BACTERIAL PRODUCTION OF DEACETOXYCEPHALOSPORIN C

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The production of β-lactam antibiotics by bacteria has been recently reported; these include monobactams[1,2] and carbapenems[3]. During a screening program developed to detect β-lactam antibiotics produced by bacteria[2], we isolated strains of *Flavobacterium* sp. and *Xanthomonas* sp. that produced deacetoxycephalosporin C (Fig. 1). This paper describes the isolation of deacetoxycephalosporin C-producers and gives a brief description of the producing strains and their fermentation conditions. The isolation and chemical identification of deacetoxycephalosporin C are also presented.

Deacetoxycephalosporin C-producing strains of bacteria were isolated infrequently from a limited number of habitats (Table 1). *Flavobacterium* sp. SC 12,154 was isolated from a soil sample collected in Caguas, Puerto Rico and plated on Brilliant green bile agar (BBL) amended with soil extract (15% v/v). *Xanthomonas* sp. SC 11,696 was isolated from decaying skunk cabbage (*Symphocarpus foetidus*) collected in West Windsor, New Jersey, and plated on YPM agar consisting of (g/liter): yeast extract 5.0, peptone 15.0, mannitol 12.5, vitamin B_{12} 0.002 and agar 15.0.

Flavobacterium sp. SC 12,154 is a Gram-negative rod, predominantly long and slender. There is no evidence of motility either by flagella or by gliding. On casein-yeast extract-peptone agar the organism forms an intracellular yellow pigment which does not diffuse into the medium. The chemical nature of this pigment has not been determined. *Flavobacterium* sp. SC 12,154 metabolizes carbohydrates oxidatively, producing acid from glucose, maltose, sucrose and lactose on DYE basal medium C[4], a peptone-free medium. It is cytochrome oxidase positive. It grows well

Fig. 1. Structure of deacetoxycephalosporin C.

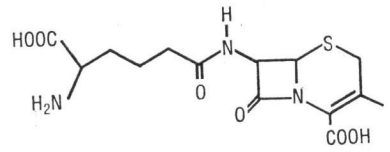

Table 1. Collection sites of *Flavobacterium* and *Xanthomonas* strains producing deacetoxycephalosporin C.

Microorganism	Sample	Site
Flavobacterium sp.	Lawn soil	Caguas, Puerto Rico
Flavobacterium sp.	Wood lawn soil	Mona Island, Puerto Rico
Xanthomonas sp.	Rotting skunk cabbage	West Windsor, New Jersey
Xanthomonas sp.	Soil under pear tree	Ringoes, New Jersey
Xanthomonas sp.	Withered blossom and seed of *Astilbe chinensil*	Germany

at 30°C but not at 37°C. Good growth is attained on Difco Marine agar, but it grows equally well in the absence of salt. The organism is thus halotolerant rather than halophilic. The mole% G+C of the DNA was 70 as determined by the method of ULITZUR[5]. The following test responses were positive: SIMMON's citrate, hydrolysis of aesculin, tributyrin, casein and gelatin. Negative tests were obtained for nitrate reduction, hydrolysis of chitin and cellulose, and production of H_2S (lead acetate method) and urease. *Flavobacterium* sp. SC 12,154 differs from members of *Acinetobacter* in that the latter are small plump rods, oxidase negative, aesculin negative and have a mole% G+C of 40~47. WEEKS[6] divides the genus *Flavobacterium* into two sections based on mole% G+C. SC 12,154 falls into Section II (high G+C) but differs from *Flavobacterium lutescens* in that it does not require sodium chloride for growth; is nitrate negative; and produces acid from lactose, sucrose and maltose. It differs from *F. capsulatum* in that it is non-cellulolytic and gelatinase positive and as already noted nitrate negative.

Xanthomonas sp. SC 11,696 is a Gram-negative polar flagellate rod. On Bennett agar a yellow, water-insoluble pigment is produced. There is no characteristic pigment on Trypticase soy agar.

Heavy mucoid growth occurs on nutrient agar with 5% glucose. The culture is cytochrome oxidase-negative and metabolizes carbohydrates oxidatively. In the weakly buffered medium of DYE[4] acid is produced from glucose, arabinose and galactose but not from mannose, trehalose or cellobiose. The following test responses were positive: citrate, gelatin, H_2S from peptone-iron agar (with lead acetate strip), ammonia from peptone (by Nessler reaction), and β-glucosidase (method of HILDEBRAND and SCHROTH)[7]. The following test responses were negative: nitrate reduction, VP, indole and urease. The yellow pigment gave a negative $SbCl_3$ test, presumptive evidence that it is non-carotinoid. The above characteristics suffice to designate SC 11,696 as a strain of *Xanthomonas campestris* in the broad sense in keeping with the treatment of this genus by DYE and LELLIOT[8].

Fermentation was initiated by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks, each containing 100 ml of the following sterilized medium: yeast extract 0.5%, and glucose 0.1% in distilled water. The flasks were incubated at 25°C on a rotary shaker (300 rpm; 5 cm stroke) for approximately 24 hours. A 1.0% (v/v) transfer of this culture growth was used to inoculate a 75-liter Fermatron fermentor (New Brunswick Scientific, Edison, New Jersey) containing 50 liters of the same yeast extract-glucose medium described above. The fermentation was continued for approximately 24 hours at 25°C at an agitation rate of 200 rpm and an air flow of 50 liters/minute. β-Lactam production (detected after extraction of the cells with methanol) and isolation was monitored using *Bacillus licheniformis* (SC 9262) as test organism. The isolation scheme is outlined in Fig. 2.

A mass spectrum of the antibiotic was obtained by the fast atom bombardment (FAB) technique, which gave peaks at m/z 358 and 380 in the positive-ion mode and at m/z 356 and 378 in the negative-ion mode, indicating a molecular weight of 357 and 379 for the free acid and the sodium salt, respectively. The antibiotic gave α-aminoadipic acid and glycine on acid hydrolysis (6 N HCl, 105°C, 15 hours). The acid hydrolysate, as *N*-pentafluoropropionyl isopropyl ester[9], was analyzed by gas chromatography using a chiral column[10] with proline as internal standard. The configuration of α-aminoadipic acid was shown to be D by peak enhancement with authentic *N*-pentafluoropropionyl-D-α-aminoadipic acid isopropyl ester. The β-lactam antibiotic was identified as deacetoxycephalosporin C by comparison of its spectral data (^{1}H NMR, IR and UV) with that of an authentic sample obtained by hydrogenation of cephalosporin C potassium salt[11].

Fig. 2. Isolation of deacetoxycephalosporin C.

Flavobacterium sp. (cells)

1. Extraction into MeOH, centrifugation and concentration
2. Filtration through Celite using acetone - H_2O (2: 1)
3. Batch absorption on Amberlite IRA-458 (OAc^-) and elution with 1 M pyridine - AcOH
4. Chromatography on BioRad AG 1×2 (OAc^-) eluting with a 0.2~2.0 M pyridine - AcOH gradient
5. Chromatography on MCI gel CHP20P at pH 3, eluting with a H_2O - MeOH gradient
6. Chromatography on QAE-Sephadex (OAc^-), eluting with a 0.2~2.0 M pyridine - AcOH gradient
7. Chromatography on cellulose powder, eluting with 4: 1~1: 1, acetone - H_2O gradient
8. Chromatography on MCI gel CHP20P at pH 3, eluting with a H_2O~MeOH gradient

Deacetoxycephalosporin C

Deacetoxycephalosporin C is present in the fermentation products of a number of fungi, including *Cephalosporium* spp. and the cephalosporin C-producing *Streptomyces* spp.[12]. LIERSCH *et al.*[13] provided the direct evidence for the role of deacetoxycephalosporin C in cephalosporin C biosynthesis by demonstrating the conversion of deacetoxycephalosporin C to deacetylcephalosporin C and to cephalosporin C in a broken-cell system. Recently[14], it has been demonstrated that deacetoxycephalosporin C is derived from penicillin N in a cell-free reaction by *Cephalosporium acremonium* mutant M-0198. The discovery of deacetoxycephalosporin C-producing strains of bacteria was unexpected. With the isolation of monobactams[2] we postulated that the "lowly" bacteria would produce only simple monocyclic β-lactam antibiotics. However, with the discovery of a carbapenem[3] and a cephalosporin of bacterial origin, it is obvious that bacteria have the biosynthetic capability to produce a range of β-lactam-containing structures comparable to those produced by streptomycetes

and fungi.

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