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Antibacterial activity of *Penicillium* spp. strains isolated in extreme environments

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Abstract *Penicillium* spp. isolated from the sediments of ponds in continental Antarctica have been studied and compared with species obtained from similar environments in the Apennines of the Liguria region, in northwestern Italy. The antibacterial activity exhibited by some strains was evaluated against standard strains using both liquid and solid media. The preliminary data suggest an antibacterial activity similar to β-lactam antibiotics.

Introduction

Fungi collected from the sediments of ponds in continental Antarctica were studied as part of the Italian National Antarctic Research Program (P.N.R.A.).

Our observations were supplemented with data referred to samples obtained from wooden baits sunk to a depth of 50 m in the Ross Sea during the 1993/1994 campaign. Several species of *Penicillium* were isolated in this extreme habitat. This finding is unusual for other habitats (Rudolph 1970 in McRae et al. 1999; Fletcher et al. 1985; Del Frate and Caretta 1990; Kerry 1990a, b; Onofri et al. 1994) and sporadic for similar habitats (Sugÿama et al. 1967; Sun et al. 1978) on the same continent. Some of the species of *Penicillium* isolated are the first recorded in the Antarctic (Montemartini Corte and Liotta 1999), while others were interesting from an ecological or systematic point of view.

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The latter group included many strains of *Penicillium chrysogenum*. Two of these, TF3/3 and TF4/1, were different from all the others, which were all similar to one another and identical to the *P. griseoroseum* neotype (MUCL 29133 NT). Following the exhaustive paper by Frisvald and Filteborg (1989b), all these *Penicillium* strains should be considered as strains of *P. chrysogenum*. Given the high variability of *P. chrysogenum* (Bridge et al. 1989) and the abundance in the Antarctic of this *Penicillium* group, in our study we concentrated more on strains of *P. chrysogenum* than on other species.

Many different species of *Penicillium* from these extreme habitats were isolated. We believe that they are adventitious world-wide species (Montemartini Corte and Gestro 1994), perhaps also to be found in the sub-Antarctic region (Corte and Daglio 1963), and that they are in the process of adapting to the Antarctic environment. We therefore decided to examine the extent of their adaptation by considering the production, if any, of secondary metabolites by comparison with type (T) strains and strains originating from the Italian Apennines (Lake Riane, Montemartini Corte and Piccardo Vajra 1988), and from a *Tamus* rhizosphere (Montemartini Corte 1989).

Materials and methods

Strains

The geographical coordinates, the height above sea level, and the size and some characteristics of the sampling areas (Guilizzoni et al. 1991) are shown in Table 1. Table 2 shows the species and characteristics of the strains.

The Antarctic strains came either from ponds or from wooden baits sunk in the Ross Sea. The strains have been isolated with a higher concentration of penicillin, streptomycin and actidione than for fungi coming from environments other than the Antarctic. For each strain the initial letters of the sites, followed by the numbers corresponding to the single strains or different isolations referred to the same site, the year of isolation for each site and the depth of sampling are indicated.

Table 1 Geographical coordinates and characteristics of collection sites (*F.s.l.* from sea level)

Collection sites	Coordinates	F.s.l	m^2	Biological characteristics
Pozza Eneide (E)	74°42′S 164°06′E	55	400	Near the Italian base frequented by skua
Gondwana (G)	74°37′S 164°13′E	85	3000	Near the German base frequented by skua
Skua Lake (S)	74°42′S 164°07′E	120	2200	Frequented by skua
Inexpres. Island (II)	74°55′S 163°40′E	26	15000	Limpid water
Tarn Flat (TF)	75°01′S 162°39′E	23	56300	Few lichens on the borders
Ross Sea (R)	74°46′S 164°02′E			Wood baits
Apennines Riane Lake (AL)	44°33′N 09°28′E	1279	10000	Advanced filling of earth
Apennines Pedemonte (AP)	44°30′N 08°57′E	140		Rhizosphere of *Tamus* in forest

Table 2 Species and characteristics of strains. *Capital letters* correspond to collection sites. In *bold type* are: type (*T*) strains of *Penicillium chrysogenum* MUCL 29079, the Raper strain isolated from cantaloup IMI 340233, from which many strains that are highly penicillin-producing have been obtained; the neotype strain (*NT*) strain of *Penicillium griseoroseum* MUCL 29133; type strain of *Penicillium melinii* MUCL 1394 T; type strain of *Penicillium spinulosum* MUCL 1394 T, MUCL 31118, MUCL 31119, AL Ca-30 of Apenninic lake; MUCL 31126 and AP 1 TN II of rhizosphere of *Tamus* are reported for comparison (*MUCL* = Mycothèque de l' Université Catholique de Louvain, Belgium; *IMI* = International Mycological Institute, Egham, UK)

Species	No. and sites of isolation	Years of isolation	Depth	*Escherichia coli*	*Staphylococcus aureus*	*Micrococcus*
Penicillium chrysogenum	MUCL 29079 T	1904			++	++
P. chrysogenum	IMI 340233	1943		−	+	+
P. chrysogenum	TF 3/3	1990–1991	−30 cm	++	++	++
P. chrysogenum	TF 4/1	1990–1991	−40 cm	−	+	±
P. chrysogenum	MUCL 29133 NT	1924		−	+	++
P. chrysogenum	MUCL 31119	1986–1987	−35 cm	−	+	+
P. chrysogenum	AL Ca-30	1986–1987	−30 cm	−	+	+
P. chrysogenum	MUCL 31118	1986–1987	−25 cm	−	+	++
P. chrysogenum	TF 3/1	1990–1991	−30 cm	+	±	+
P. chrysogenum	TF 4/5	1990–1991	−40 cm	−	+	+
P. chrysogenum	II 4/2	1990–1991	−40 cm	+	+	±
P. chrysogenum	R9	1993–1994	−50 m	−	++	+
P. chrysogenum	II 6/1	1990–1991	−60 cm	+	±	±
P. chrysogenum	En 1	1990–1991	−10 cm	−	−	−
P. chrysogenum	G3/2	1993–1994	−30 cm	−	+	+
P. chrysogenum	R23	1993–1994	−50 m	±	+	++
P. chrysogenum	R28	1993–1994	−50 m	±	++	++
P. chrysogenum	R31	1993–1994	−50 m	±	++	++
P. chrysogenum	R34	1993–1994	−50 m	±	++	++
P. chrysogenum	R36	1993–1994	−50 m	±	+	++
P. chrysogenum	R38	1993–1994	−50 m	±	++	++
P. citrinum	S1/4R	1990–1991	−10 cm	±	±	+
P. citrinum	S1/4 bis	1990–1991	−10 cm	±	±	++
P. roseopurpureum	S1/3 bis	1990–1991	−10 cm	±	±	±
P. waksmanii	S1/5	1990–1991	−10 cm	±	+	±
P. waksmanii	II/3	1990–1991	−60 cm	±	±	±
P. waksmanii	G3/17	1993–1994	−30 cm	±	±	±
P. waksmanii	G3/18	1993–1994	−30 cm	±	+	±
P. montanense	S3/9	1990–1991	−10 cm	±	±	±
P. montanense	En/11	1990–1991	−20 cm	+	+	±
P. melinii	MUCL 29082 T	1930		+++	+++	++
P. melinii	R55	1993–1994	−50 m	++++	++++	+++
P. jensenii	G3/10	1993–1994	−30 m	+	−	−
P. aurantiogriseum	R14	1993–1994	−50 m	−	−	−
P. miczynskii	MUCL 31126	1986–1987	−30 m	−	−	−
P. spinulosum	MUCL 1394 T	1910		+	++++	+++
P. spinulosum	AP 1 TN II	1986–1987	−30 cm	+	+++	++++

The Italian strains came from a pond in the Ligurian Apennines: a first strain, MUCL 31119, came from Lake Riane; a second strain, AL-Ca-30, was isolated in a transition zone, and a third strain, MUCL 31118, from a moist meadow. Two more strains, MUCL 31126 and AP-1 Tn II, came from a *Tamus* rhizosphere in a deciduous forest in the Apennines. Determination of the Antarctic strains using the usual methods (Pitt 1979) sometimes encountered some difficulties due to their physiological and morphological adaptation. *P. citrinum*, for instance, well known as a species unable to survive at 5°C, has instead always developed, albeit slowly, at this temperature, with some slight microscopic modifications as compared with the original type and description. Twelve strains of *P. griseoroseum*, five strains of *P. citrinum* and three strains of *P. purpurescens* have been omitted from the table since their activity was found to be very weak.

P. miczynskii strains do not produce this kind of secondary metabolite, as already shown with some strains studied by Christensen et al.

The following standard strains were employed to test the antibacterial activity: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29212, *Micrococcus luteus* ATCC 4698. Some clinical isolates producing extended spectrum β-lactamase were also used.

Media

The fungus strains were cultivated in czapek yeast extract agar (CYA) (Pitt 1979) and carrot agar (CA) (Montemartini Corte 1991) and incubated at 19°C. The bacterial strains were grown in a Brain Heart Infusion Broth (BHIB) and agar (BHIA) (Difco), Muller Hinton Agar (MHA) (Difco) and a specific medium for inducing Penicillium Broth; Penicillium Agar and beta-lactamase genes (Sylvester and Coghill 1954; Martin and Demain 1980; Turner 1994).

Methods

Two different methods were used to assess the antibacterial activity of the fungus products.

Method 1

The initial screening of the fungus-producing secondary metabolites with antibacterial activity was carried out according to Crueger and Crueger (1989). The fungi being tested were inoculated onto the surfaces of PA plates and incubated for 8 days at 19°C. At the end of this period 20 µl of a stationary bacterial culture (optical density 0.800 at 650 nm) was added to 3 ml of soft agar and poured onto the fungus culture. The clear halo (ø) surrounding the fungal spot was measured and the antibacterial activity was then evaluated semi-quantitatively as follows: ($\varnothing \leq 2$ mm) no activity; +/– ($2 > \varnothing \geq 10$ mm) very slight activity; + ($11 \geq \varnothing \geq 15$ mm) slight activity; ++ ($16 \geq \varnothing \geq 25$ mm) moderate activity; +++ ($26 \geq \varnothing \geq 31$ mm) good activity; ++++ ($32 \geq \varnothing$ mm) excellent activity.

The results are reported in Table 2.

Method 2

The production of fungus metabolites in liquid cultures was determined as follows. Sixty-five milliliters of PB medium in a 300-ml flask was inoculated with a 10-day-old fungus culture grown in a CA or CYA medium. The flasks were incubated at 19°C and samples were tested at different intervals in order to determine the inhibitory activity of the fungus metabolites (Sylvester and Coghill 1954). Before plating, the samples were filtered through a 0.2-nm filter. Bacterial soft agars were performed as before using 5-µl cultures, and minimal inhibitory concentrations (MICs) of liquid samples were performed according to Lorian (1991). The results were read after 24 h at 37°C (Table 3).

Results and discussion

The results of the preliminary screening of *Penicillium* strains are given in Table 2. It can be seen that strains *P. chrysogenum* TF 3/3 and *P. melinii* R 55 inhibit *E. coli* as well as gram-positive bacteria. The *P. spinulosum* AP 1TN II strain has an excellent activity on gram-positive bacteria, while the majority of the other strains show greater activity on gram-positive bacteria even if not at high levels. The antibacterial activity of liquid fungus cultures (Sylvester and Coghill 1954) was assessed after 11 and 21 days. These intervals were chosen on the basis of the vegetative multiplication of the single fungus strain rather than the pH values, which were very variable even within the same species (*P. chrysogenum*).

MICs on liquid samples were also performed for some strains, the results of which are shown in Table 3. It can be seen that their efficacy is greater on gram-positive strains. In many tests the inhibitory activity was

Table 3 Minimal inhibitory concentrations (MIC) of liquid cultures of *Penicillium* strains (*0* no activity)

Species	Strains	*Escherichia coli*	*Staphylococcus aureus*	*Micrococcus*
Penicillium chrysogenum	MUCL 29079 T	0	0	1:32
P. chrysogenum	TF 3/3	1:4	1:4	1:4
P. chrysogenum	MUCL 29133 NT	0	0	1:32
P. chrysogenum	MUCL 31119	0	1:64	1:64
P. chrysogenum	Al Ca-30	0	1:64	1:64
P. chrysogenum	MUCL 31118	0	1:4	1:32
P. chrysogenum	G 3/2	0	1:64	1:32
P. chrysogenum	R 9	0	1:4	1:8
P. chrysogenum	R 28	0	1:4	1:16
P. chrysogenum	R 31	0	1:4	1:32
P. chrysogenum	R 34	0	1:8	1:32
P. chrysogenum	R 36	0	1:2	1:16
P. chrysogenum	R 38	0	1:4	1:32
P. citrinum	S1/4R	0	1:16	1:16
P. citrinum	S1/4 bis	0	1:8	1:16
P. roseopurpureum	S1/3 bis	0	1:16	1:32
P. waksmanii	S1/5	1:2	1:4	0
P. waksmanii	II/3	1:2	1:2	1:2
P. waksmanii	G3/17	1:2	1:4	0
P. waksmanii	G3/18	1:4	1:2	0
P. montanense	En 11	0	0	1:8
P. melinii	MUCL 29082 T	1:4	1:4	1:32
P. melinii	R 55	1:8	1:4	1:64
P. spinulosum	MUCL 1394 T	1:2	1:2	1:2
P. spinulosum	AP 1 Tn II	1:2	1:2	1:2

found to be very low, but we decided to exclude a non-specific toxic activity, since this would have been evident for all the strains tested.

The TF 3/3 strain of *P. chrysogenum* differed from the type strain in that it produced a red-brown pigmentation, and also in the presence of activity in respect of gram-negative bacteria. These preliminary data suggest, for this activity, the production of beta-lactam antibiotics. The prevalence in the Antarctic environment of gram-negative bacteria could have led to selective pressure on this pattern of activity.

The *P. waksmanii* strains exhibited a very weak antibacterial effect.

The excellent activity of *P. melinii* might be attributable to the production of patulin, as already seen for the type strain (Frisvald and Filteborg 1989a). The Apenninic strain *P. spinulosum* also showed an antibacterial activity, but it was different in some ways from that of the type strain.

These preliminary data enable us to conclude that, except for two strains, there are no remarkable differences among the strains coming from a temperate environment (Ligurian and type strains) and those coming from an extreme environment. The reason might be that strains isolated from the Antarctic could have been brought there by birds or in foodstuffs, or they could be part of a spore bank (Corte and Daglio 1963) which reached this continent from a place characterised by milder temperatures.

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