resting cells of *S.griseus*, supplemented with glucose (50 mg/ml)

studied in more detail (Fig.4). Maximal stimulation of candicidin biosynthesis by propionate was found at 40 mM (4 mg/ml) after 37 h of incubation. One hundred mM propionate was inhibitory. The stimulatory effect was dependent upon the length of incubation of the cells in the resting cell system with little stimulation occurring in the first 12 h of incubation. It appears that the intracellular pool of propionate carried over by the cells when suspended in the phosphate-limited system has to be reduced or depleted before the stimulatory action of exogenous propionate may take effect. Acetate also stimulated candicidin synthesis but the levels required for maximal stimulation were higher than with propionate. Increased synthesis was observed in flasks with 62, 125 and 250 mM sodium acetate, with maximal effect at 250 mM (20 mg/ml). Inhibition occurred at 625 mM acetate.

Stimulation of Candicidin Production by Acetate and Propionate Analogs. A number of acetate and propionate analogs and derivatives were tested for stimulation of candicidin synthesis in the resting cell system (Table 3). Isopropanol, (2,2)-dimethyl malonate, (1,3)-propanediol and malonate dimethylester were all as stimulatory as propionate or more so when used at the same concentration. Sodium butyrate was equally stimulatory; 1-butanol was not. The greatest stimulation was obtained with 1-propanol and

Table 3
Stimulation of candicidin production by acetate and propionate analogs

Analogs	Final concentration		Candicidin (µg/ml)		
	mg/ml	(mM)	8 h[a]	22 h[a]	Increase (8 - 22 h)
None (Control)	0	(0)	410	604	194
Sodium propionate	4	(41)	364	703	339
Sodium methyl malonate	4	(24)	413	805	392
Sodium (2,2)-dimethyl malonate	4	(23)	392	752	360
Malonate dimethyl ester	4	(30)	364	733	369
1-Propanol	4	(66)	402	825	423
Isopropanol	4	(66)	387	702	315
(1,3)-Propanediol	4	(52)	383	710	327
Sodium butyrate	4	(36)	385	766	381
1-Butanol	4	(54)	378	581	203

Duplicate flasks containing resting cells of *S.griseus* were supplemented with the analogs indicated and incubated as indicated in Materials and Methods. Values are averages of four determinations.
[a] Time after addition of the analogs.

sodium methyl malonate. Both increased by 100 % the amount of candicidin produced in the 14 h period following precursor addition. Ethanol at 85 and 170 mM was found to be slightly inhibitory for candicidin formation.

DISCUSSION

Washed cells of different microorganisms have been frequently utilized in biosynthetic studies. Washed cell systems are especially useful in the study of the biosynthesis on non-growth-associated antibiotics and other secondary metabolites since in such systems the effect of growth on antibiotic formation can be eliminated (Majumdar & Majumdar, 1971). The main disadvantage of the washed cell systems is the low antibiotic yield when cells are suspended in buffer or mineral salts.

Our results indicate that little or no candicidin is produced in the absence of glucose. The addition of glucose, however, supports growth. This problem was overcome by limiting growth by phosphate starvation.

Inorganic phosphate is a negative effector of polyene antibiotic biosynthesis (Liu et al., 1975). As shown in Figure 1 starvation for phosphate in the presence of glucose limits growth, favoring antibiotic biosynthesis.

Disaccharides, especially lactose, were poor substrates for antibiotic production, even though slowly-utilizable disaccharides are useful in many cases for bypassing catabolite repression (Demain, 1972). Apparently the cells lack or upon resuspension in lactose fail to induce the enzymes for lactose utilization.

The lack of candicidin biosynthesis in acetate, propionate or malonate, all of them direct biosynthetic precursors of candicidin, is not surprising, since in addition to acetate and propionate, reduced cofactor NADPH required for reduction of the highly oxidized polyketide chain is provided by glycolysis. Glucose is also a better source of intermediates for biosynthesis of the aromatic moiety of candicidin through the pentose phosphate cycle.

The stimulation of candicidin biosynthesis by acetate and propionate in the presence of glucose supports the conclusion that they are direct precursors, a conclusion arrived at by incorporation studies with labelled compounds. The fact that there is stimulation where the requirements for cofactors and for the aromatic moiety are met by glucose, indicates that under these conditions the intracellular concentrations of acetate and propionate are limiting for candicidin biosynthesis. The higher requirement for acetate than for propionate probably reflects the diversion of acetate into the Krebs cycle and intermediary metabolism of the cell. The requirement for relatively high

levels of both acetate and propionate is indicative of a precursor effect rather than a regulatory role on the biosynthetic enzymes for producing the antibiotic.

Malonate dimethyl ester was found to be stimulatory although sodium malonate was not. This effect may be due to better permeability of the cell to the dimethyl ester or to lower toxicity as compared with the disodium salt.

The large stimulation by methyl malonate confirms previous findings which indicated that it is an intermediate in the biosynthesis of propionate-derived polyene macrolides. Methyl malonate formed by carboxylation of propionate is an intermediate in the biosynthesis of the non-polyene macrolide, erythromycin (Corcoran & Chick, 1966), but its involvement in polyene macrolide biosynthesis has not been established before. Since labelled propionate is actively incorporated into candicidin (Martin & Liras, submitted), the high stimulatory effect of methyl malonate is indicative of its role as intermediate in candicidin biosynthesis.

The stimulatory effect of propionate analogs on candicidin biosynthesis appears to be related to their conversion into propionate or to their utilization in place of propionate in the biosynthetic pathway.

1-Propanol is a well known stimulatory precursor in the biosynthesis of erythromycin (Raczynska-Bojanowska et al., 1970). As in the case of erythromycin, the specific stimulation of candicidin formation by propanol but not by ethanol or butanol suggests that it is a precursor effect rather than a non-specific effect due to its solvent properties. Propanol isomers like isopropanol and analogs like propanediol are less stimulatory, presumably because they are more distantly related to propionate than is propanol itself.

The stimulatory effect of butyrate is interesting since butyl groups exist as side chains of the macrolide rings of some polyene antibiotics and 4-carbon units have been reported to be used in the biosynthesis of some macrolides (Omura & Nakagawa, 1975). Butyrate has been shown to stimulate the biosynthesis of the polyether antibiotics X537 and monensin, both of which contain ethyl group side chains (Day et al., 1973).

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