Production of Cephalosporin C by *Paecilomyces persicinus* P-10

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After the growth of *Paecilomyces persicinus* P-10 in a glucose-peptone medium, filtrates were collected and analyzed for antibiotic activity. Activities against *Salmonella gallinarum* ATCC 3030 and *Alcaligenes faecalis* ATCC 8750 (penicillin N-resistant strain) were obtained. Part of the former activity was readily inactivated by penicillinase. The fraction active against *A. faecalis* was isolated by passage through Amberlite XAD-2 and Amberlite IRA-68. The powder eventually obtained was subjected to paper chromatography followed by bioautography, and the activity obtained corresponded to that of a sample of cephalosporin C. Thin-layer chromatography was also employed to verify the presence of cephalosporin C in the P-10 powder. The active solids were further purified by means of paper chromatography in a solvent system consisting of n-butanol-acetic acid-water (60:15:25, vol/vol). The material obtained from this procedure yielded an infrared absorption spectrum identical to that of cephalosporin C. Similarly, the ultraviolet absorption of the purified preparation coincided with that of cephalosporin C. Exposure of the purified solids to cephalosporinase resulted in rapid inactivation of the antibiotic. In addition to penicillin N and cephalosporin C, filtrates of *P. persicinus* P-10 also contained deacetylcephalosporin C, deacetoxycephalosporin C, and cephalosporin P.

The genus *Paecilomyces* includes several species known to synthesize substances exhibiting antimicrobial activity. Wilkins and Harris (15) provided one of the earliest reports that related antibiotic production to a species of *Paecilomyces*, *P. burci*. The synthesis of an antifungal antibiotic, variotin, was attributed to *P. variotii* var. *antibioticus* (16). Pisano et al. (14) were the first to report the production of an antibiotic by *P. persicinus*. The identity of the antibiotic with penicillin N was shown by means of paper chromatography, electrophoresis, and various chemical procedures. Subsequently, Fleischman and Pisano (6) developed a chemically defined medium for the production of penicillin N by *P. persicinus*. Neither of the latter two investigations presented evidence for cephalosporin C synthesis by *P. persicinus*.

The present study was undertaken to determine the nature of the antimicrobial activity elaborated by a recently acquired isolate of *P. persicinus* (P-10). Analytical procedures suggested the presence of more than one active component. Substantiation of these initial findings and the indentification of one of the active components as cephalosporin C are presented in this report.

MATERIALS AND METHODS

Culture. The cultural characteristics of *P. persicinus* P-10 are identical with those of *Acremonium persicinus* CBS295.70 D according to Gams (7). The fungus was maintained on Sabouraud dextrose agar (Difco) slants at 28.5 C.

Fermentations. Initial fermentations were carried out in 250-ml Erlenmeyer flasks containing 50 ml of the desired medium. All flasks were incubated at 28.5 C on a rotary water bath shaker (model G-77, New Brunswick Scientific Co.) operated at 240 rpm. Inocula were prepared by growing *P. persicinus* P-10 in flasks containing a seed medium identical to fermentation medium A (see Table 1). After 72 h, additional flasks of seed medium were inoculated with 10% of the growth suspension from the original flasks and incubated for 24 h. Two-milliliter portions from the second-stage seed flasks were used to inoculate flasks containing various fermentation media.

Large scale fermentations were carried out using 30-liter fermentors. Agitation was maintained at 800 rpm, whereas the aeration rate was 1:1 (vol/vol). The fermentors were maintained at 23 C for 40 to 60 h.

Isolation of cephalosporin C. Cephalosporin C was isolated according to the following procedure. Approximately 23.5 liters of the culture filtrate was acidified to pH 3.0 and then passed through a 9-liter bed of Amberlite XAD-2 contained in a 4-inch (about 10 cm) column. This was previously washed with 4.5 liters of water. Elution was carried out with 20 liters of water containing 10% (vol/vol) isopropanol. The flow rate was adjusted to 20 liters per h. Three hundred milliliters of Amberlite IRA-68 was used to adsorb the compound from 14 liters of active eluate. The resin bed, contained in a 1-inch (about 2.5 cm) column, had been washed with water (600 ml) prior to adsorption. Elution of the activity was accomplished with 1.5
liters of pyridine-acetate buffer (0.44 M:0.2 M) at a flow rate of 300 ml/h. Approximately 700 ml of active eluate was concentrated under vacuum in a rotary evaporator at 30 to 35°C. The concentrated fraction (50 ml) was treated with 600 ml of isopropanol, and the precipitate which developed was collected by filtration, washed with 50 ml of isopropanol, and dried under vacuum at 45°C. Approximately 30.8 g of a yellow tinted powder was collected. Its cephalosporin C activity corresponded to 1.5% of the standard.

Higher-purity preparations were obtained by subjecting the yellow tinted powder to paper chromatography in solvent system no. 2. The active cephalosporin C areas were excised from replicate strips after development for approximately 15 h at 25°C. The individual papers thus collected were pooled and eluted with water. The water solutions were then lyophilized and stored at −20°C prior to use. This preparation of highest purity was used in the identification procedures.

Antibiotic assay. The antimicrobial activity of fermentation broths and purified preparations of cephalosporin C was determined by agar diffusion assays employing a bacterial isolate which had been selected from the parent culture of *Alcaligenes faecalis* ATCC 8750. The isolate selected is resistant to penicillins N and G as well as the cephalosporin C group. It is susceptible to cephalosporin C as well as deacetoxycephalosporin C and deacetylcephalosporin C. The assay medium was prepared by adding 0.85% ion agar no. 2 (Colab) to Penassay broth no. 3 (Difco). Four plates were prepared by seeding 100-ml quantities of the assay medium with 0.4 ml of an 18-h culture of *A. faecalis*. Active samples were applied to paper disks 12.7 mm in diameter which were then placed on the freshly seeded agar substrate. Plates were incubated at 37°C for 18 h. The potency of antibiotic preparations was determined by the use of a standard curve prepared with authentic cephalosporin C. Linearity was obtained within the range of 25 to 200 μg of the cephalosporin C standard per ml. The presence of antibiotics other than cephalosporin C was detected by means of microbiological assays which employed the following organisms: *Neisseria catarrhalis* ETH, *Salmonella gallinarum* ATCC 3030, and *Staphylococcus aureus* ATCC 2400.

Identification procedures. Paper chromatograms were developed in one or more of the following solvent systems: system no. 1, n-butanol-ethanol-water (4:1:5; vol/vol), Whatman no. 1 paper, descending; system no. 2, n-butanol-acetic acid-water (60:15:25; vol/vol), Whatman no. 1 paper, descending (3); system no. 3, 70% propanol, Whatman no. 1 paper, ascending (5). Developed chromatograms were subjected to bi- oautography on plates containing the antibiotic assay medium seeded with the test organisms. Appropriate standard reference compounds were also applied to the same plates. All standards, which were supplied by CIBA-Geigy, Ltd., Basle, Switzerland, were between 93 to 95% pure. These compounds, listed in order of least to greatest mobility in solvent system no. 2, were deacetylcephalosporin C, cephalosporin C, deacetoxycephalosporin C, penicillin N, and cephalosporin P. In terms of relative activities of the cephalosporin C-related compounds against the test bacteria employed, deacetylcephalosporin C and deacetoxycephalosporin C exhibited 20 and 10%, respectively, of the cephalosporin C activity found.

Cephalosporin C was also identified by means of thin-layer chromatography with Silica Gel H plates (Applied Science Lab.). A solution of the P-10 solids (10 mg/ml) was spotted in 5- and 10-μliter amounts. Development was effected in a solvent system consisting of 85% aqueous acetone over a 60-min period. A ninhydrin spray was employed for the detection of cephalosporin C.

The procedure described by Marelli (12) was followed to allow the P-10 powder and hydroxyamine to react. The standard preparation of cephalosporin C was also subjected to hydroxylation, and then the cephalosporin C content of the purest P-10 preparation was determined.

The action of cephalosporinase on the high purity material was tested with enzyme prepared from cells of *Enterobacter cloacae* PSAL 969 ruptured by sonic vibration. After chromatography on diethylaminoethyl-cellulose, active fractions were pooled and lyophilized. The enzyme (50 μg) and the P-10 powder (100 μg) were dissolved in 1 ml of 0.01 M phosphate buffer (pH 7.0), and the reaction was followed by measuring the change in absorption at 260 nm.

The infrared absorption spectrum of the *P. persicus* P-10 material was determined using a Beckman infrared spectrophotometer, model IR 8. One-milligram samples of the antibiotic were incorporated into KBr (0.55%) disks. Absorption in the ultraviolet region of the P-10 sample was measured in a model 202 Perkin-Elmer spectrophotometer. The solution employed contained 200 μg of the purified material per ml.

RESULTS

Fermentations. *P. persicus* P-10 was cultivated in the three media listed in Table 1 in shake-flasks. All culture filtrates were found to be active against one or more of the test bacteria. Culture filtrates from medium A yielded greatest activity 5 days after inoculation, whereas the filtrates from medium B and medium C were most active after 3 days of incubation. Filtrates from medium A exhibited inhibitory activity against *S. aureus* and *S. gallinarum*, but not against *A. faecalis*. Bioautography revealed the presence of an active spot which corresponded to that of penicillin N. The latter activity was destroyed by treatment with penicillinase. A fast-moving component, which inhibited the growth of *Staphylococcus aureus*, had a mobility identical to that of a standard preparation of cephalosporin P. Culture filtrates obtained from medium B contained penicillin N but failed to inhibit *A. faecalis*. Activity against this microorganism was evident in filtrates from medium C. Spots
inhibitory to A. faecalis has an average $R_f$ value of 0.04 in solvent system no. 1, which was identical to $R_f$ values obtained with the cephalosporin C standard. The latter $R_f$ value agrees with that reported by Loder et al. (11) who employed the same solvent system. The activity exhibited against A. faecalis was not destroyed by treatment with penicillinase. Penicillin N and cephalosphorin P production by P. persicus in medium C was also detected by means of bioautography. In addition, trace quantities of activities, apparently equivalent to those of deacetylcephalosporin C and deacetoxyccephalosporin C, were occasionally found. The latter compounds were detected only in medium C, occurring in amounts which represented approximately 2% of the cephalosporin C activity found. The presence of deacetylcephalosporin C and deacetoxyccephalosporin C in fractions prepared from active filtrates of medium C was verified by J. Nüesch (CIBA-Geigy, Ltd.), by means of high-pressure liquid chromatography according to the procedure of Konecny et al. (10).

Attempts to enhance the synthesis of cephalosporin C by P. persicus P-10 by supplementation of medium C with DL-methionine, employing concentrations that ranged from 0.05 to 0.5%, did not succeed. Some improvement in yields, however, was obtained by varying the concentration of the components of medium C. In general, higher yields of cephalosporin C activity occurred when glucose levels were increased and the concentration of peptone decreased. A formulation consisting of 2% glucose, 0.5% peptone (Difco), and 0.5% CaCO$_3$ allowed production of from 50 to 75 μg of cephalosporin C per ml as compared to 25 to 40 μg/ml obtained with medium C.

Production of cephalosporin C in 30-liter fermentors was readily accomplished. The pH of the medium fell from a value of 7.5 to 7.0 in approximately 24 h. After this, the pH rose and at the time of harvest (approximately 48 h) it had attained a value of 8. The activity remained stable in the fermentor for approximately 100 h despite the relatively high pH. Approximately 20 μg of cephalosporin C per ml was found in culture filtrates. Relatively high recoveries (approximately 50%) were obtained despite the low activity in the culture filtrates.

**Identification of cephalosporin C.** The highly purified solids were subjected to paper chromatography by using primarily solvent systems no. 2 and 3. A single spot active against A. faecalis was obtained with both systems. In system no. 2, the active material had an average $R_f$ value of 0.39, which was identical to that obtained with the cephalosporin C standard. Treatment with cephalosporinase destroyed activity. The active component exhibited greater mobility in solvent system no. 3, the average $R_f$ value being 0.58. Thin-layer chromatography on Silica Gel H also revealed the presence of one spot which was inhibitory to A. faecalis. The active solids had an average $R_f$ value of 0.11 which coincided with that of the cephalosporin C standard. The highly pure P-10 solids and hydroxylamine were allowed to react, and a positive reaction was indicated by the formation of a colored complex, the absorbance of which was determined at 515 nm. Comparison of the results obtained with those calculated for a standard preparation of cephalosporin C revealed that the P-10 material was equivalent to the standard in cephalosporin C content.

Exposure of the solids to cephalosporinase brought about complete inactivation within 2 min at 25 C. With the concentrations of enzyme and active material described above, an average decrease of 0.35 optical density U per min was observed. The rate of inactivation was readily increased by using higher concentrations of enzyme.

Figure 1 presents a comparison of the infrared absorption spectrum of the cephalosporin C standard as well as the high-purity preparation obtained from P. persicus P-10. Both materials exhibited identical absorption spectra.

The ultraviolet absorption spectrum of the
P-10 material is shown in Fig. 2. Maximal absorption occurred at 260 nm, which corresponds to the value reported by Abraham and Newton (2) for cephalosporin C.

**DISCUSSION**

The production of cephalosporin C by *P. persicinus* P-10 was carried out by cultivation of the fungus in a medium consisting of glucose, peptone, and CaCO₃. Media containing components that originate from plants were ineffective for cephalosporin C synthesis, although the production of penicillin N and cephalosporin P did occur. The failure of plant supplements to enhance cephalosporin C production by *P. persicinus* P-10 is in contrast with the use of such materials for the production of the antibiotic by a strain of *Cephalosporium* (13). Demain and Newkirk (4) employed a medium that contained crude materials derived from both plant and animal sources for cephalosporin C synthesis by a mutant of *Cephalosporium*. In the present investigation, *Cephalosporium* ATCC 14553 was found to yield activity inhibitory to *A. faecalis* after cultivation in both medium A and medium B. From the foregoing, it appears obvious that *P. persicinus* P-10 differs in some respects from *Cephalosporium* ATCC 14553 in the utilization of substrates for cephalosporin C formation. This point was further illustrated by the failure of DL-methionine to stimulate cephalosporin C production by *P. persicinus* P-10; this fact contrasts with the beneficial effect of this amino acid on cephalosporin C synthesis by strains of *Cephalosporium* (4, 13). Conceivably, variations in nutritional requirements for cephalosporin C production may explain the failure to detect evidence of such activity in a previously investigated isolate of *P. persicinus* (14).

The isolation procedure employed in this investigation proved suitable for the recovery of active solids containing cephalosporin C. Most of the activity was confined to the eluates collected from the Amberlite columns used. It is of interest to note, however, that the partially purified active material did not display infrared spectra which were exactly comparable to the cephalosporin C standard. These solids, for example, did not show absorption bands in the region of 5.62 μm, which are attributable to the carbonyl stretching vibration of the lactam group in the fused β-lactam thiazolidine ring system (2). After further purification of the P-10 solids by means of paper chromatography, however, the material obtained showed typical absorption in the region of 5.62 μm. In fact, as determined by the infrared spectra, the latter preparation proved to be identical to the standard.

Other identification procedures utilized indicated that *P. persicinus* P-10 produces cephalosporin C under the prescribed conditions. Paper chromatography and thin-layer techniques confirmed the presence of cephalosporin C in test materials. The ultraviolet absorption studies...
demonstrated the expected activity associated with the cephalosporin C nucleus (2). The inactivation of the P-10 solids by the E. cloacae cephalosporinase provided further evidence that P. persicinus P-10 produces cephalosporin C. Lastly, the formation of hydroxamic acid after exposure of the active material to hydroxylamine established the presence of a β-lactam ring. This reaction provided further evidence of the purity of the P-10 solids obtained.

Of special interest was the detection of deacetylcephalosporin C and deacetoxycephalosporin C in preparations of active material obtained from P. persicinus P-10. Deacetylcephalosporin C formation in the cephalosporin C fermentation has been reported for Cephalosporium (9), and deacetoxycephalosporin C is found in a variety of fungi and streptomycetes (8).

Relatively few microorganisms are known to synthesize cephalosporin C. Among the fungi, this biosynthetic capacity appears to be restricted to certain species of Cephalosporium and Emericellopsis (1). The strain of Cephalosporium originally isolated by Brotz was considered to be similar to Cephalosporium acremonium, but Gams (7) has since replaced the genus Cephalosporium (and other genera) with Acremonium, and the Brotz strain was reclassified as A. chrysogenum. As indicated previously, certain species of Paecilomyces have been known to synthesize antibiotics for many years. It was not until 1961, however, that the production of a β-lactam antibiotic was reported for this genus (14). In the latter case, penicillin N but not cephalosporin C was detected. In view of the data reported in the present investigation, it is obvious that nutrition plays a major role in the antibiotics that are elaborated by P. persicinus. It is possible, therefore, that variations in nutritional requirements may have delayed the recognition of this fungus and perhaps other microorganisms as sources of cephalosporin C. This fact has suggested additional studies, beyond the scope of the present report, which are being pursued in our laboratory.

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LITERATURE CITED