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Studies on a strain of *Kitasatospora* sp. paclitaxel producer

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Abstract - The strain P&U 22869, which produces paclitaxel and related taxanes, discovered during the course of endophytic actinomycetes screening on *Taxus baccata* plants, was classified as *Kitasatospora* sp. on the basis of its morphological characteristics observed by light and scanning electron microscopy, the presence of the major constituents of its cell wall and the 16S rDNA sequence. The morphophysiological profile of the strain was compared with those of the valid and invalid species described for this genus. The paclitaxel production detected by means of monoclonal antibody assay (CIEA) was confirmed by LC-MS analysis and its concentration determined by HPLC. The *de novo* paclitaxel biosynthesis operated by strain P&U 22869 was demonstrated by biosynthesis experiments with labeled precursors.

Key words: *Kitasatospora*, *Taxus*, paclitaxel, 16S rDNA sequence.

INTRODUCTION

Paclitaxel is a anti-microtubule drug used for the treatment of ovarian and metastatic breast cancer, which shows unique antitumor properties promoting the polymerization of tubulin and inhibiting the depolymerization of microtubules back to tubulin (Rowinsky *et al.*, 1992; Safavy, *et al.*, 1999). It is a complex diterpenoid produced by many *Taxus* species such as *T. brevifolia*, *T. baccata*, *T. canadensis*, *T. cuspidata*, *T. wallichiana* and related *Taxaceae* (Kerns *et al*., 1994; Das *et al*., 1998; Rao and Juchum, 1998; Baloglu and Kingston, 1999).

Originally paclitaxel was isolated and purified from the bark of the Pacific yew tree, but this source was considered ecologically unsuitable as it required the distruction of many yew trees. Over the past few years, other renewable sources such as the needles of *Taxus* species or cell cultures were utilized (Witherup *et al.*,

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1990; Roberts and Shuler, 1997). Alternative synthetic methodologies for the conversion of the precursor 10-deacetylbaccatin III, which is more easily isolated from yew, to paclitaxel have been developed (Gabetta *et al*., 1998).

A new possibility to produce paclitaxel in a cheaper way via industrial fermentation has come from the discovery that some endophytic fungi belonging to different genera such as *Taxomyces, Pestalotiopsis, Alternaria, Periconia* (Stierle *et al*., 1993; Dahiya, 1996; Li *et al*., 1998a, 1998b) and some endophytic bacteria such as *Brevibacterium, Sphingomonas, Bacillus* (Tahara *et al*., 1995; Page *et al*., 1999) are able to produce paclitaxel and related taxanes. Interesting results in terms of improvement of paclitaxel production by microorganisms and knowledge of resistance mechanisms in fungi (Mu *et al*., 1999) has been recently achieved that prompt us to investigate the possibility to produce paclitaxel also by endophytic actinomycetes. The current study focuses on the identification and characterization of an actinomycete strain able to produce paclitaxel.

MATERIALS AND METHODS

Investigated actinomycete. Strain P&U 22869 (=CECT 4991, Spain) was isolated from a wood sample of *Taxus baccata*, collected in Bologna (Italy), by an isolation procedure previously described (Caruso *et al*., 2000). It was detected on a plate of tap water agar WA25 (2.5% agar) containing the selective antibiotic cycloheximide to suppress the growth of fungi (50 ppm) incubated for 14 days at 27 °C.

The strain was grown on ISP Medium 3 (Difco, USA) at 27 °C for 10-12 days and stored as spores in water-glycerol (10:1) at –80 °C or lyophilized.

Cultural and morphological characterization. Cultural characteristics were determined after 7, 14 and 21 days of incubation at 27 °C. The following media were used: tap water 2.5% agar (WA25), ISP Medium 1, 2, 3, 4, 5 (Difco), Nutrient agar Difco (NA), Bennett's agar (BA), Czapek solution agar Difco (CA).

Growth intensity, reverse colour of vegetative mycelium, production of soluble pigment and sporulation were noted. The morphological characteristics were examined by light and scanning electron microscopy (SEM). For SEM observation, small blocks of culture were removed from plates and prepared by the technique previously described (Locci and Petrolini Baldan, 1971). Observations were carried out with a Stereoscan 250 (Cambridge Sci. Instr. Ltd., UK).

Physiological characterization. Carbohydrate utilisation tests were done by the API 50 CH system (BioMérieux SA, France), utilizing spores collected from a culture on WA25 incubated for 14 days at 27 °C and resuspended in YNB (Yeast nitrogen base, Difco) medium neutralised with 20% (vol/vol) 1% K_2HPO_4 solution.

Enzymatic activities were tested by applying the API ZYM system (BioMérieux SA) to colonies of the strain P&U 22869 grown on ISP Medium 3 incubated for 14 days at 27 °C.

Antibiotic resistance was also tested on solid medium using susceptibility test discs (Oxoid, UK).

Cell chemistry. Cell-wall composition, including diaminopimelic acid isomers and sugars, was determined by two methods: the first reported by Becker *et al*. (1965) and the second one by Omura *et al.* (1981). Mycelium without spores for chemical analysis was obtained from liquid cultures grown on Bennett's broth cultivated at 27 °C in flasks on a rotary shaker (250 rpm). Spores were collected on plates of Bennett's agar, which was washed by means of sterile water to remove aerial growth. In both cases the material was harvested by centrifugation, washed with sterile water and lyophilized before being chemically analyzed.

16S rDNA analysis. A spore suspension of strain P&U 22869 was prepared and used to inoculate 250 mL Erlenmeyer flask containing 50 mL of YEME/TSB 1:1 media with 1% glycine added (Hopwood *et al.*,1985). The inoculated flask was incubated for 3 days at 28 °C on a rotary shaker at 250 rpm and 50 mg of mycelium, harvested by centrifugation and washed with sterile water, were treated with 500 µL of TSE buffer containing lysozyme (2 mg/mL) at 37 °C for 1 hour. Chromosomal DNA was isolated by the rapid small scale procedure of Hopwood *et al.* (1985).

The 16S rDNA from the strain P&U 22869 was amplified by PCR with a Gene Cycler (Bio Rad Laboratories, Italy) with a final volume of 50 µL containing 2 µL bacterial DNA solution, 5 µL 10x Taq Polymerase Reaction buffer (Amersham Pharmacia Biotech, Italy), 100 mM each dNTP, 1 µM each primer (forward 27F AGAGTTTGATCCTGGCTCAG, reverse 1494R CTACGGCTACCTTGTTACGA) and 2.5 U Taq polymerase (Amersham Pharmacia Biotech).

Termal cycles were performed with the following profile:

– initial denaturation 94 °C for 4 min;
– five cycles: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min;
– five cycles: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min;
– twenty five cycles: 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min;
– final extention was at 72 °C for 4 min.

The amplified band was visualised on a 1.5% (wt/vol) agarose gel stained with ethidium bromide.

The 16S rDNA was subjected directly to cycle sequencing according to the protocol described by Sanger *et al*. (1977). Primer extension was obtained using a Gen Amp PCR System 9.600 (Perkin-Elmer, USA). In addition to primers 27F and 1494R, the following sequencing primers, complementary to the internal region of the 16S rRNA gene, were used: 8005 F (ACTGAGACACAGCCCAGACTCCTA), 8006 F (TTCGATACGGGCAGGCTAGAGT), 8007 F (CACAAGCAGCGGAGCATGTGGCTT), 8008 F (CACGTGCTACAATGGTCGGTA). The products were purified using Centri-Sep spin columns (Perkin-Elmer) and analysed with an ABI Prism 373 A Genetic Analyser (Applied Biosystems, USA).

To find the nearest relative of strain P&U 22869, its sequence was compared with sequences of microorganisms in the EMBL and GenBank databases using the program FASTA (Pearson and Lipman, 1988).

The 16S rDNA sequence was then compared with the nine valid *Kitasatospora* species and three other strains belonging to invalid *Kitasatospora* species *(K. kifunense, K. brunnea* and *K. melanogena*): their sequences were obtained from the EMBL and GenBank databases using the program ENTREZ. Multiple align-

ment of sequences was carried out by using the CLUSTAL W program package (Thompson *et al*., 1994).

Paclitaxel production. Fermentations were carried out in 500 L fementers containing 300 L of the following production medium: Morsuit 25 g/L, dextrin 10 g/L, soytone 15 g/L, MOPS 5 g/L, K_2HPO_4 0.5 g/L. Two stages of the seed phase were used: for the first stage four 14 days cultures of strain P&U 22869 on slants of ISP Medium 3 were used to inoculate 2 L baffled glass bottles containing 0.5 L of the following medium: cornsteep liquor 10 g/L, caseine 10 g/L, dextrin 20 g/L, $(NH_4)SO_4$ 1 g/L, $CaCO_3$ 5 g/L, K_2HPO_4 0.1 g/L, deionized water to 1000 mL. The bottles were incubated at 27 °C for 48 h on a rotary shaker, 130 rpm. The seed cultures obtained were pooled and 3.5 L of this pool were transferred into a 200 L fermenter containing 100 L of the same medium. The process was carried out at 27 °C for 48 h, with agitation at 150 rpm remote control and an air flow at a rate of 68 ml/min. 15 L of the second seed phase were used to inoculate the production medium. After 120 h of incubation at the same conditions previously described, the mycelium was removed from the culture by continuous centrifugation with a centrifugal separator and 100 L of the clear supernatant applied onto a XAD-2 column (30 L).

After the absorption, the resin was washed with 90 L of water (discarded) and eluted with ethanol (60 L). The ethanol extract was concentrated under reduced pressure to a volume of 10 L and extracted twice with n-hexane (discarded) and twice with dichloromethane.

The dichloromethane crude extract was dried, resuspended to a 30 mL volume with methanol and partially purified using C18 disposable cartridges containing 10 g of sorbent. Aliquots of the extract (6 mL) were loaded on each cartridge. The cartridges were eluted with 50 mL of Milli-Q grade water and 100 mL of methanol at increased concentrations (20%, 50%, 80% and 100%).

Paclitaxel and taxanes quantification. Paclitaxel and taxanes in the extracts were detected and assayed by enzyme immunoassay kits (Indirect Competitive Inhibition Enzyme Immunoassay - CIEA, Hawaii Biotechnology Group Inc., USA) according to the procedure described by the suppliers (Grothaus *et al*., 1995; Caruso *et al*., 2000).

To quantify the putative paclitaxel in methanol samples, HPLC analyses were performed on a reversed-phase C18 column-5µm-25×0.45 cm using the following elution program:

– from 0 to 20 min isocratic at 30% HPLC grade acetonitrile in Milli-Q grade water;
– from 20 to 25 min linear gradient from 30% to 50% acetonitrile in water;
– from 25 to 40 min isocratic at 50% acetonitrile in water;

The column was maintained at 32 °C and eluted at a flow rate of 2 mL/min.

A standard curve of paclitaxel concentration versus peak area was used to quantify paclitaxel in samples.

Spectroscopic analysis. Two millilitres of the methanol sample containing the putative paclitaxel were dried, resuspended in 20 mL of methanol and further purified by HPLC. Fractions were collected every minute from the outlet of the HPLC system; the resulting 40 fractions were monitored for paclitaxel content

with the monoclonal antibody assay, that showed paclitaxel in one single fraction eluted from the HPLC column between 27 and 28 minutes with an estimated concentration of 1.3 µg/L referred to the original culture volume (300 L). The sample was subjected to RP-HPLC using a 2.0x250 mm, pentafluorophenyl 5 µm column. Mobile phases A and B were respectively:

– phase A: Milli-Q grade water containig 0.1% of acetic acid glacial, 99.99%;
– phase B: acetonitrile HPLC grade containing 0.04% of acetic acid glacial, 99.99%.

Elutions were carried out with a step gradient of B from 46.7% to 57.3% in 47 minutes at a flow rate of 0.165 mL/min. Separations were performed at room temperature and elution profiles were monitored with a diode array detector at the wavelength of 227 nm.

On-line RP-HPLC/electrospray mass spectrometry was performed on a single quadrupole instrument equipped with an electrospray interface. Eluates from RP-HPLC were directly injected into the ion source of the mass spectrometer. The electrospray potential was approx. 6 kV. The quadrupole mass analyzer was set up to scan over a mass-to-charge ratio (m/Z) from 150 to 920 Da, at 0.5 s per scan. The sum of data acquired constituted the final spectrum. Mass calibration was performed with a mixture of valine, tri-tyrosine and hexa-tyrosine.

Synthesis experiments with ^{3}H-Baccatin. The radioactive paclitaxel precursor ^{3}H-Baccatin (0.74 MBq, specific activity 1.83 MBq/mg) was added to 50 mL of liquid productive culture of the strain P&U 22869 after 48 h of incubation in 300 mL Erlenmyer flask under aerobic conditions at 27 °C, 250 rpm. After additional 120 h of incubation, the culture was centrifuged and the mycelium extracted three times with MeOH. The organic solvent was evaporated under reduced pressure and the aqueous residue was added to the clear supernatant. The mixture was loaded onto two Extrelut 20 (diatomaceous earth) columns (E. Merck, Germany). Each column was extracted with 100 mL of n-hexane (discarded) to remove the fatty components and then with 100 mL of CH_2Cl_2. The CH_2Cl_2 extract was dried under reduced pressure, added with unlabeled paclitaxtel (50 µg) then submitted to chromatographic purification on TLC plates silica gel 60 (E. Merck) in two TLC systems. The first run was in n-hexane:acetone (1:1). The band corresponding to paclitaxel (Rf 0.45) was scraped, eluted with MeOH:CH_2Cl_2 (1:1), concentrated and submitted to the second chromatographic run in CH_3CN:CH_2Cl_2 (35:65). The band corresponding to paclitaxel (Rf 0.35) was eluted and concentrated. The paclitaxel-containing band was further purified by injection in HPLC. The peak corresponding to paclitaxel was recovered and its radioactivity determined by liquid scintillation.

Synthesis experiments with L-[2,3,4,5,6-^{3}H] phenylalanine. The labeled precursor L-[2,3,4,5,6-^{3}H] phenylalanine (4.44 MBq, specific activity 5.11 TBq/mmol) was added to the same strain culture after 48 h of incubation, as previously described. After additional 48 h of incubation the culture was centrifuged and the supernatant extracted by Extrelut column as previously described.

Unlabeled Baccatin III and paclitaxel 10 µg each were added to the crude extract which was then submitted to two steps of TLC purification. First system: TLC plates silica gel 60 (E. Merck) developed in n-hexane:acetone (1:1).The bands corresponding to Baccatin III (Rf 0.55) and paclitaxel (Rf 0.55) were

scraped, eluted and submitted to a second purification step on TLC run in CH_3CN:CH_2Cl_2 (35:65). Radioactivity of the Baccatin III (Rf 0.45) and paclitaxel (Rf 0.35) spots were determined by liquid scintillation counting.

RESULTS

Morphological and physiological characteristics

Substrate mycelium of strain P&U 22869 developed with densely branched hyphae 0.5 – 0.8 μm in diameter that do not bear spores. Aerial growth consisted of straight or slightly flexuous unbranched long spore chains developed on the colony surface (Fig. 1 and 2). Emerging hyphae from substrate mycelium start the spore differentiation: the spores are globose, oblong, of irregular length (0.8 – 1 × 0.8 – 1.2 μm) with smooth surface (Fig. 3).

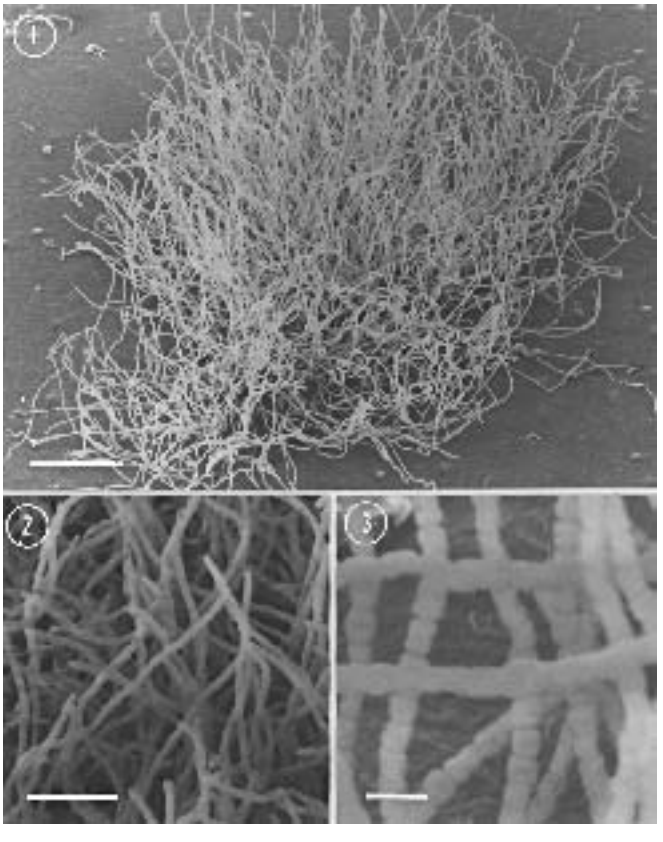

FIG. 1-3 – Morphology of *Kitasatospora* sp. P&U 22869: colony grown on water agar for 30 days (Fig. 1), evidence of spore chain length (Fig. 2) and particular of spore morphology (Fig. 3). Marker equals to 40 μm, 10 μm and 2 μm respectively in Fig. 1, 2 and 3.

TABLE 1 – Cultural and morphological characteristics on different media of strain P&U 22869

Medium	Growth	Spore mass	Substrate mycelium
Water agar (WA25)	++	gray	colourless
Yeast extract-malt extract agar (ISP2)	+++	gray	brown
Oatmeal agar (ISP3)	+++	gray	gray
Inorganic salts-starch agar (ISP4)	+++	gray	beige
Glycerol-asparagine agar (ISP5)	+++	gray	light gray
Tyrosine agar	+++	gray	light gray
Nutrient agar (NA)	+++	gray	white
Bennet's agar (BA)	+++	gray	beige
Czapek agar (CA)	+++	gray	gray

++ = good; +++ = abundant.

The cultural characteristics on tested media are shown in Table 1. The strain grew well on all organic and synthetic media. Substrate mycelium was variable in colour and the reverse colour of colonies was not distinctive. Aerial sporulation was present on all substrates, the spore mass was gray in every condition, the melanin and the diffusible pigments were not produced in different media. The optimun range of growth temperature was 24-28 °C.

Glycerol, D-xylose, galactose, glucose, mannose, N-acetyl-glucosamine, amygdalin, arbutin, cellobiose, maltose, sucrose, trehalose, starch, glycogen, gentobiose and gluconate were utilized as carbon sources.

The enzymatic activities detected in strain P&U 22869 mycelium, by API Zym assay, were the following: phosphatase alcaline, esterase, esterase lipase, leucine arylamidase, phosphatase acid, naphthol-AS-B1-phosphoydrolase, β-galactosidase, β-glucosidase.

The strain growth was inhibited by hygromycin, chloramphenicol, spectinomycin, streptomycin, thiostrepton, apramycin, eritromycin, neomycin, daunomycin and tylosin and not by ampicillin and novobiocin.

The analysis of the cell wall showed L-diaminopimelic acid in aerial spores and meso-diaminopimelic acid in vegetative mycelia. Whole-cell hydrolysates were characterized by the presence of galactose and absence of madurose, arabinose and xylose.

Table 2 shows some morphocultural and physiological characteristics of the strain P&U 22869 and the representative strains of the nine valid *Kitasatospora* species and three other strains belonging to invalid *Kitasatospora* species *(K. kifunense, K. brunnea* and *K. melanogena*). The strain P&U 22869 morpho-physiological profile does not correspond exactly to any one of the described species. Morphologically the species with the highest similarity to P&U 22869 is *K. griseola.* This shows rectiflexibiles spore chains and a gray spore mass color but pro-

TABLE 2 – Comparison of significative morpho-physiological characteristics and percentage of 16S rDNA sequence identity between strain P&U 22869 and *Kitasatospora* species

Characteristics	ceppo P&U 22869	*K. brunnea* IFO 14627[4]	*K. paracochlea* IFO 14769[4]	*K. griseola* IFO 14371[4]	*K. cochleata* IFO 14786[4]	*K. phosalacinea* IFO 14372[4]	*K. setae* IFO 14216[4]	*K. cheerisanensis* KCTC 2395[1]	*K. melanogena* IFO 14327[4]	*K. cystarginea* IFO 14836[2]	*K. kifunense* IFO 15206[4]	*K. mediocidica* IFO 14789[3]	*K. azatica* IFO 13803[4]
Spore chain morphology	rf	rf	s	rf	s	rf	rf	rf	rf	s	hs	ra	rf
Spore mass color	gy	w	gy	gy	gy	w	w	g/y	w	gy	gy	gy	w
Soluble pigment	–	yb	yb	pink	yb	–	–	–	br	–	–	br	–
Reduction of nitrate	+	+	+	–	–	+	+	+	+	–	–	–	+
Hydrolysis of starch	+	+	+	+	+	+	+	+	+	+	–	–	+
Liquefaction of gelatin	–	–	–	–	–	–	–	+	–	–	–	–	–
Formation of melanoid pigment	–	–	+	–	+	–	–	–	+	–	+	–	–

rf = *rectifexibiles*; s = *spirales*; ra = *retinaculiaperti*; gy = gray; w = white; g/y = green / yellow; yb = yellow brown; br = brown.
1= data from Chung *et al*. (1999); 2= data from Kusakabe and Isono (1988); 3= data from Labeda (1988); 4= data from Nakagaito *et al.* (1992).

(*Continued*)

TABLE 2 – Comparison of significative morpho-physiological characteristics and percentage of 16S rDNA sequence identity between strain P&U 22869 and *Kitasatospora* species (*follow the previous page*)

Characteristics	ceppo P&U 22869	*K. brunnea* IFO 14627[4]	*K. paracochlea* IFO 14769[4]	*K. griseola* IFO 14371[4]	*K. cochleata* IFO 14786[4]	*K. phosalacinea* IFO 14372[4]	*K. setae* IFO 14216[4]	*K. cheerisanensis* KCTC 2395[1]	*K. melanogena* IFO 14327[4]	*K. cystarginea* IFO 14836[2]	*K. kifunense* IFO 15206[4]	*K. mediocidica* IFO 14789[3]	*K. azatica* IFO 13803[4]
Utilization of carbohydrate:													
L-arabinose	–	+	–	+	+	+	+	+	+	–	+	+	+
D-fructose	–	+	+	–	–	+	–	–	+	–	–	–	+
inositol	–	–	–	–	–	–	–	–	–	–	–	–	–
D-mannitol	–	–	–	–	–	–	–	–	–	–	+	–	–
raffinose	–	–	–	+	+	+	–	–	–	–	–	–	–
L–rhamnose	–	–	–	–	–	+	–	+	–	–	–	–	–
sucrose	+	+	–	–	–	+	–	–	+	–	+	–	–
D-xylose	+	+	–	+	+	+	+	+	+	–	+	–	+
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Accession number		U93314	U93328	AB022870	AB022871	AB022869	U93332	AF050493	U93326	AB022872	U93322	U93324	U93312
Identity of 16S rDNA sequence		98.98%	98.91%	98.57%	98.57%	98.37%	97.84%	97.69%	96.83%	96.96%	96.56%	96.42%	96.29%
n° bases compared		1476	1476	1476	1476	1476	1483	1477	1483	1483	1483	1483	1483

5’AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATG
CAAGTCGAACGGTGAAGCCCTTCGGGGTGGATCAGTGGCGAACGGGTGAG
TAACACGTGGGGAATCTGCCCTGAACTCTGGGACAAGCCTTGGAAAC
GAGGTCTAATACCGGATACGACCTTCTCCTGCATGGGGGTTGGTG
GAAAGCTCCGGCGGTTCAGGATGATCCCGCGGCCTAT
CAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGG
TAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCT
GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTG
TAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAA
GAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGC
GAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCG
TAGGCGGCCTGTCGCGTCGGATGTGAAAGCCCGGGGCT
TAACCCCGGGTCTGCATACGATACGGGCAGGCTGGAGTGTGGTAGGGGA
GATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAA
CACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGC
GAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCG
CAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGC
TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAG
CATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACA
TACGCCGGAAACTGGTAGAGATATCAGCCCCCTTGTGGTCGGTGTA
CAGGGTGGTGCATGGTTGTCGTCAAGCTCGTGTCGTGAGATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCGAG
TAATGTCGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAG
GAAGGTGGGGACGGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTGC
CACGTGCTACAATGGCCGGTACAAAGGGCTGCGATGCCGCGAGGCGGAGC
GAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCC
CATGAAGTTGGAGTTGCTAGTAATCGCAGATCAGCATGCTGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAA
CACCCGAAGCCGGTGGCCTAACCCGTAAGGGGAGGAGCCGTCGAAGGTGG
GACCAGCGATTGGGACGAAGTCGTAACAAGGGCCCC3’

FIG. 4 – Strain P&U 22869 16S ribosomal RNA gene, partial nucleotide sequence (1480 nucleotides).

duces a pink diffusible pigment typical of this species and absent in the studied strain. This latter showed also a very different physiological profile from *K. griseola* and all the other considered *Kitasatospora* species.

16S rDNA gene sequence

An almost complete 16S rDNA sequence was determined for the strain P&U 22869 (Fig. 4): this sequence compared to the others included in the EMBL and GenBank databases showed that among the 30 nearest neighbours strains, 26 belong to the *Kitasatospora* genus. The first strain belonging to a different genus was a *Streptomyces* sp. placed 17th (97.80% identity in 1456 nucleotide overlap).

The strain P&U 22869 16S rDNA sequence did not correspond exactly to any one of the described valid and invalid *Kitasatospora* species (Table 2), showing the highest identity to *K. brunnea* (98.98% in 1476 nucleotide overlap).

Strain P&U 22869 can be assigned to the genus *Kitasatospora*, but none of the morphological, physiological or genomic profiles corresponds exactly to any described species of the genus.

Paclitaxel production by fermentation

The presence of paclitaxel in the actinomycete extract has been confirmed by comparative chromatographic behaviour with yew paclitaxel, mass spectrometry and reactivity with paclitaxel-specific monoclonal antibodies. At end of the extraction procedure of the supernatant from 100 L of the culture obtained in 500 L fermenter the analysis with the polyclonal antibodies confirmed that all taxanes had selectively been eluted from the C-18 cartridges in the 80% methanol extracts.

By further purification of this methanol extract by HPLC, the presence of paclitaxel was revealed in one fraction eluted from the HPLC column between 27 and 28 minutes. A concentration of 1.3 µg/L of paclitaxel in the original culture volume (300 L) was estimated with the monoclonal antibody assay.

Liquid chromatography-mass spectrometry (LC-MS) analysis of this fraction confirmed the presence of paclitaxel, by evidentiating a peak with the same RT, UV spectrum and mass spectrum of authentic paclitaxel. In particular the MS analysis evidentiated the fragment at *m/z* 268, 286 (C-13 side-chain fragments), 509, 551, 569 (taxane ring fragments), 776 and 854 [MH^+]. The concentration of paclitaxel determined in HPLC in comparison with a paclitaxel calibration curve corresponded to 1 µg/L referred to the total volume of the culture (300 L).

Evidence of *de novo* paclitaxel biosynthesis

The low quantity of paclitaxel produced by the strain P&U 22869 required further investigation to demonstrate this particular biosynthesis and two experiments with the radioactive precursors L-[2,3,4,5,6-^{3}H] phenylalanine and ^{3}H-Baccatin demonstrated the *de novo* biosynthesis of paclitaxel.

In the first experiment where the labeled precursor L-[2,3,4,5,6^{3}H] phenylalanine was supplemented to the cultures of the microorganism, the incorporated radioactivity determined by liquid scintillation in the paclitaxel peak corresponded to 312 dpm; in the same experiment baccatin III was extracted and purified with the same procedure. Radioactivity found in the HPLC peak corresponded to the background (28 dpm). The recovery of unlabeled baccatin was significant because it excluded the possibility that paclitaxel could be labeled by exchange with the tritiated water eventually present in the medium. In the second experiment with the ^{3}H-Baccatin supplied to the culture, the radioactivity incorporated in paclitaxel was 46.6 Bq/50 mL of culture, corresponding to an incorporation of 510 ng/L of labeled baccatin III and to a production of 742 ng/L of paclitaxel. The recovery of the added unlabeled paclitaxel was 73% and the final estimated production of paclitaxel was of 942 ng/L.

A 50 mL-culture supplemented of unlabeled paclitaxel (100 mg), submitted to extraction and purification procedures as previously described, showed a paclitaxel recovery of 73 mg, determined in comparison with a calibration curve.

DISCUSSION

The strain P&U 22869 was isolated from the inner cortical tissues of a yew-tree and gave positive results during a screening program, based on immunological tests, looking for microorganisms producing taxane (Caruso *et al.*, 2000). Since until now no strain belonging to actinomycete group was described as microor-

ganism able to produce paclitaxel, this strain was selected to be thoroughly examined in terms of taxonomic characteristics and metabolic behaviour.

The strain P&U 22869 on the basis of morphologic characterization, cell wall composition and 16S rDNA sequence, was assigned to the genus *Kitasatospora*. The genus was proposed by Omura *et al.* in 1982 for actinomycete strains which were phenotypically similar to *Streptomyces* strains but contained LL-DAP in aerial spores and meso-DAP in the vegetative mycelium (Takahashi *et al*., 1983). The *Kitasatospora* species are also characterized by their resistance to wide host range *Streptomyces* phages, by the formation of aerial growth constituted only by chains of spores and by the formation of submerged spores also in liquid culture, three features rarely observed in *Streptomyces* species. The morphological characteristics of the *Kitasatospora* species are similar to *Streptomyces* ones and the type strains were described adopting the same criteria.

During past years the name of this genus was also subjected to some distortions and some species were wrongly described as belonging to *Kitasatosporia*. At present, after some taxonomic reviews, the genus *Kitasatospora* includes nine valid species (Takahashi, *et al.,* 1999; Zhang *et al*., 1997), some of which produce particular antibiotics among them, the strain MF730-N6, classified as *Kitasatosporia griseola*, also produces a diterpenoid antitumor antibiotic (Tamamura *et al*., 1985).

The attempt to identify the strain P&U 22869 at the species level only on the basis of the 16S rDNA sequence gave no positive results because of the small differences existing among the type strains and the considered strain. In seven cases the identity level was greater than 97% consequently requiring the DNA-DNA homology tests to identify the strain P&U 22869 at the specie level (Stackebrandt and Goebel, 1994).

K. brunnea is the more similar species to the strain P&U 22869 but considering some differences of the morphocultural characteristics they probably belong to different species. The studied strain can be clearly differentiated also from the other nine valid *Kitasatospora* species (Table 2). Nevertheless, further analyses (DNA-DNA homology, menaquinone contents, mol% G+C. etc.) to propose the strain P&U 22869 as a new species can be done.

Also considering the low level of paclitaxel production ascertained for the strain P&U 22869 it is important to point out the particular metabolic activity showed by an endophytic actinomycete of *Taxus* as already reported for endophytic fungi isolated from the same source. These results can be considered the starting-point for further research directed to improve by different approaches, including genetic engineering technologies, the production of a typical vegetal compound by an actinomycete.

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