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Radamycin, a Novel Thiopeptide Produced by *Streptomyces* sp. RSP9

I. Taxonomy, Fermentation, Isolation and Biological Activities

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The newly isolated strain *Streptomyces* sp. RSP9 produces two thiopeptides; one of them is methylsulfomycin I, which shows potent antibiotic activity against several Gram-positive bacteria such as *Micrococcus luteus* and *Staphylococcus aureus*. The other is a new thiopeptide named radamycin. In the present work, this compound was purified and tested against several microorganisms and no antibiotic activity was detected in the assays. However, it does have a very strong capacity as an inducer of the *tipA* promoter, and indeed is the first reported molecule with *tipA* promoter induction capacity without detectable antibiotic activity. Induction of the *tipA* promoter also occurs with methylsulfomycin I.

Cyclic thiopeptide antibiotics such as promoinducin, promothiocin, sulfomycin, thiopeptin, thiostrepton *etc*, are composed of oxalazole and/or thiazole rings with modified amino acids and a linear peptide containing dehydroalanines[1]. These antibiotics are active against Gram-positive bacteria and their mechanism of action is not well understood. The one most studied, thiostrepton, acts by inhibiting translation through its binding to the large subunit of the prokaryotic ribosome. Resistance to thiostrepton is generated by a methylase and has been an important selection marker in *Streptomyces* cloning vectors (compiled in[2]). Most of these antibiotics induce the expression of the *tipA* promoter, isolated from *Streptomyces lividans*, which has been used to develop usefull expression systems in these microorganisms[3]. Promoter induction "*in vivo*" has also been used to isolate new thiopeptides such as geninthiocin, promothiocin and promoinducin[4~6]. Thompson's group has reported the formation of irreversible thiopeptide-antibiotic-protein complexes with the TipAS protein and have suggested that the minimal motif for TipA recognition is the linear peptide that extends the thiazoles and oxazoles attached to positions 4 or 5 of the pyridyl group[1].

Although the main use of these antibiotics has been as growth promotants for animals[7,8], they have lately attracted the interest of researchers and pharmaceutical companies as new tools against methicilin-resistant (MRSA) and/or vancomycin-resistant (VR) *Staphylococcus aureus* infections[9].

Here we describe the isolation and identification of strain *Streptomyces* sp. RSP9, its fermentation conditions, the isolation and biological activities of two thiopeptide compounds produced by this strain, and the capacity for *tipA* promoter induction by these compounds. One of

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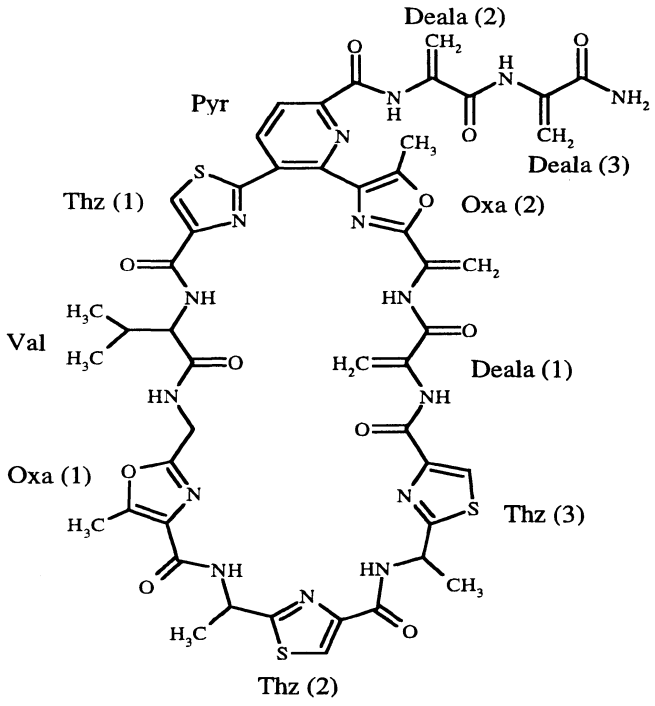
the molecules is the recently described antibiotic methylsulfomycin I[9] and the other is a novel that we have named radamycin (Fig. 1). The structure of both compounds was determined and that of radamycin is described in the accompanying paper[10].

Fig. 1. Structure of radamycin.



Materials and Methods

Microorganisms and Media

Streptomyces sp. RSP9 spores were harvested from R2YE or from SFM solid media[2] and used to inoculate (10^6 spores/ml) different liquid media to study antibiotic production. Fermentations were carried out in different modifications of medium YES (1% Yeast extract, 10.3% sucrose [pH 7.2] supplemented with 0.5% xylose and 5 mM $MgCl_2$). Cultures were incubated at 28°C for 4~7 days on a rotary shaker at 200 rpm. Other media, such as SPG[11] or Trypticase Soy Broth (TSB)[2], were used in some experiments. Normally, three baffled 100 ml flasks containing 10 ml of media were used.

Taxonomy

Streptomyces sp. RSP9 was identified by morphological, physiological and molecular characterisation methods. Total DNA was obtained from cells grown on YES for 24 hours[12] and the 16S rDNA was amplified using the Expand High Fidelity PCR System (Roche) and the primers 27f (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1522r (5′ AAGGAGGTGATCCANCCRCA 3′), which amplified almost the complete gene[13]. f, indicates the forward strand and r the reverse one. N indicates any nucleotide and R indicates A or G.

Sequencing was done using the following primers: 27f (5′ AGAGTTTGATCCTGGCTCAG 3′); 339r (5′ ACTGC-TGCCTCCCGTAGGAG 3′); 358f (5′ CTCCTACGGGAG-GCAGCAGT 3′); 519f (5′ CAGCAGCCGCGGTAATAC 3′); 849f (5′ GCCTGGGGAGTACGGCCGCA 3′); 1112f (5′ AGTCCCGCAACGAGCGCAAC 3′); 1150f (5′ GGG-GATGACGTCAAGTCATCA 3′); 1500r (5′ AAGGAGGT-GATCCAGCCGCA 3′).

Antibiotic Production

The time-course of antibiotic production was studied in duplicate in 500 ml Erlenmeyer flasks (baffled) with 100 ml of YES medium. These flasks were inoculated with 1 ml of 24 hours-old culture in the same media and incubated at 28°C. Samples (5 or 1.5 ml) were collected every 24 hours up to 10 days and the antibiotic activity present in culture supernatants and cells was tested. The antibiotic present in the cells was extracted by adding acetone and vortexing for 30 seconds, followed by centrifugation at 10,000×*g* for 10 minutes. Overall growth was monitored as dry weight (cells were washed twice with 10 mM phosphate buffer, pH 6.8 and dried at 50°C for 72 hours).

For antibiotic purification, three liters of YES medium supplemented with 0.5% xylose and 2 mM $MgCl_2$ were inoculated with 10^6 spores/ml of *Streptomyces* sp. RSP9 and aliquoted into 500 ml four baffled Erlenmeyer flasks at 150 ml each. Cultures were incubated for four days in a shaker at 200 rpm and the whole cultures were mixed with 3 litres of *n*-butanol and vortexed for 30 seconds several times. Phases were separated by centrifugation at 10,000×*g* for 10 minutes and the organic layer was harvested and concentrated under reduced pressure, giving an oily brownish residue. The residue was dissolved in 500 ml of a mixture 10% aqueous NaCl - MeOH 1 : 1 and the hydro-alcoholic layer was extracted twice with 500 ml of $CHCl_3$ and the active $CHCl_3$ extracts were concentrated and chromatographed on silica gel.

Analytical Procedure

Analytical TLC was accomplished on pre-coated silica gel 60 F_{254} plates (0.2 mm thick, 20×20 cm, Merck) and the spots were detected under UV light (254 nm).

Silica gel 60 (70~230 mesh, Merck) and Lichroprep RP-18 (40~63 mm, Merck) were used for column chromatography. HPLC analysis was performed on a Waters 991 apparatus with a photodiode-array detector and a Rheodyne injector, using a Resolve C18 analytical radial pack cartridge (10 μ, Waters Chromatography), using CH_3CN - H_2O, 96 : 4, as a mobile phase at a flow rate of 2.0 ml/minute and detection at 250 nm.

Biological Assays

Antimicrobial activity against different bacteria and yeasts grown on YEPD-agar[14] was studied in diffusion assays on agar plates. Wells of 0.5 cm diameter were bored on plates inoculated with the different microorganisms and several quantities of supernatant or pure antibiotic were assayed. Activity was observed and measured after 24 or 48 hours of incubation at 28°C.

tipA promoter (*tipA*p) induction by the purified thiopeptide molecules was studied in *S. lividans* transformed with the multicopy plasmid pAK114 or with pGG016, a single-copy integrative plasmid. In pAK114, thiostrepton-inducible *tipA*p controls the expression of a kanamycin resistance gene[15], and in pGG016 *tipA*p controls the expression of a truncated xylanase gene derived from the *xysA* gene from *S. halstedii* JM8[16,17]. In this work, pGG016 was constructed by cloning the DNA fragment of the *xysA* gene (*xysA*Δ), which encodes the catalytic domain of xylanase under the control of the *tipA* promoter, into the integrative plasmid vector pIJ8600[18].

S. lividans transformed with pAK114 was used to study the *tipA* induction effect in culture supernatants or pure compounds (5 to 30 ng). Spores of this strain were inoculated as a lawn onto R2YE plates containing 15 μg/ml neomycin. Different quantities of filter-sterilized supernatant or purified compounds were deposited in wells and the plates were incubated at 28°C. The induction capacity of total supernatant or pure compounds was detected as growth halos around the wells. Halo size depended on the kanamycin concentration and on the concentration and effectiveness of the inducer.

tipA induction on liquid media was studied by inoculating spores of *S. lividans* transformed with pGG016 in YES medium supplemented with different amounts (2 to 10 μg/ml) of the purified compounds.

Cultures with the same amounts of thiostrepton were used as controls of induction. Samples of the induced supernatants were collected at different times and analyzed by denaturing polyacrylamide gel electrophoresis(SDS-PAGE). The truncated ΔXys1 produced was detected after Coomassie Blue staining as a band of 30 kDa or by Western blot with anti-Xys1 antibodies[16].

Results and Discussion

Streptomyces sp. RSP9 Isolation and Taxonomy

The strain *Streptomyces* sp. RSP9 was isolated in a screening procedure for *Streptomyces* protease-producing strains (GONZÁLEZ HOLGADO and SANTAMARÍA, unpublished). Morphologically, this strain grows abundantly in complex and synthetic media. The spores are green-grey on R2YE and SFM media. Mycelia (back side of the plates) are yellowish-brown. This strain does not liquefy agar, indicating undetectable agarase activity, but does produce several extracellular enzymes such as amylases, cellulases, xylanases and proteases. A study of these enzymes is currently underway.

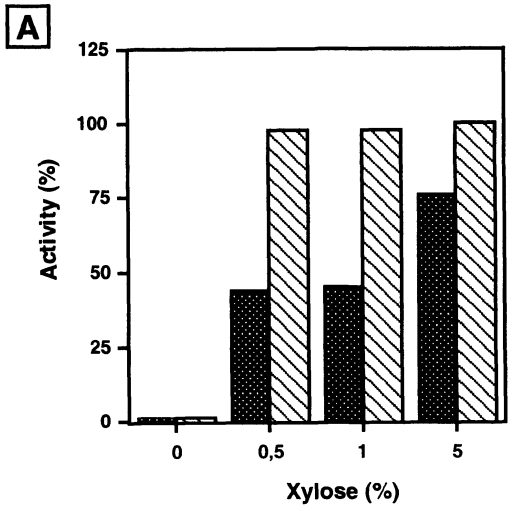
Streptomyces sp. RSP9 was identified on the basis of its 16S rDNA sequence. Comparison of the sequence obtained with those found in the databases showed 99.87% identity to *S. griseus* and *Streptomyces* sp. AA8321.1 (only two nucleotides out of 1518 are different). Thus, we tentatively consider *Streptomyces* sp. RSP9 as a new *S. griseus* sub-species.

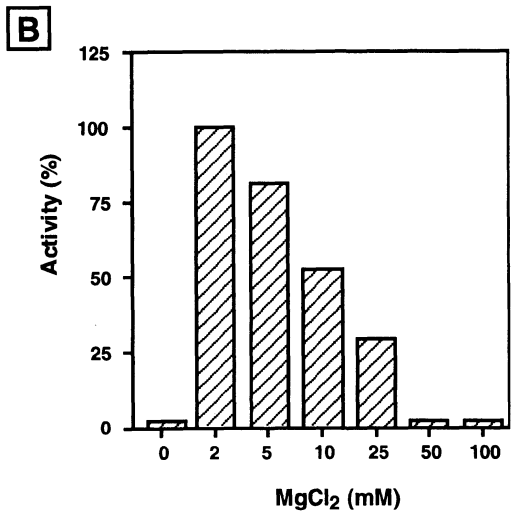
Antibiotic Production

Further characterization permitted us to observe that *Streptomyces* sp. RSP9 culture supernatants displayed strong antibiotic activity against several Gram(+) microorganisms such as *Micrococcus luteus*, *Bacillus subtilis*, *Brevibacterium lactofermentum* and *Staphylococcus aureus*, but no activity was detected against *Mycobacterium smegmatis*, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae* or animal cells. The results of the experiments described here were obtained with *M. luteus* as the test strain.

Antibiotic production was studied in several complex media, YES giving the best results. Other media, such as SPG, also gave an acceptable production (57.14% of the production obtained in YES). In order to optimize the production in YES medium, we studied the effect of the addition of glucose or xylose. Concentrations ranging from 0 to 5% of each monosaccharide were assayed on YES medium with and without 5 mM $MgCl_2$ and production after 96 hours was evaluated in plate diffusion bioassays. A clear positive effect was observed when increasing concentrations of xylose were added to YES without $MgCl_2$. However, the best production was obtained in YES

Fig. 2.



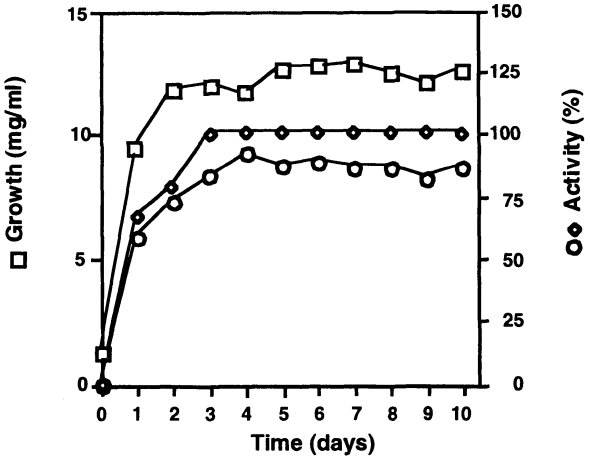


A) Antibiotic production in the presence of different quantities of xylose. ▩ YES without $MgCl_2$. ▨ YES containing 5mM $MgCl_2$.

B) Antibiotic production ▨ in the presence of different amounts of $MgCl_2$.

with $MgCl_2$ supplemented with xylose. In this medium, production was very similar at all the concentrations of xylose used, although it was slightly higher with 5% xylose, Fig. 2A. With glucose, the best production was obtained at 1%, slightly lower than that obtained with xylose (data not shown). From this experiment onwards, all fermentations were carried out in YES supplemented with 0.5% xylose and $MgCl_2$.

Fig. 3. Time-course of antibiotic production in YES.



□ Growth, ◇ antibiotic in the supernatant, ○ antibiotic in the cells.

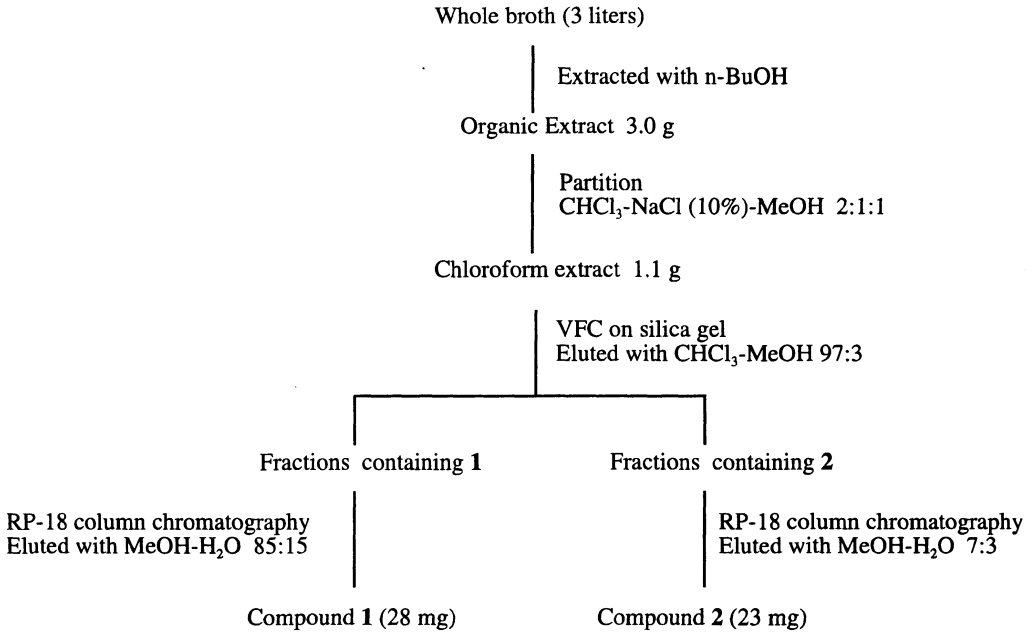
The effect of different concentrations of $MgCl_2$ (0 to 100 mM) was evaluated in YES media supplemented with 0.5% xylose. The best production was obtained on media supplemented with 2 mM $MgCl_2$ Fig. 2B. All successive fermentations were thus carried out in modified YES media (1% Yeast extract, 10.3% sucrose [pH 7.2] supplemented with 0.5% xylose and 2 mM $MgCl_2$).

A fermentation time-course study was performed in 500-ml Erlenmeyer flasks as indicated in Materials and Methods and the antibiotic present in the supernatant and in the cells was evaluated in bioassays. After 24 hours, the presence of antibiotic was detected in both fractions, and was found to be more abundant in the supernatant (58%) than in cells (42%). Maximum production was observed at 72 hours, after which it reached a plateau and remained stable for up to 10 days of incubation Fig. 3. Since both supernatant and cells contained considerable activity, we decided to process both together for antibiotic purification.

Antibiotic Isolation and Purification

The procedure used for isolating antibiotic activity is summarized in Fig. 4. After 96 hours, the culture (3 liters) was mixed with 3 liters of *n*-butanol. After centrifugation the organic layer was harvested and concentrated under reduced pressure, giving a brownish oily residue (3.0 g).

Fig. 4. Procedure for the isolation of radamycin.



The residue was dissolved in 500 ml of a mixture 10% aqueous NaCl - MeOH, 1 : 1, and the hydro-alcoholic layer was extracted twice with 500 ml of $CHCl_3$. The active $CHCl_3$ extracts were concentrated to yield 1.1 g of oil. The extract was chromatographed on silica gel using a VFC (Vacuum Flash Chromatography) system with $CHCl_3$ - MeOH as the eluting solvent. Fractions containing compound **1** (60 mg) and compound **2** (55 mg) were eluted with $CHCl_3$ - MeOH, 97 : 3, in different fractions. The final purification of both compounds was carried out by C18 reversed phase chromatography, affording 28 mg of pure compound **1** eluted with MeOH - H_2O, 85 : 15, and 23 mg of compound **2** eluted with MeOH - H_2O, 70 : 30. The purity of each preparation was confirmed by TLC visualized with vanillin in concentrated H_2SO_4 and by analytical HPLC with a photo-diode array detector.

Structural elucidation of both compounds was obtained; the compound 1 corresponding to radamycin is described in the accompanying paper[10]. The structure of compound 2 corresponds to methylsulfomycin I, an antibiotic recently patented and published[9].

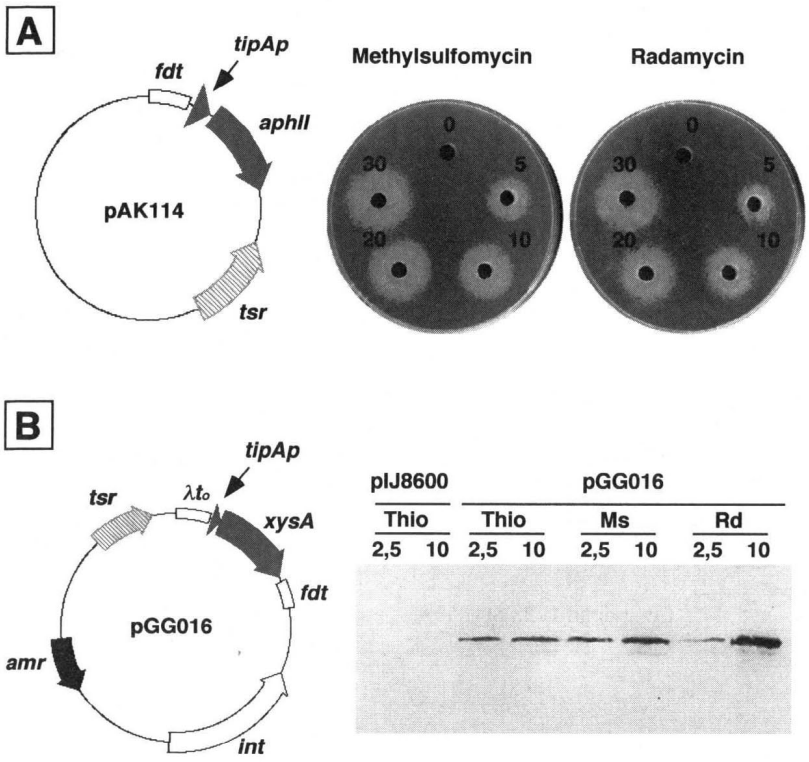
Biological Properties

After purification, we tested the antibiotic activity of both pure compounds against *M. luteus*. While methylsulfomycin I exhibited powerful inhibitory activity, we were unable to observe any effect caused by radamycin. This suggests that the antibiotic activity detected in supernatants and cells is due to methylsulfomycin I alone, although inactivation of an active radamycin precursor during the purification procedure cannot be ruled out. Radamycin itself appeared to be fairly stable since, even after 6 months in a refrigerator, it showed no signs of modification in its HPLC pattern. We therefore consider radamycin to be a final, stable product rather than an intermediate compound. A mutant strain defective in methylsulfomycin I production would answer this question.

Antimicrobial activity was also tested against 30 different clinical isolates of *Staphylococcus aureus*. Again, methylsulfomycin I showed a strong inhibitory effect against all the isolates in plate diffusion assays. Amounts as low as 0.3 μg/well were enough to give halos of 1.55 cm. However, none of the radamycin samples (25 ng to 3 μg per well) inhibited *S. aureus* growth.

In another set of experiments *S. lividans* was found to be sensitive to methylsulfomycin I but not to radamycin. However, it is worth noting that *S. lividans* sensitivity decays with time and that the mycelia eventually overgrow the area of initial inhibition. In addition, the external border of the inhibition area (in the transition to the fully-grown mycelium) showed accelerated aerial mycelium formation

Fig. 5.



A) Expression of the *aphII* (neo) gene under the control of *tipAp*. *fdt* is a transcriptional terminator; *tsr* is a gene encoding resistance to thiostrepton. The numbers indicate the amount (in ng) of methylsulfomycin I or radamycin deposited in each well.

B) Expression of the *xysA* gene under the control of *tipAp*. λto and *fdt* are transcriptional terminators. *amr*, apramycin resistance; *int* ΦC31 integrase. The numbers indicate, in μg/ml, the amounts of thiostrepton (Thio); methylsulfomycin (Ms) or radamycin (Rd) added to the cultures. pGG016 is a pIJ8600[18] derivative.

and sporulation. This effect and the inhibition of growth were more clearly seen when supernatants rather than pure compounds were used for the plate diffusion assays. This suggests that better solubility, higher stability or some kind of synergism might occur in the supernatants that are absent in the purified compounds. With radamycin, a slightly increased acceleration in sporulation was observed.

A known biological property of many thiopeptide molecules (first seen with thiostrepton) is their ability to induce the production of several proteins (thiostrepton induced proteins, Tip) by *Streptomyces lividans* and other strains apparently unable to produce their own thiopeptides. The *tipA* gene corresponding to one of these proteins-TipA-has been cloned and characterized. TipA is a transcription factor that, upon binding the thiopeptide, activates its own expression and probably that of other *tip* genes. The actual function of this thiopeptide-sensing system is unknown, but could be linked to low resistance levels to the cognate thiopeptide antibiotics[19].

We tested the ability of both methylsulfomycin I and radamycin to induce the *tipA* promoter (*tipA*p) in *S. lividans*. Two reporter systems were used. First, *S. lividans* was transformed with pAK114, a derivative of the multicopy vector pIJ486, where the *tipA* promoter drives the expression of the Tn5 neomycin resistance gene[15]. 5 ng of methylsulfomycin I or radamycin were able to activate the *tipA* promoter and allowed the pAK114 transformants to grow on plates containing 15 μg of neomycin (Fig. 5A). Growth, measured as halos around wells where the molecules had been deposited was clearly dose-dependent (Fig. 5A).

In another set of experiments, we used the *xysAΔ* gene of a truncated secreted xylanase (Xys1Δ) from *Streptomyces hastedii* JM8[17] as reporter. The *xysAΔ* gene was cloned

downstream from the *tipA* promoter in the single copy *E. coli*-*Streptomyces* shuttle vector pIJ8600[18] to give plasmid pGG016. This construction integrates at the ΦC31 attachment site in the chromosome. We made up duplicated master liquid cultures (50 ml of YES medium with 1% Glucose, 5 mM $MgCl_2$) of *S. lividans* transformants carrying either pIJ8600 (empty vector) or pGG016 (*xysAΔ*). After 18 hours of growth, the cultures were split into four aliquots. Three of them were supplemented with 2.5 or 10 μg/ml of thiostrepton, methylsulfomycin I or radamycin. The fourth aliquot was left unsupplemented as an uninduced control. Incubation was continued and 1 ml samples were withdrawn at different times after induction and analyzed to detect the xylanase protein Xys1. Xys1 was detected by Western-blot using anti-Xys1 antibodies. All three compounds were able to induce the *tipA* promoter (Fig. 5B). By the intensity of the protein band induced by 10 μg/ml of radamycin that radamycin looks more powerful than thiostrepton and methylsulfomycin I.

The ability of radamycin to induce the *tipA* promoter in the absence of detectable antibiotic activity would permit the construction of inducible *tipAp* vectors lacking a thiostrepton resistance gene, *tsr*. On the other hand, the lack of antibiotic activity in radamycin would allow its use as a *tipAp* inducer for the industrial scale production of proteins without the need for antibiotic addition with a view of preventing the selection of thiopeptide antibiotic-resistant strains.

The fact that radamycin lacks antibiotic activity while at the same time preserving *tipA* induction capacity may allow its use in the study of structure-function relationships in thiopeptide antibiotics.

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