APPLIED MICROBIOLOGY, Nov. 1975, p. 807-810

Vol. 30, No. 5
Printed in U.S.A.

Prodiginine (Prodigiosin-Like) Pigments from *Streptoverticillium rubrireticuli*, an Organism That Causes Pink Staining of Polyvinyl Chloride

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Received for publication 1 April 1975

Red pigments were extracted from *Streptoverticillium rubrireticuli* strain 100-19, an organism frequently incriminated in pink staining of polyvinyl chloride. These pigments were identified as undecylprodiginine and butylcycloheptylprodiginine.

Pink discoloration of polyvinyl chloride due to microbial growth has frequently plagued the plastics industry (6, 18). Such discoloration occurs on vinyl-covered furniture, vinyl wall covering, and vinyl doors.

In an attempt to induce artificially the pink discoloration of vinyl plastic, Yeager (17) buried vinyl in soil and observed production of pink spots on the vinyl. Of the many organisms isolated, only one, *Streptoverticillium rubrireticuli* (formerly *Streptomyces rubrireticuli*), produced the pink discoloration of vinyl.

Because of the lack of information in the literature on the nature of the pink pigments produced by *S. rubrireticuli*, a study was initiated to characterize these substances and to determine cultural conditions suitable for their formation. The evidence presented indicates that the pink pigment is actually a mixture of two prodiginine pigments, undecylprodiginine and butylcycloheptylprodiginine.

MATERIALS AND METHODS

Organism. *S. rubrireticuli* strain 100-19, an organism kindly provided by G. Tirpak, Tenneco Chemicals, Inc., was used in this study. It was originally isolated from pink-stained vinyl.

Solid media. Five different media solidified with 15 g of agar per liter (USP no. 1, Meer Corp., New York, N.Y.) were used. These included (i) Bennett's medium (5); (ii) yeast-Czapek medium (5a); (iii) Pablum medium containing 60 g of Pablum mixed cereal per liter (Canadian Pablum, Mead Johnson Co., Toronto, Ont.) in tap water, no pH adjustments; (iv) malt medium containing 10 g of malt extract per liter (Fisher Chemical Co., Pittsburg, Pa.) in tap water; and (v) nutrient glycerol medium containing 60 ml of glycerol, 5 g of Wilson's peptone 851, 5 g of NaCl, and 3 g of meat extract (Difco Laboratories, Detroit, Mich.) per liter of tap water.

Liquid media. The five liquid media used were (i) Bennett's, (ii) yeast-Czapek, (iii) modified yeast-Czapek in which the phosphate salt was replaced by calcium carbonate (10 g/liter), (iv) yeast-dextrose (5), and (v) soybean media (5). The soybean meal in the latter medium differs from that used previously (5) in that it contains 49% solvent-extracted soybean meal from Central Soya (Fort Wayne, Ind.). It contains not less than 49% crude protein; the ingredients are solvent-extracted dehulled soybean meal with added kaolin.

Cultural conditions. Slant cultures on solid media were maintained at 28 C. Growth in liquid media was in 50-ml batches in 250-ml Erlenmeyer flasks shaken on a New Brunswick model V rotary shaker at 180 to 220 rpm. When the organism was grown for extraction and purification of the pigments, a slant culture was inoculated into soybean medium or Bennett's broth, shaken 3 days at 28 C, and then transferred into 10 flasks of soybean medium. After 3 days, the cells were harvested by filtration.

Extraction and purification of the prodiginine pigments. The following steps were taken. (i) The cells were shaken overnight with acetone. Spectrophotometric assay at 530 nm (5), the wavelength at which maximum absorbance occurred, indicated about 4 mg of prodiginine pigments in the acetone extract. (ii) This acetone extract was concentrated and poured into water, and the mixture was extracted twice with chloroform. Before the second extraction the aqueous layer was made strongly acid and the shaken mixture was allowed to stand overnight before the lower, pigment-containing chloroform layer was withdrawn. This procedure was known from previous work with other microorganisms to result in higher yields of chloroform-extractable pigments. (iii) The pigment mixture was further purified and resolved into two fractions by column chromatography on alumina and silica gel (5).

Techniques for characterization of the pigments. Procedures for spectrophotometric analysis, thin-layer chromatography (TLC), and mass spectroscopy were described previously (5). Mass spectra were obtained at 2 kV with an inlet temperature of 240 C.

RESULTS

Pigmentation and cultural conditions. *S. rubrireticuli* grown on five solid media, previously found useful for eliciting prodiginine pigments, showed strong red nondiffusing pigmentation on nutrient-glycerol agar only. Weak red pigmentation was observed in the growth on Bennett's agar, but it was nearly obscured by the intense dark diffusing pigment that was also produced. Only the dark diffusing pigment was observed when this organism was grown on malt, Pablum, or yeast-Czapek agar. During the past 2 years one of us (N.N.G.) has studied 12 *Streptomyces* or *Streptoverticillium* strains that produce prodiginine pigments. *S. rubrireticuli* strain 100-19 is the first such strain that failed to show red pigment on yeast-Czapek agar.

In shaken broth cultures, strain 100-19 grew well in yeast dextrose and soybean media, less well in Bennett's broth, and poorly in the two types of yeast-Czapek broth. Red pigmentation was most noticeable in cells grown in soybean medium. Some pigmentation was apparent in cells from Bennett's and yeast-dextrose media, but it was absent from cells in the two yeast-Czapek media.

More pink pigment was produced at 28 to 31 C than at 22 C. At 38 C pigment production was slight to none. Maximum pigment accumulation occurred during the early stationary phase of growth.

Characterization of the prodiginine pigments. The pigments extracted from cells were purified and resolved into two fractions (A and B) as described in Materials and Methods. The purified pigments were compared with authentic samples of the nine known, fully characterized, naturally occurring prodiginines.

Pigment A exhibited visible absorption maxima at 528 nm in acid-$CHCl_3$ and at 525 nm in acid-ethanol. These maxima as well as the observed TLC behavior (color, fluorescence, and R_f) were identical with those of authentic samples of undecylprodiginine and nonylprodiginine (4). Mass spectroscopy indicated that pigment A was undecylprodiginine (Fig. 1A; Fig. 2). The molecular ion was clearly 393 mass units, and the spectrum closely resembled that of authentic undecylprodiginine (8). Nonylprodiginine has a molecular ion at 365 mass units.

Pigment B was identical with authentic butylcycloheptylprodiginine (Fig. 1B) in visible absorption maxima (542 nm in acid-$CHCl_3$ and 536 nm in acid-ethanol) and TLC behavior (5a). On the TLC plate, spots of pigment B were slightly behind those of authentic metacycloprodigiosin (15) and pinker (less orange). Spots of pigment B were the same color as those of authentic prodigiosin (10, 16) but slightly ahead. The structure was verified by mass spectroscopy (Fig. 2). The mass spectrum was similar to that obtained previously for butylcycloheptylprodiginine (5a) and clearly different from that of metacycloprodigiosin, which does have the same molecular formula but a different structure.

DISCUSSION

Two prodiginine pigments have been identified as products of *S. rubrireticuli*, undecylprodiginine and butylcycloheptylprodiginine. These pigments were recently found by Gerber (5a) to also be produced by *Streptomyces* sp. Y-42, a strain isolated from leaf and grass compost. Several other members of the order *Actinomycetales* have been found to be capable of producing prodiginine pigments (1–5a, 7–9, 11–15).

It is probable that the "pink staining" of polyvinyl chloride caused by *S. rubrireticuli* is due to the prodiginine pigments produced by the organism. Yeager (18) reported that "the presence of the organism in the vinyl system is not necessary to produce the stain. Instead, the discoloration is caused by a migration of the stain from a nearby substrate on which the organism is growing." The prodiginine pigments of *S. rubrireticuli* are soluble in diisodecyl adipate, one of the plasticizers used commercially by some vinyl manufacturers. Exposure of the plasticizer to red mycelia resulted in extraction of the pigment (Stahly, unpublished data). Thus, the plasticizer is the probable vehi-

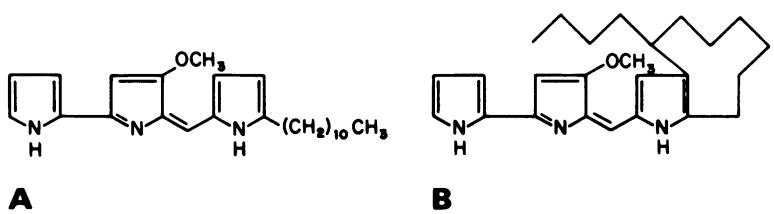

FIG. 1. *Prodiginine pigments of S. rubrireticuli. (A) Undecylprodiginine; (B) butylcycloheptylprodiginine.*

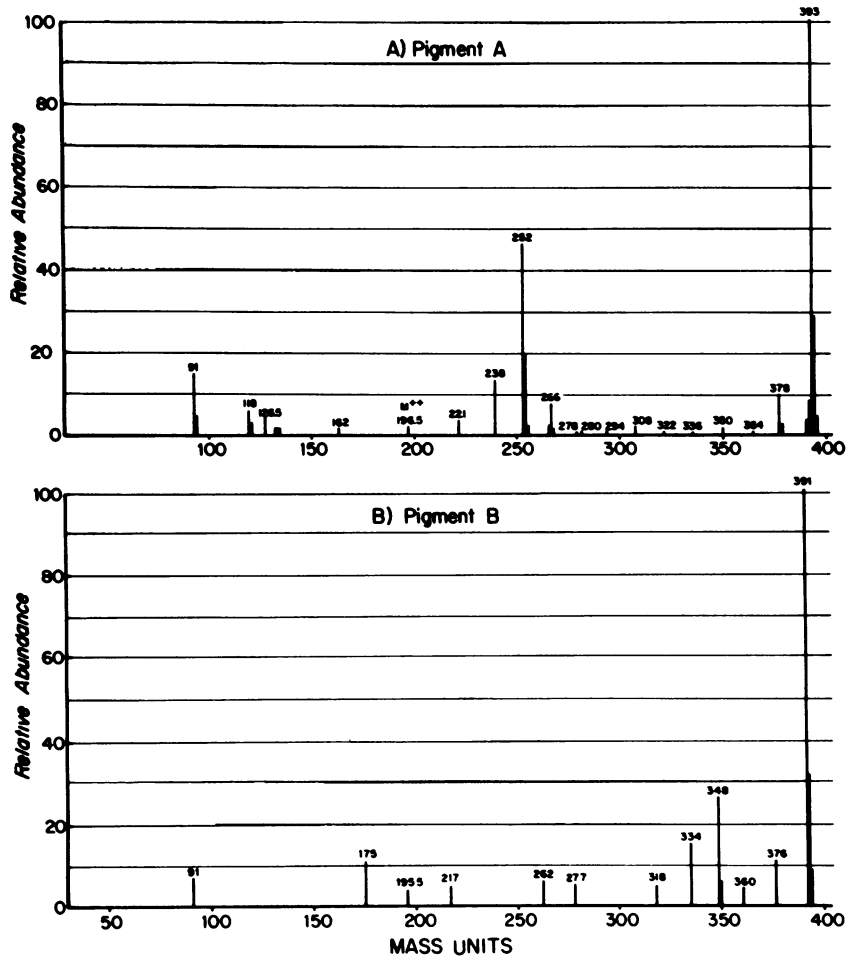

FIG. 2. *Mass spectra of prodiginine pigments of S. rubrireticuli. (A) Pigment A, undecylprodiginine; (B) pigment B, butylcycloheptylprodiginine.*

cle through which the pigment is transported from *S. rubrireticuli* throughout the vinyl system.

The mass spectrum of pigment A with its strong molecular ion at 393 mass units is clearly different from that of nonylprodiginine, the molecular ion of which is 28 mass units less. Both show the 252 mass unit peak caused by β cleavage of the aliphatic side chain. Although both metacycloprodigiosin and butylcycloheptylprodiginine have strong molecular ions at 391 mass units, the next largest peaks are 307 and 320 for the former and 348 and 334 for the latter.

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