ENHANCED PRODUCTION OF THE MINOR COMPONENTS OF GLIDOBACTINS IN *POLYANGIUM BRACHYSPORUM*

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(Received for publication March 25, 1988)

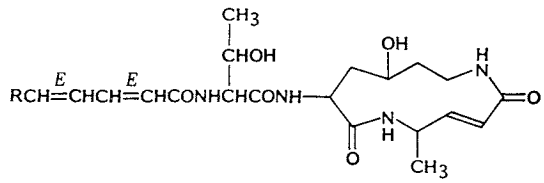
Polyangium brachysporum sp. nov. strain ATCC 53080 produces a novel type of antifungal and antitumor antibiotic complex, glidobactins A, B and C. Enhanced production of minor components, glidobactins B and C, was achieved by medium modification. The addition of soybean oil or corn oil, which are rich in unsaturated C_{18} fatty acids, to the fermentation medium led to an increased production of components B and C. Productivity of component C was selectively enhanced by the addition of oleic acid-rich oils, olive oil and Tween 80 (polyoxyethylene sorbitan mono-oleate). Furthermore, precursing palmitoleate, linoleate and oleate permitted the direct biosynthesis of components A, B and C, respectively. The fermentation with 3% addition of an appropriate oil at initial time provided an optimal production of component B or C.

Glidobactins A, B and C, produced by *Polyangium brachysporum* sp. nov. strain ATCC 53080, were isolated as new antifungal and antitumor antibiotics.[1] The structure determination revealed their unique peptide nucleus and unusual fatty acyl side chain moiety, as shown in Fig. 1, through chemical and enzymatic degradations and subsequent spectral analyses. The structural difference among glidobactins A, B and C was found only in their acyl side chain moiety.[2,3]

The therapeutic activity of the main component A against systemic fungal infections was marginal, but it showed potent *in vivo* antitumor activity against P388 leukemia in mice. Component C showed better therapeutic index than that of component A in the antitumor testing.

Directed biosyntheses of acyl side chains of lipopeptide antibiotics were reported in the polymyxin B[4] and A21978 C[5] fermentation by the addition of lower fatty acids, or their precursor amino acids. The modification of acyl side chains was supposed to be due to the alteration of biosynthetic pool size of odd number or branched chain primers. On the other hand, the oil addition led to enhanced production of polyether antibiotics[6,7] which were found to biosynthesize through the polyketide pathway. The polyketide intermediate was elongated in the similar fashion seen in the fatty acid biosynthesis. Therefore, predominant formation of an appropriate component and increased total production could be controlled in the glidobactin fermentation by feeding fatty acid precursors. In fact, medium improvement by precursing lipids led to the enhanced

Fig. 1. Chemical structures of glidobactins A, B and C.



Glidobactin A R=$CH_3(CH_2)_6$–
Glidobactin B R=$CH_3(CH_2)_4CH{=}CH(CH_2)_2$–
Glidobactin C R=$CH_3(CH_2)_8$–

production of minor glidobactins B and C.

This paper deals with studies on optimal fermentation production of each component of glidobactins, and the directed biosynthesis of glidobactins A, B and C.

Materials and Methods

Chemicals

Tweens 20, 40, 60 and 80 were purchased from Tokyo Chem. Ind. Co., Ltd. Polyethylene glycol 600 and Triton X-100 were from Wako Pure Chemical Industries, Ltd. Hardened oil, lard and tallow (Nippon Yushi Co., Ltd.), corn oil and olive oil (Sigma Chem. Co., Ltd.), soybean oil (Kimura Sangyo Co., Ltd.), Adekanol (Asahidenka Co., Ltd.) and others were all purchased from the respective commercial sources.

Microorganism

P. brachysporum sp. nov. ATCC 53080 was first isolated from the soil sample collected in Greece. The morphological variant R-4-2, selected as a highly producing strain by the monospore isolation, was used throughout the study.

Cultivation

The stock culture of the variant R-4-2 was propagated for 3 days at 28°C on a slant of the modified BENNETT's agar medium (rB) composed of soluble starch 0.5%, glucose 0.5%, meat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.6% (pH 7.0). A well grown slant was used to inoculate a vegetative medium containing the same ingredients without agar as the above medium. After incubation for 48 hours at 28°C on a rotary shaker (200 rpm), 5 ml of the growth was transferred into a 500-ml Erlenmeyer flask containing 100 ml of a production medium. The medium FR-10-1 composed of soluble starch 2%, beet molasses 1%, soybean meal 1% and $CaCO_3$ 0.5% (pH 7.2, before sterilization) was used as a basal production medium.

Determination of Productivity

The productivity of each component was determined by HPLC. The harvested broth (2 ml) was extracted by 3 ml of BuOH by 15-minute vigorous shaking and centrifuged for 20 minutes at 5,000 rpm. The solvent layer of the supernatant was applied to Waters QA-1 Analyzer with Radialpak C_{18}. When 78% MeOH was used as a mobile phase at flow rate 2 ml/minute the components A, B and C were eluted at the following retention times: 5.09, 6.48 and 10.13 minutes, respectively.

Results and Discussion

Effect of Various Nitrogen Sources on the Glidobactin Production

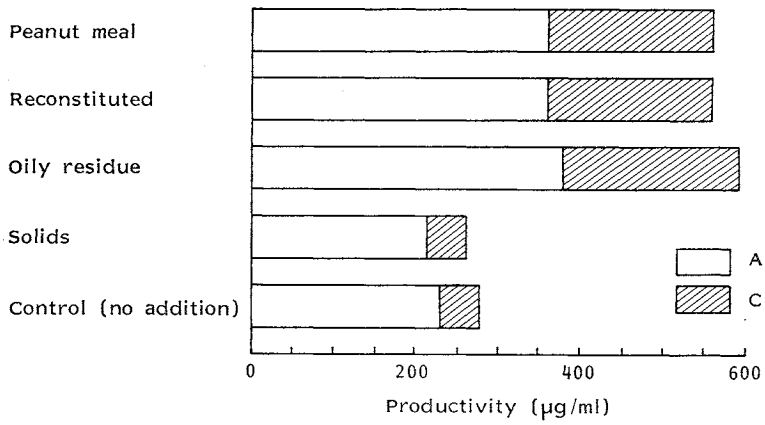
In the original production medium No. 132 containing corn starch 2.0%, soybean meal 3.0%, $CaCO_3$ 1.0% and $MgSO_4 \cdot 7H_2O$ 0.33%, *P. brachysporum* strain ATCC 53080 produced 50 to 100 μg/ml of glidobactin complex with the following ratio of the three components: 85% of component A, trace amount of component B and 15% of component C. The medium FR-10-1 was selected as a basal medium for this study after the random screening of production medium. The production of glidobactins in the medium FR-10-1 reached 200 to 300 μg/ml after 5-day fermentation. Soybean meal (1%) in the basal medium FR-10-1 was replaced for various ingredients (1%) in order to examine the effect on the productivity of each component. The culture broths after 5-day fermentation were analyzed by HPLC. When soybean meal was used as a nitrogen source, the production ratio of each component accounted for 85% of A, trace amount of B and 15% of C. This production ratio of each component was remarkably altered, when used peanut meal as a nitrogen source as shown in Table 1. The production ratio of component C reached 41% of the total production (139 μg/ml).

Table 1. Effect of various nitrogen sources on the glidobactin production.

Additive (1%)	Total production (μg/ml)	Component (%)		
		A	B	C
Soybean meal	230	85	Trace	15
Fish meal	160	84	Trace	15
Pharmamedia	158	74	7	19
Peanut meal	139	59	0	41
Linseed meal	60	86	0	14
Corn meal	16	92	4	0
Corn steep liquor	8	100	0	0
Distiller's solubles	7	100	0	0

Medium: FR-10-1 without soybean meal. Total productivity was determined on day 5.

Fig. 2. Effect of peanut meal and its ether extract on productivity.



Medium FR-10-1 with 1% of the respective additive were used. Productivity was determined on day 5.

Furthermore, increased production of component B (10 μg/ml, 7% of the total glidobactins) was observed, when Pharmamedia was used as a nitrogen source.

Effect of Peanut Meal and its Fractions on Productivity

Since the alteration of the production ratio as described above were considered to be depended on the lipid component of materials used as the nitrogen source from the structural difference of each component, the effect of peanut meal and its fractions on the glidobactin production was examined. Peanut meal (10 g) was extracted twice with 500 ml of diethyl ether. After separation of solid material with filtration, the ether fraction was concentrated *in vacuo* to obtain 3.6 g of oily material. The solid fraction was desiccated under reduced pressure and used as a reference additive.

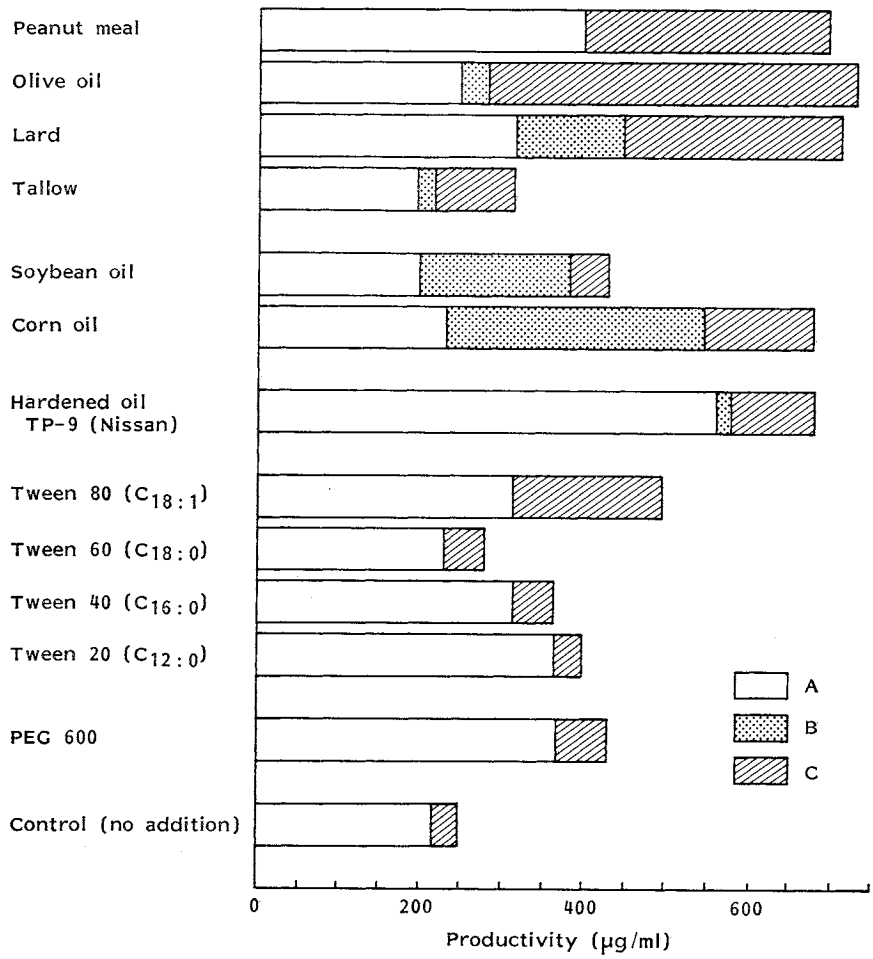
The medium FR-10-1 was chosen as the basal medium because of its high productivity as described above. The oily residue, solid fraction and reconstituted peanut meal, which were prepared by simply mixing the oily residue and the solid fraction with the original weight base, were added to the basal medium at the beginning of fermentation. Total productivity and the ratio of each component were examined comparatively with those of raw peanut meal. The addition of the oily residue, the raw and reconstituted peanut meal stimulated similarly the total productivity and percentage of component C, while addition of the solid fraction affected neither as shown in Fig. 2. The results in-

dicate that some components of the oily residue of peanut meal can be functioned as the precursors or as regulators of the glidobactin biosynthesis.

Effect of Different Oils and Surfactants on Productivity

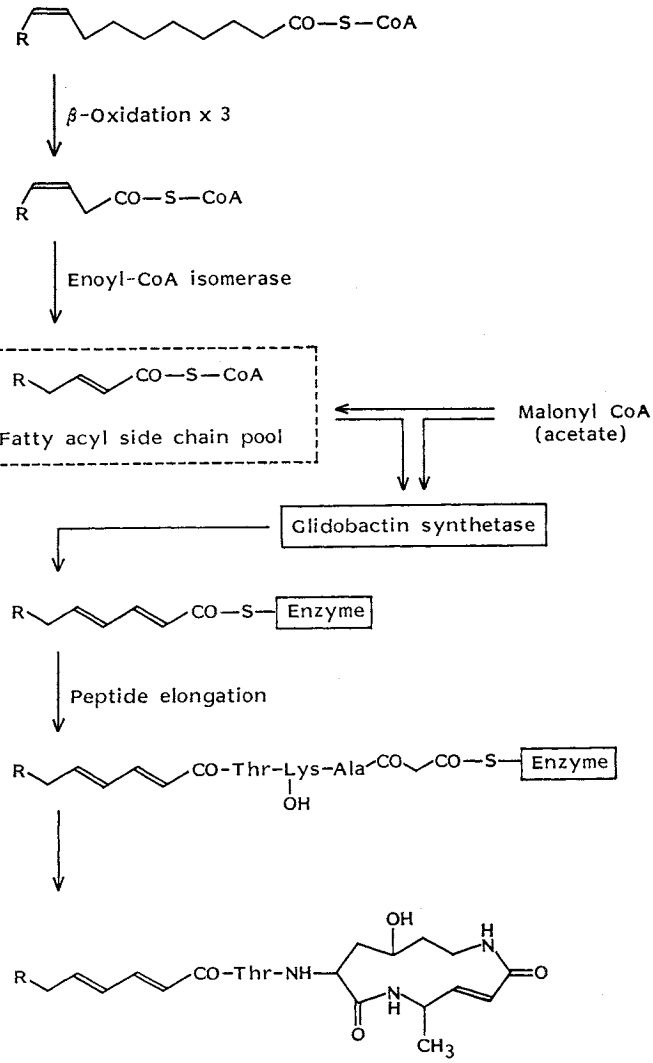
The ether-extractable oily residue from peanut meal was supposed to contain mainly lipids. The effect of various oils on the glidobactin production was examined. The 1% supplement of olive oil, corn oil or tallow to the medium FR-10-1 was enhanced the production of component C (90~450 μg/ml). The addition of soybean oil and corn oil permitted the increased production of components B (170~320 μg/ml) and C (70~130 μg/ml). However, the addition of hardened oil stimulated only the total production of glidobactins without alteration of component ratio (Fig. 3). These results indicated that the different oils functioned as precursors of glidobactins rather than as a regulatory factor of membrane functions, as observed in the neomycin formation and glucosyltransferase secretion.[8,9] This consideration was further supported the supplement study using various surfactants, polyoxyethylene derivatives, Triton X-100 and Adekanol. While the addition of Triton X-100 and Adekanol at sub-growth inhibitory concentrations to the medium FR-10-1 depressed the productivity

Fig. 3. Effect of various oils and surface active agents on productivity.



Medium FR-10-1 with 1% each additive was used. Productivity was determined on day 5.

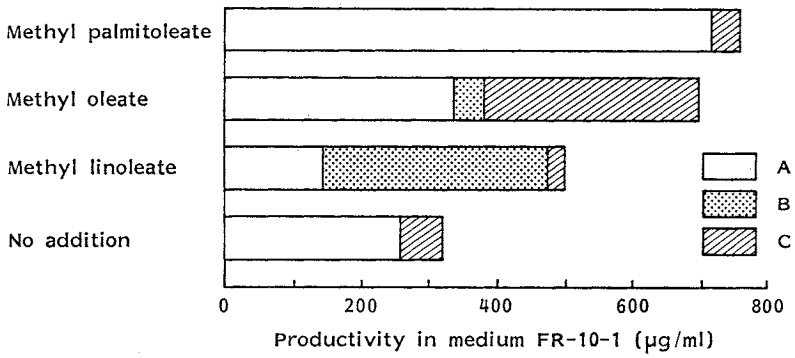
Fig. 4. Hypothetical biosynthetic pathway of the glidobactin side chain.



without any change of the ratio of each component (data not shown), all of the 1% addition of polyoxyethylene derivatives stimulated the total productivity of glidobactins. Among the polyoxyethylene sorbitan derivatives, only Tween 80 (containing primarily oleic acid) markedly enhanced the production of component C (190 μg/ml), as shown in Fig. 3.

The oils which enhanced the productivity of components B and C were found to contain linoleic acid ($C_{18:2}$) and oleic acid ($C_{18:1}$) as the main ingredients, and the degree of enhancement seemed to be proportional to their contents. Furthermore, the unsaturated bond between C-12 and C-13 positions in linoleic acid could be deduced to retain in the fatty acyl side chain moiety of component B. A hypothetical biosynthetic pathway of the side chain (Fig. 4) was partially clarified by precursing an appropriate fatty acid to the medium. Addition of methyl palmitoleate ($C_{16:1}$), methyl linolate ($C_{18:2}$) and methyl oleate ($C_{18:1}$) permitted the directed biosynthesis of glidobactins A, B and C, respectively (Fig. 5). Formation of component B (5%) was observed, when methyl oleate was added. This

Fig. 5. Effect of unsaturated fatty acids on productivity.



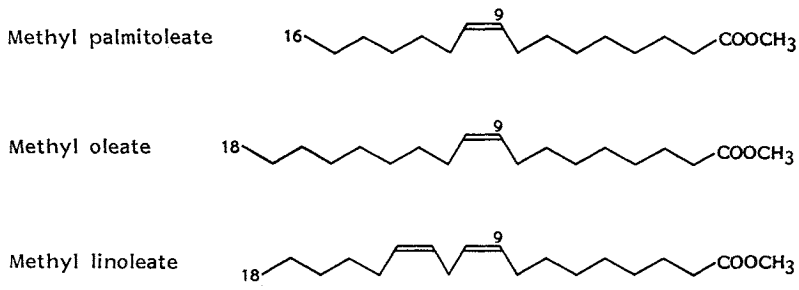


Table 2. Effect of the additive concentration on productivity.

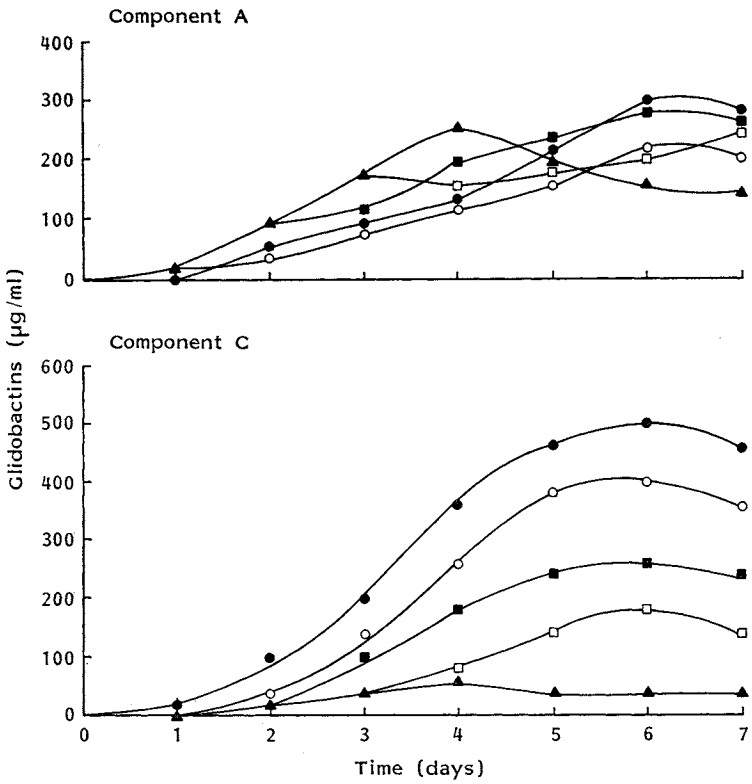
Additive	Conc (%)	Total production (μg/ml)	Component (%)		
			A	B	C
Soybean oil	0.3	494	62	19	19
	1	428	43	36	21
	3	552	20	56	23
	10	508	19	63	19
Olive oil	0.3	394	55	2	43
	1	566	39	2	59
	3	640	19	3	78
	10	439	25	8	67
Tween 80	0.3	311	72	0	28
	1	452	67	0	33
	3	477	49	0	51
	10	460	48	0	52
Control	—	304	85	0	15

Each oil was initially added to the medium FR-10-1.
The data shows productivity on day 5.

phenomenon would be due to the fact that the commercial preparation of methyl oleate was 70% of purity, and some amounts of methyl linoleate might be contained. Although the directed biosynthesis of fatty acyl side chains in several types of antibiotics was reported,[4,5,10,11] our observation would be the first example that such higher fatty acids could be incorporated into the acyl moiety as the nearly intact form through the β-oxidation pathway.

Fig. 6. Time cource of the glidobactin production with addition of olive oil.

● Oil added initially, ○ oil added on day 1, ■ oil added on day 2, □ oil added on day 3, ▲ control (no addition).



Basal medium (FR-10-1): Soluble starch 2%, beet molasses 1%, soybean meal 1%, $CaCO_3$ 0.5%.

Optimal Condition for Selective Production of Components B and C

Effect of concentration of oils was examined to determine optimal conditions for selective accumulation of glidobactins B and C. As shown in Table 2, 3% addition of olive oil to the basal medium FR-10-1 provided an optimal production of glidobactin C. Furthermore, initial addition of the oil was found to be the most effective to increase the production of component C as shown in Fig. 6. The later addition of oil resulted in the reduced accumulation of component C. Repression of the component A biosynthesis was observed when olive oil was added before day 4 as compared with the control (without the addition of oil).

Similarly, 3% addition of corn oil to the medium at the beginning of fermentation was found to be suitable for the specifically enhanced production of component B.

These results suggested that the *de novo* biosynthesis of precursor fatty acids of glidobactin might be completed before day 4 and that the repression of their incorporation might have occurred in competition with the salvaged precursors derived from the added oils. Further studies on glidobactin biosynthesis will provide a novel mechanism of the regulation of fatty acyl side chain of the antibiotic, and artificial production of new glidobactin analogs.

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