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Improved Production of Bioactive Glucosylmannosyl-Glycerolipid by Sponge-Associated *Microbacterium* Species

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Abstract: The marine *Microbacterium* species HP2 (DSM 12583), isolated from the sponge *Halichondria panicea*, is able to produce a glucosylmannosyl-glycerolipid when grown on a complex medium with glucose. Optimizing the carbon sources in shake flask experiments has shown that glycerol affords the highest specific glycoglycerolipid production. The product yield approached 300 mg/L or 25 mg/g biomass upon scaling up in a 40-L bioreactor volume. The native diglycosyl-glycerolipid GGL.2 strongly inhibited growth of the tumor cell lines HM02 and Hep G2 (50% inhibition at 0.4 to 3 μg/mL), while the related deacylated compound (GG.2) showed a potent anti-tumor-promoting activity.

Key words: marine bacterium from sponge, glycoglycerolipid, glycoglycerol backbone, bioreactor cultivation, antitumor and anti-tumor-promoting activity.

INTRODUCTION

Marine invertebrates are rich sources of new bioactive compounds (Li et al., 1994; Miyaoka et al., 1998; Constantino et al., 2000; Faulkner, 2001, 2000). Microorganisms are often associated with these and are considered to be of particular importance because metabolites previously thought to arise from the invertebrates may be biosynthesized by their endobionts (Stierli et al., 1988; Bewley and Faulkner, 1998). As a result of this, marine natural product research has focused on marine bacteria and fungi, which can be cultivated more easily than the marine invertebrates.

Examples of fungi isolated from sponges are *Exophilia pisciphila* (Doshida et al., 1996), *Microsphaeropsis olivacea* (Yu et al., 1996), *Trichoderma longibrachiatum* (Sperry et al., 1998), and *Microspaeropsis* sp. and *Coniothyrium* sp. (Höller et al., 1999), which produce new metabolites with interesting bioactivities. An example of a bacterial metabolite is the novel cytotoxic macrolide produced from *Micromonospora* sp., originally isolated from a sponge collected from the Indian Ocean near the coast of Mozambique (Cañedo et al., 2000; Fernández-Chimeno et al., 2000). Another *Micromonospora* sp. strain (origin: sponge *Clathrina coriacea*) was reported to produce new indo-

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locarbazole alkaloids (Cañedo Hernández et al., 2000). Unfortunately, no reference was made to a comparison of natural products from the different systems of the host and inhabitant.

Recently we cultivated *Microbacterium* sp., isolated from the Mediterranean sponge *Halichondria panicea*, in a glucose-rich seawater medium and found a new glucosyl-mannosyl-glycerolipid (Wicke et al., 2000). The goals of the present studies were to improve the specific production of this glycoglycerolipid, from 16 mg/g biomass to higher values, and to determine in detail its antitumor and anti-tumor-promoting activities.

Materials and Methods

Strain

Microbacterium sp. HP2 (DSM 12583) was isolated from a homogenate of the sponge *Halichondria panicea* (provided by W.E.G. Müller, University of Mainz, Germany), which had been collected from the Adriatic Sea in Croatia. Morphologic and physiologic studies, together with sequencing of the 16S ribosomal RNA, were used to classify the bacterium (German collection of microorganisms and cell cultures [DSMZ], Braunschweig; Wicke et al., 2000).

Cultivation of *Microbacterium* Species

Initial growth was achieved on marine broth. Slants were stored at 4°C and transferred at 2-month intervals. Liquid cultures (2000-ml Erlenmeyer flasks, 500-ml broth volume) were incubated at 30°C in a shaking incubator rotating at 100 rpm until cells reached the stationary phase (1 to 3 days).

The medium contained basic peptone/YE medium of (per liter) NaCl 23.0 g, KCl 0.75 g, $CaCl_2 \cdot 2\ H_2O$ 1.47 g, $MgCl_2 \cdot 6\ H_2O$ 5.08 g, $MgSO_4 \cdot 7\ H_2O$ 6.16 g, NH_4Cl 5 g, peptone 3.5 g, yeast extract 3.5 g, $Na_2HPO_4 \cdot 2\ H_2O$ 0.89 g; and either glucose (20 g) or glycerol (20 g). For batch cultivations on artificial seawater medium or marine broth with glucose, a 50-L bioreactor (Braun) was used under the following conditions: 40-L working volume, inoculation with 10% (v/v) of a 72-hour preculture; temperature at 30°C, stirring at 500 rpm, aeration rate of 0.4 v/vm, pH adjusted at 7.5. The bioreactor was equipped with a Rushton turbine, and the physiologic activity was followed by the use of a pO_2-electrode, and by oxygen and carbon dioxide gas analyzers (Oxygor and Unor).

General Experimental Procedures

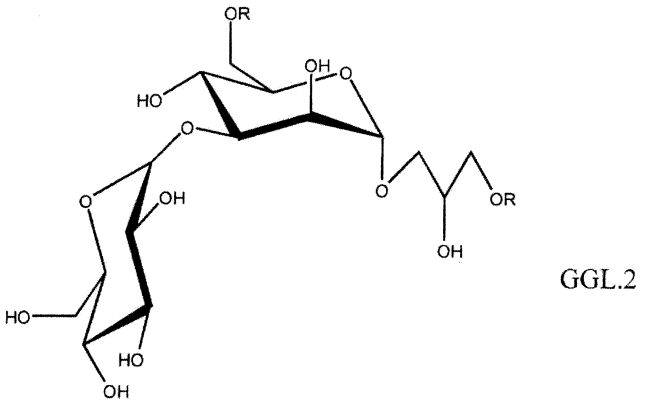
For biomass measurement, 10 ml of whole broth was centrifugated for 30 minutes at 13,000 rpm dried at 105°C, and biomass was determined gravimetrically. The glycerol concentration was measured enzymatically (test combination no. 148270, LaRoche). The glucose concentration was measured with the test combination no. 716251 (LaRoche).

To determine the content of glycoglycerolipid, 100 ml of culture broth was centrifuged for 30 minutes at 13,000 rpm. After freeze-drying, the cells were extracted with 100 ml CH_2Cl_2/CH_3OH (2:1, v/v) for 12 hours. Quantitative measurements were performed by thin-layer chromatography (TLC), densitometer CD 60 with Si gel 60 as stationary phase, $CHCl_3/CH_3OH/H_2O$ (65:15:2, v/v/v) as solvent system, and α-naphthol/sulfuric acid as detecting reagent at 580 nm.

The crude glycoglycerolipid was purified using liquid chromatography with a stationary phase of Si gel 60 and a solvent system consisting of various proportions of $CHCl_3/CH_3OH$ (from 90:10 to 50:50).

Alkaline hydrolysis of the native glycoglycerolipid was carried out as follows: 150-mg samples of both microbial products were incubated separately in 10 ml of boiling aqueous 0.5 N NaOH solution, which was subsequently cooled and neutralized with 0.5 N sulfuric acid. Addition of 20 ml methanol caused precipitation of Na_2SO_4, which was filtered off. The resulting free fatty acids were extracted 3 times with 20 ml methyl-*t*-butylether. The methanol of the aqueous phase was evaporated, and subsequently the residual aqueous phase was freeze-dried. The pure hydrolysis product, the diglycosylglycerol, was purified by subsequent chromatography over a cationic exchanger (DOWEX 50WX8) and an anionic exchanger (DOWEX 2X8; Serva).

The antitumor activity of the test compounds was determined in the human cancer cell lines HM02 (stomach adenocarcinoma) and Hep G2 (hepatocellular carcinoma), according to National Cancer Institute guidelines (Grever et al., 1992). Cells were grown in 96-well microtiter plates of RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 in air. After 24 hours of incubation, the test compounds (0.1 to 10 μg/ml) were added to the cells. Stock solutions of the test compounds were prepared in dimethylsulfoxide (DMSO). The final DMSO concentration in the medium was 0.1%. After 48 hours of incubation in the presence of the test drugs, the cells were fixed by addition of trichloroacetic acid, and the cell protein was as-



GGL.2

Figure 1. Glucosylmannosyl-glycerolipid (GGL.2) from *Microbacterium* sp. HP2. R indicates anteiso-C15:0, -C17:0, iso-C16:0. GG.2 has corresponding diglycosylglycerol backbone (without fatty acids) after alkaline hydrolysis.

sayed with sulforhodamine B (Skehan et al., 1990). For each compound tested the drug concentration causing 50% growth inhibition (GI_{50}) and drug concentration causing 100% (total) growth inhibition (TGI) were determined. The inhibition tests (anti-tumor-promoting activities), determined using a short term in vitro test for Epstein-Barr virus activation in Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), are described by Shirahashi et al. (1993).

Results and Discussion

Microbacterium Species HP1 (DSM 12583): Production of Glucosylmannosyl-Glycerolipid

Recent studies have shown that *Microbacterium* sp. HP2, isolated from the Mediterranean sponge *Halichondria panicea*, is able to synthesize the glucosylmannosyl-glycerolipid GGL.2 (Figure 1), with R_F value of 0.45 in TLC, and also some byproducts (Wicke et al., 2000). For cultivation, the authors used marine broth or artificial seawater supplemented with peptone-yeast extract and glucose, respectively.

Using 500-ml shake flask experiments the carbon source spectrum has now been extended. Considering the carbohydrate-glycerol backbone of the glycoglycerolipid, it seemed appropriate to use not only glucose but also mannose, glycerol, and mixtures of these. Compared to the above initial results, we found that use of glycerol as the carbon source improved the yield of biomass and glycoglycerolipid production considerably. As shown in Figure 2,

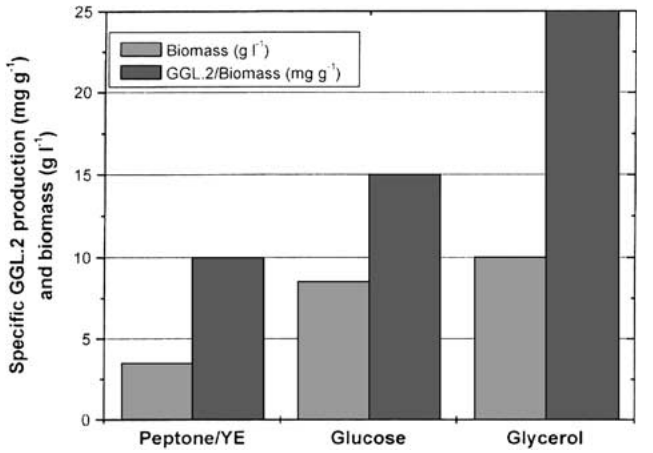


Figure 2. Influence of major carbon sources on biomass and the specific glucosylmannosyl-glycerolipid production of *Microbacterium* sp. HP2. Conditions: 500-ml cultures; artificial seawater. Peptone/YE (in total), 7 g/L; glucose, 20 g/L and 7 g/L peptone/YE; glycerol, 20 g/L and 7 g/L peptone/YE. Initial pH 7.5; 30°C; 100 rpm; cultivation time, 1 to 3 days.

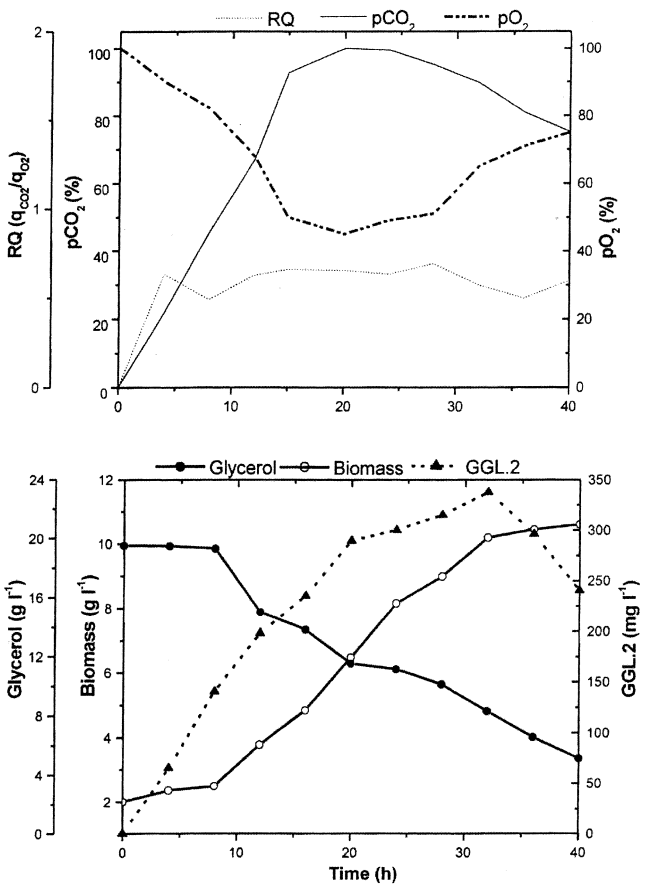


Figure 3. Batch (40-L) cultivation of *Microbacterium* sp. HP2. Conditions: 50-L bioreactor; artificial seawater; 20 g/L glycerol, 7.0 g/L of complex carbon sources (peptone-yeast extract); pH adjusted at 7.5; 30°C; 500 rpm; aeration rate, 0.4 v/vm.

Table 1. Activities of GGL.2 and GG.2 Against Selected Human Tumor Cell Lines

	GI_{50} [a] [μ ml^{-1}]		TGI[b] [μg ml^{-1}]	
Compound	HM02	Hep G2	HM02	Hep G2
GGL.2	0.38	2.7	>10[c]	>10[d]
GG.2	>10	>10	>10	>10

[a]Drug concentration causing 50% growth inhibition.
[b]Drug concentration causing 100% growth inhibition.
[c]77% growth inhibition at 10 μg ml^{-1}.
[d]70% growth inhibition at 10 μg ml^{-1}.

20 g/L glycerol and 7 g/L peptone-yeast extract yielded 10 g/L of biomass and a specific production of 25 mg/g biomass. The product was identical to that from the glucose cultivation.

Scaling up to a 40-L batch cultivation, which included pH adjustment at 7.5 and on-line monitoring of oxygen partial pressure and inlet/outlet gas content, confirmed the results of the shake flask experiments. Figure 3 presents typical data of the cultivation: while consuming glycerol *Microbacterium* sp. grew and reached 12 g/L biomass after 32 hours. The glycoglycerolipid formation was growth-associated, leading to approximately 300 mg/L or 25 mg/g biomass at this time. Later on, the product concentration decreased, probably because of enzymatic degradation of the glycosidic linkages. The physiologic activity indicated by the data on pO_2 and pCO_2 (calculated from Vol% CO_2 on the basis of outlet-air analysis), were in agreement with cell growth. The minimum pO_2 at 20 hours, and at the same time the maximum of the pCO_2 indicate the end of the exponential phase. Upon nutrient limitation (no detailed analysis data), a long transition phase occurred in which growth continued but at a reduced specific growth rate.

Finally, the fatty acids of the native glycoglycerolipid were successfully cleaved by alkaline hydrolysis to furnish the glucosylmannosyl-glycerol (GG.2).

The limited amount of sponge material did not allow comparable studies of the metabolite spectra of host and the associated bacterium to be carried out.

Bioactive Properties of Native Glycoglycerolipid and Its Hydrolysis Product

The natural product GGL.2 strongly inhibited growth of the tumor cell lines HM02 and Hep G2 (GI_{50}: 0.38 and 2.7 μg/ml, respectively), whereas the glycoglycerol GG.2 failed to inhibit tumor cell growth (Table 1). In addition, the native glycoglycerolipid GGL.2 and, particularly the free glycoglycerol GG.2, showed effective anti-tumor-promoting activities during inhibition of the TPA-induced activation of Epstein-Barr virus early antigen tests (Table 2).

Table 2. Anti-Tumor-Promoting Activity of GGL.2 and GG.2: Inhibition of TPA-Induced Activation of Epstein-Barr Virus Early Antigen

	% to control (% viability of Raji cells)[a]			
Compound	1000	500	100	10
GGL.2	17.5 (50)	62.5	88.2	100
GG.2	7.1 (60)	42.0	71.6	93.8

[a]Values are EBV-EA activation (%) in the presence of different concentrations of the test compound (mol ratio/TPA), relative to the control (100%). Activation was attained by treatment with 32 pmol TPA.

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