Antagonistic Effects of *Myxococcus xanthus* on Fungi: II.
Isolation and Characterization of Inhibitory Lipid Factors

BÖRJE NORÉN, Department of Microbiology, University of Lund, Sweden, and
GORAN ODHAM, Lipid Chemistry Laboratory, University of Göteborg, Sweden

**ABSTRACT**

The chemical composition of the lipophilic excretion of *Myxococcus xanthus* inhibitory to the germination of fungal spores and growth has been investigated. The inhibitory effect was attributed to a mixture of fungistically acting fatty acids and a component of antibiotic character. The fatty acid mixture has been fully characterized and found to constitute a mixture of saturated (68%) and unsaturated (32%) structures in the C13-C17 range. The major part is methyl-branched of the iso-type, with 13-methyl-tetradecanoic acid being the main component (33% of the total). The fungistic activity of the fatty acid mixture on spore germination is attributed to the structures with iso-configuration. The presence of unsaturation is of minor importance. Observed morphological changes of the spores and hyphae in the presence of iso-fatty acids suggest that they act on the plasma membrane.

**INTRODUCTION**

Myxobacteria are terrestrial organisms which are ubiquitous inhabitants of normal soil, bark, and decaying plant material (1). As described in a previous paper (2), *Myxococcus xanthus* secretes into the culture medium organic substances inhibitory to the germination of spores and the growth of fungi. The isolation and characterization of these excreted factors are important, as they possibly may be involved in the phenomenon known as soil mycostasis.

In an early stage of the investigation of the chemical background of these findings, it was found that the responsible components readily dissolved in organic solvents such as ethyl acetate, chloroform or benzene. Recently, Schroder and Reichenbach (3) reported an analysis of the fatty acids of vegetative cells and myxospores of *Stigmatella aurantiaca*. A saturated branched C15 acid (~35%) and a mono-unsaturated C16 acid (~20%) were identified as the main fatty acids. No effort was made to locate the position of the branch (assumed to be a methyl group) or the double bond.

This paper deals with extracellular material in the culture medium and not with extracts of cells.

**EXPERIMENTAL PROCEDURES**

The cultivation of *M. xanthus* has been described previously (2).

**Germination experiments:** Two wetted and sterilized strips (10 x 10 mm) of cellophane (PT 300) were placed on a sterile glass filter (Jena G1) in a small petri dish half filled with 3 ml test solution. The nutrient medium consisted of glucose (2.0 g), KNO3 (2.0 g), KH2PO4 (2.5 g), MgSO4·7H2O (1.26 g) and Tween 80 (0.1 ml) in distilled water (1000 ml). The preparations to be examined were added to the medium as methanol solutions. All media had a methanol concentration of 1.0% and pH 6.5. The strips were inoculated with conidia of *Fusarium roseum* (40,000 conidia per strip) from cultures grown for 10 days at 25°C. The strips were removed after 3 and 4 hr incubation at 25°C, mounted on slides, and treated with lactophenol to stain and kill the conidia. The proportions of germinated and nongerminated conidia were then determined in the microscope. On each strip at least 400 randomly chosen conidia were examined.

**Analytical gas chromatography (GC):** A 50 m long stainless steel capillary tube, ID 0.01 in. (Golay capillary column type R) coated with polypropylene glycol was used. A Perkin-Elmer model 900 gas chromatograph equipped with a standard capillary injector block, split no. 3, and flame ionization detectors was employed. Helium was used as carrier gas, and the gas flow was ca. 3 ml/min at room temperature.

**Preparative GC:** The column used had a length of 8 m and an internal diameter of 8 mm. It was filled with Versamid 900 (6%) on Gaschrom Z (80-100 mesh) and was initially kept at 275°C during 24 hr in a stream of nitrogen. The chromatography was performed at 190°C on a modified Aerograph (model A-700) gas chromatograph operated manually under isothermal conditions. The flow of helium carrier gas was 100 ml/min, and the vapors were condensed in standard Autoprep flasks filled with stainless steel turnings. The turnings were moistened with 0.5 ml chloroform and the flasks immersed in a cold bath at -80°C.
Mass spectrometry (MS): The GC-MS combination instrument described by Stallberg-Stenhagen et al. (4) was used. The temperature of the ion source was 200 C, and the electron energy 70 eV.

Catalytic hydrogenation: In a typical experiment, 4.5 mg of the unsaturated methyl ester mixture was dissolved in 2 ml n-heptane in a 25 ml flask. One milligram of Adam's catalyst (PtO2·H2O) was added and the flask filled with hydrogen, stoppered, and shaken for 15 min at 25 C. The suspension was centrifuged prior to analysis.

Oxidative degradation: The procedure was illustrated by the oxidative degradation of component XIV; 0.5 mg XIV was dissolved in 50 µl glacial acetic acid in a small test tube. One and eight-tenths milligrams finely pulverized potassium permanganate was added and the mixture heated for 15 min at 40 C. One-tenth milliliter of a solution of sulphur dioxide in water (10%) was added, followed by one drop of diluted sulphuric acid (5%). The mixture was heated on the waterbath until colorless. One-tenth milliliter dichloromethane was added; after shaking and subsequent centrifugation the aqueous layer was discarded and the organic phase washed with water. After evaporation the residue was esterified with diazomethane in ether. The reaction product was investigated on the combined GC-MS.

Preparation of branched chain reference material. Synthesis of 12-methyltridecanoic acid: This previously known acid, see e.g. (5), was prepared via a mixed anodic coupling (Kolbe electrosynthesis) of 4-methylpentanoic acid (Fluka AG, Bucks, Switzerland) and methyl hydrogen decan-1,10-dioate (6). There was obtained 13.5 g of the desired methyl ester of bp 136-140 C, 1 mm, from 64.0 g half ester and 34.4 g 4-methylpentanoic acid. The methyl ester was hydrolyzed and the free acid recrystallized from light petroleum (bp 60-85 C). Yield 10.7 g, mp 52.2-52.8 C of free acid.

13-Methyltetradecanoic acid: This previously known acid (7) was prepared from 12-methyltridecanoic acid by chain lengthening (Arndt-Eistert reaction). The diazoketone obtained from 5.0 g of 12-methyltridecanoic acid was rearranged in methanol in the presence of commercial silver oxide. The crude methyl 13-methyltetradecanoate (5.1 g) was chromatographed on 50 g silicic acid (Mallinckrodt, 100 mesh) with ether-light petroleum (bp 40-60 C) (1:50 v/v). Free acid (2.1 g) mp 49.7-50.3 C was obtained after hydrolysis and crystallization from light petroleum (bp 40-60 C).

RESULTS

Fractionation of the crude inhibitory material: After 4 days of incubation at 30 C, the cells (5.2 g dry weight) were removed from 3000 ml of culture solution by centrifugation. The solution was shaken immediately with five 200 ml portions of ethyl acetate (Analar grade), and the combined organic extracts evaporated to dryness at reduced pressure (25 C). One hundred thirty-five milligrams of brownish viscous residue with a strong odor characteristic of Myxobacteria was obtained. The extract was triturated with 25 ml benzene for 1 hr and the suspension centrifuged. The benzene soluble material weighed 94 mg (69.7%). This was

FIG. 1. Liquid chromatogram of the lipophilic excretion of M. xanthus on silicic acid. Tube contents are shown as a function of tube number. a benzene-ethyl acetate (3:1 v/v), b benzene-ethyl acetate (1:1 v/v), c ethyl acetate, d ethyl acetate-acetone (1:1 v/v), e acetone, f methanol. Three liters of culture medium.

FIG. 2. Relative number of germinating conidia of F. roseum after 3 hr incubation at 25 C. Control = 100. Supplement to F-solution: o lipophilic extract of cell free culture solution of M. xanthus, o natural fatty acid mixture,  a saturated moiety of natural fatty acid mixture,  unsaturated moiety of natural fatty acid mixture,  neutral fraction of the lipophilic extract.