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ISOLATION AND PURIFICATION OF CANADAPHORE, A SIDEROPHORE PRODUCED BY HELMINTHOSPORIUM CARBONUM

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A new siderophore was isolated and purified from the spent growth medium of the fungus Helminthosporium carbonum by solvent extraction and reverse phase high pressure liquid chromatography. This new molecule has been assigned the name Canadaphore. Canadaphore was detected in culture filtrates after 15 days of growth and production was maximal after growth of H.carbonum to maximal stationnary phase in modified Fries basal medium. Production of Canadaphore was completely suppressed when the organism was grown in medium supplemented with iron. Mass spectral analysis yielded a molecular mass of 680 Daltons for the iron-Canadaphore complex and 627 Daltons for the iron-free molecule. Spectroscopic analysis indicates that Canadaphore is a siderophore of the hydroxamate type.

Iron is essential for all living organisms with the exception of some Lactobacilli species (1,2). The importance of the metal stems from its key role in a variety of vital metabolic processes including DNA synthesis and electron transport. However, iron is relatively unavailable to microorganisms owing to its profound insolubility at neutral pH. Thus, in order to satisfy their iron requirements, a variety of aerobic and facultative anaerobic microbial species produce low molecular weight iron-chelating molecules collectively called siderophores(3). After synthesis, siderophores are secreted in the environment where they solubilize iron to subsequently return to the cell as ferric complexes.

In an agricultural ecosystem, the struggle for growth-essential iron is observed not only among microorganisms but also between microbes

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and their plant hosts (4-6). Hence, the production of siderophores provides pland pathogens with a unique and highly specialized way to compete with their host and is thus an important virulence factor (1,7). The fungus Helminthosporium carbonum has been recognised as a pathogen of maize for many years (8). This study reports on the isolation and purification of an as yet unidentified siderophore produced by H.carbonum and should provide a better understanding of host-parasite interactions in agricultural systems.

MATERIALS AND METHODS

Organism and growth conditions : Helminthosporium carbonum race 1 Ullstrup was used throughout this study. The strain was maintained on potato dextrose agar slants (Oxoid, England). The organism brought into culture for siderophore production was grown to maximal stationnary phase in modified Fries basal medium (8). Cultures were grown at room temperature under aerobic conditions.

Isolation and Purification of Canadaphore : H.carbonum was grown for 25 days in large acid-washed bottles containing 250 ml of modified Fries basal medium. The mycelia were removed by filtration and the culture filtrate mixed with $FeCl_3$. The orange filtrate was then saturated with $(NH_4)_2SO_4$ with stirring at room temperature for 2 h. The precipitate was removed by centrifugation and the supernatant was extracted with an equal volume of benzyl alcohol. The benzyl alcohol extract was then treated several times with 10 volumes of diethyl ether and 0.1 volume of deionized water. Canadaphore immediatly entered the aqueous phase which was collected and freeze-dried. The crude preparation was resuspended in water and Canadaphore was purified from the mixture by reverse phase high pressure liquid chromatography (HPLC) carried out on a Dupont Zorbax C18 column (25 cm by 4.6 mm inner diameter). The flow rate was kept constant at 1 ml/min and Canadaphore was eluted using a gradient of water and acetonitrile (0-100%). Materials eluted from the column were detected by their absorption at 240 nm or 430 nm.

Time course of production of Canadaphore : To examine the time course of production of Canadaphore, H.carbonum cultures were sampled every 5 days over a 30 day growth period. The culture filtrates were assayed for the presence of Canadaphore by adding an excess of $FeCl_3$ followed by measurements of visible absorption spectrum from 700 nm to 350 nm on a Hitachi Perkin Elmer double beam spectrophotometer. The culture filtrates were also assayed for the presence of Canadaphore using the Folin-Ciocalteu reagent method as described by Subramanian et al (9). The same experimental procedure was used to measure production of Canadaphore by H.carbonum grown in modified Fries basal medium supplemented with 2 uM iron.

Mass spectral analysis : Fast atom bombardment (FAB) spectrometry was performed on a VG Micromass ZAB1F mass spectrometer at room temperature using a neutral beam of xenon atoms from a saddle field discharge current of 14 uA. Samples were mixed with thioglycerol:glycerol (3:1 v/v) and the sample was deposited directly on the probe tip.

Chromatography : Thin layer chromatography (TLC) was performed on silica-coated glass plates using 100% methanol as the solvent system. Spots were detected visually or after spraying of the plates with ceric ammonium sulfate (2% ceric ammonium sulfate in 50% H_2SO_4) or a solution of $FeCl_3$ (1 mg/ml $FeCl_3$ in 0.1 M HCl).

Preparation of iron-free Canadaphore : Iron-free Canadaphore was prepared either by alkaline precipitation of the iron (3) or by purification of the siderophore without prior saturation with iron. In the latter method, progress in the purification process was followed by determining the reactivity of a small sample of each fraction with $FeCl_3$.

RESULTS AND DISCUSSION

Isolation and Purification of Canadaphore

When grown in modified Fries basal medium, H.carbonum produced an iron-binding molecule to which we have assigned the name Canadaphore. Canadaphore was isolated from the culture filtrates as described in the section Materials and Methods. Extraction of the spent growth medium with benzyl alcohol and water yielded a crude mixture of red brown hygroscopic powder. Canadaphore was purified from the mixture by reverse phase HPLC with monitoring at 430 nm. Canadaphore characteristically had a retention time of 11 minutes (Fig.1) under the gradient conditions used. Rechromatography of this fraction by HPLC yielded a single peak when a water-acetonitrile or a water-methanol gradient was used (data not shown). The purity of Canadaphore obtained in this fraction was confirmed by FAB spectrometry and TLC. Two other fractions possessing iron-binding activity were eluted shortly after the Canadaphore peak. The

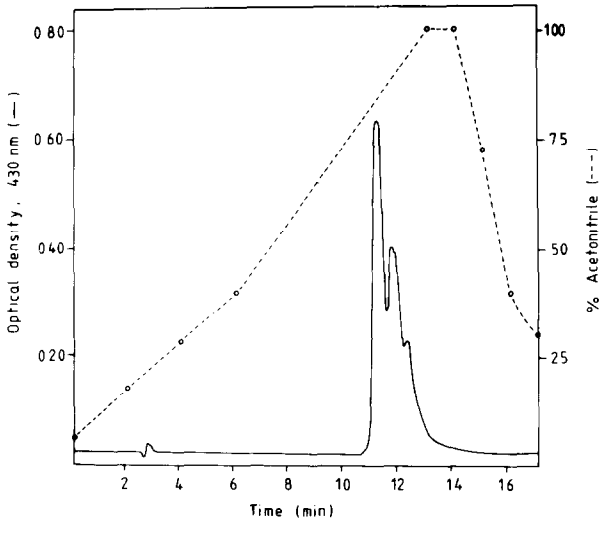

Figure 1. Purification of Canadaphore by reverse phase HPLC. The crude mixture obtained by solvent extraction of culture filtrates was lyophilized resuspended in water, and 100 uls were applied on a C18 column. Canadaphore was eluted using a water-acetonitrile gradient.

identity of the component(s) of these peaks was not investigated further in this study. They may be other siderophores produced by H.carbonum. The production of a few siderophores by the same organism has been reported previously (7,10).

Time course of production of Canadaphore.

The production of Canadaphore by H.carbonum was monitored as a function of time in modified Fries basal medium. The pattern of production obtained by measurements of the absorption of culture filtrates at 430 nm correlated with that obtained when Canadaphore production was measured using the Folin-Ciocalteu reagent method (Fig.2). The presence of Canadaphore in culture filtrates was first detected after 15 days of growth which indicates that Canadaphore synthesis starts during the active growth period of the organism and not after entry into stationnary phase. Siderophore production was maximal after 25 days of growth (data not shown). The presence of 2 uM iron in the medium completely suppressed the biosynthesis of Canadaphore (Fig.2), a response typical of siderophores in general (3,11).

Physical and Chemical properties of Canadaphore.

The iron-Canadaphore complex was very soluble in highly polar solvents such as water and methanol but was insoluble in less polar solvents such as

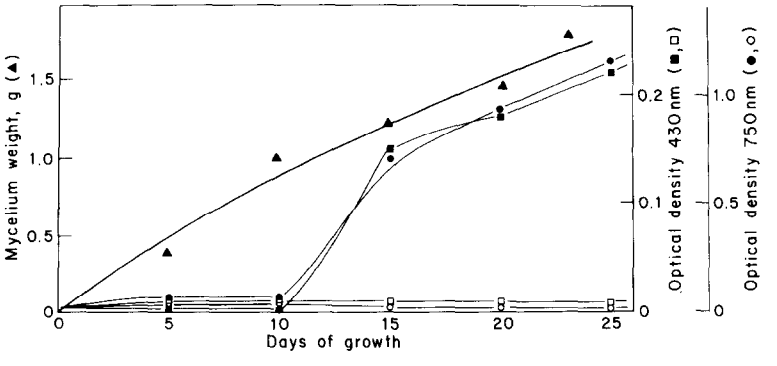

Figure 2. Time course of production of Canadaphore by H.carbonum. Culture filtrates of H.carbonum were obtained at regular time intervals and tested for the presence of Canadaphore by measurements of absorption at 430 nm (■) and by the Folin-Ciocalteu reagent method (●). The filtrates of H.carbonum grown in medium supplemented with 2 uM iron were assayed using the same procedures (open symbols). Growth of H.carbonum is shown as weight of mycelia (▲).

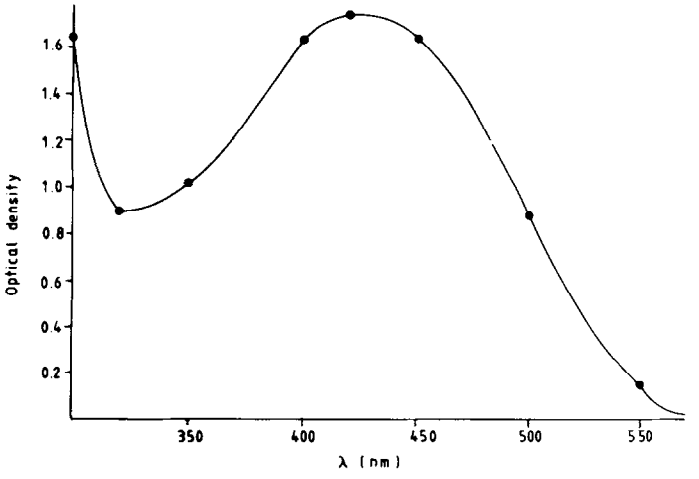

Figure 3. Visible absorption spectrum of Canadaphore in water. Pure Canadaphore obtained from HPLC was redissolved in water and used for spectroscopic analysis.

chloroform and diethyl ether. Mass spectral analysis yielded a molecular mass of 680 Daltons for the iron-Canadaphore complex and 627 Daltons for the iron-free siderophore. Solutions of ferric Canadaphore had a bright orange color and the complex had an absorption maximum at 430 nm ($E^{1\%}_{1\ cm}$=35.0) (Fig.3). This is typical of siderophores of the hydroxamate type which absorb maximally between 420 nm and 450 nm (3,11). Results obtained from TLC provided more evidence that Canadaphore belongs to the hydroxamate group of siderophores. Ferric Canadaphore had an R_f value of 0.38 when methanol was used as the solvent system and was readily detected as a bright orange spot. The iron-siderophore complex stained burgundy upon spraying of the plates with a $FeCl_3$ solution, a color reaction characteristic of hydroxamate-containing siderophores (12).

As expected from a molecule with iron-chelating properties, iron could be removed reversibly from Canadaphore under alkaline conditions where competition of the hydroxide ion caused precipitation of ferric hydroxide with resulting loss of the typical orange color of the complex. The maximal iron-binding capacity of Canadaphore obtained by alkaline precipitation of the iron was examined by adding Fe^{3+} in small increments to a 1 mg (1.6 μmole) sample of iron-free Canadaphore at pH 5.5. No further increase in optical

density at 430 nm was observed after the addition of 1.6 μmole of iron. This value suggests a ligand to iron ratio of 1:1 and a saturation level of Canadaphore with iron of approximately 8% at pH 5.5. The precise chemical structure of Canadaphore is currently under investigation. It is likely that the production of Canadaphore by H.carbonum will be shown to contribute significantly to the pathogenic potential of this organism.

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