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An Unexpected Inhibitory Effect of Rapamycin against Germination of Spores of *Bacillus brevis* Strain Nagano

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Rapamycin is used in medicine as an immunosuppressive agent (Sirolimus; Rapamune™) although discovered as an antifungal agent. It is thought not to have antibacterial activity. Surprisingly, we found that rapamycin inhibits the germination of *Bacillus brevis* Nagano spores, but is inactive against *Bacillus brevis* Nagano vegetative cells. Surprisingly rapamycin did not show antimicrobial activity against other *Bacillus* strains, including other gramicidin S-producing *Bacillus brevis* strains such as ATCC 9999 and BI-7, whether tested as spores or vegetative cells.

Rapamycin, a nitrogen-containing triene macrolide with a very large (31-membered) lactone ring[1,2], was first isolated from soil samples collected on Easter Island[3]. Rapamycin was originally discovered to be an antifungal antibiotic produced by *Streptomyces hygroscopicus* which did not show antimicrobial activity against any Gram-negative or Gram-positive bacteria tested[3,4]. Subsequent studies of rapamycin for clinical use demonstrated its antitumor and immunosuppressant activities[5~8].

Rapamycin, structurally similar to another immunosuppressant FK506 (Tacrolimus), binds to the FK506-binding protein 12 (FKBP12) encoded by the *fpr1* gene. The complex inactivates protein FRAP which is a phosphatidylinositol kinase, and then inhibits T cell activation and also growth of filamentous fungi and yeast[9,10]. However, since rapamycin showed no activity against bacteria, bacterial species are considered to lack FKBP12 and/or FRAP.

We have previously described the antifungal activities of rapamycin[11,12]. The present work reports a surprising activity that the compound exhibits against spore germination of a single strain of *Bacillus brevis*.

Materials and Methods

Microorganisms Used

Gram-negative strains: *Escherichia coli* ESS, *E. coli* ZK4, *Proteus vulgaris*, *Pseudomonas aeruginosa* ATCC 27853, and *Zoogloea ramigera* 115. Gram-positive strains: *Bacillus subtilis* K, *B. subtilis* Marburg, *B. subtilis* 168, *B. subtilis* JH642, *Bacillus licheniformis* 6346, *Bacillus cereus* 9139, *B. brevis* ATCC 9999, *B. brevis* Nagano, and *B. brevis* BI-7. Yeasts: *Saccharomyces cerevisiae* and *Candida albicans*. Filamentous fungus: *Aspergillus niger*.

Preparation of Spores

Spores were produced in sporulation medium LBS+3AA containing (per liter) 10 g tryptone, 5 g yeast extract, 10 g L-arginine, 0.05 g L-methionine, 1.0 g L-phenylalanine, 100 mg $CaCl_2 \cdot 2H_2O$, 10 mg $MnCl_2 \cdot 4H_2O$, 200 mg $MgCl_2 \cdot 6H_2O$, and 16 mg $FeCl_3 \cdot 6H_2O$. The pH of the medium was adjusted to 7.0 with 6 N HCl. Cells were inoculated into 50 ml sporulation medium in 500 ml baffled Erlenmeyer flasks and cultured at 37°C, and 220 rpm for 4 to 6 days. The spores were examined under the microscope

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and were stored at −20°C in 30% (v/v) glycerol.

Media

For bacteria, LB agar medium consisted of (g/liter) tryptone 10, yeast extract 5, sodium chloride 10, and agar 8. LB liquid medium was the same minus agar. For *A. niger* and yeasts, YM agar medium consisted of (g/liter) malt extract 3, yeast extract 3, peptone 5, glucose 10, and agar 8. LB-glucose agar medium consisted of (g/liter) tryptone 10, yeast extract 5, sodium chloride 10, glucose 10, and agar 8. LB-glucose liquid medium was the same minus agar.

Agar-diffusion Assay

20 μl samples of different rapamycin concentrations (in 50% methanol) were placed on 6.3 mm paper discs and dried for 1 hour at room temperature. 20 μl samples of 30% glycerol stock cultures of each organism (previously frozen at −80°C) were mixed with 10 ml agar medium in 100×15 mm sterile Petri dishes. For the study of antimicrobial activity of rapamycin against a stationary phase culture of *B. brevis* Nagano vegetative cells, 20 μl samples of a stock culture of *B. brevis* Nagano were inoculated into LB liquid medium and the medium was incubated at 37°C at 220 rpm for 16, 24, 36 and 42 hours. 20 μl samples of the cultures were used for assay.

Two discs containing the same sample were used for each plate. The agar plates were placed in a cold room for 2 hours to allow diffusion of the rapamycin from the disc into the agar and then incubated at 37°C (bacteria) and 30°C for yeast and the fungus for 24 hours before measuring diameters of the resulting inhibitory zones surrounding the discs. These clear zones were compared to a standard curve of clear zones obtained with pure rapamycin.

Liquid Assay

Rapamycin (10 mg) was dissolved in 1 ml of 100% methanol. The rapamycin was serially diluted to 100, 10, and 1 μg/ml concentrations with 100% methanol. As a negative control, 100% methanol was used. 20 μl samples of spores and vegetative cells of *B. brevis* Nagano and vegetative cells of *S. cerevisiae* were inoculated into 5 ml LB plus 1% glucose medium and the culture tubes (12×75 mm) were incubated at 37°C on the 220 rpm shaker. Growth was measured both by Klett units using a Klett Colorimeter (Bel-Art Products, Pequannock, NJ) and with a spectrophotometer (Shimadzu, Kyoto) at 660 nm wavelength after cultivation for 16 hours.

Results

Antimicrobial Activity of Rapamycin against Bacteria

Gram-positive and Gram-negative bacteria, yeast, and a filamentous fungus were initially studied with the agar-diffusion assay. As shown in Table 1, rapamycin surprisingly inhibited the germination of spores of *B. brevis* Nagano strain whereas growth of vegetative cells of the other bacteria was not affected by rapamycin. As expected, growth of the yeasts *C. albicans* and *S. cerevisiae*, and the filamentous fungus, *A. niger*, was inhibited by rapamycin (Table 1).

Further Examination of *Bacillus* Species

Since we had found activity of rapamycin against growth from a spore suspension of *B. brevis* Nagano, we further tested its activity against spores and vegetative cells of other *Bacillus* species. Table 2 shows the results of agar-diffusion assays of rapamycin against growth inoculated from spore and vegetative cell suspensions of various *Bacillus* species. Surprisingly, growth from vegetative cell suspensions of *B. brevis* Nagano was not inhibited although growth from spores was inhibited. Furthermore, rapamycin failed to inhibit growth from spores of other *B. brevis* strains such as ATCC 9999 and BI-7. It should be noted that *B. brevis* ATCC 9999 is a gramicidin S producer, just as *B. brevis* Nagano. Mutant *B. brevis* BI-7 was derived from *B. brevis* Nagano by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) treatment, lacks D-phenylalanine activating and/or racemizing enzyme, and is a non-producing mutant[13,14].

The activity of rapamycin against growth from spores of *B. brevis* Nagano was next tested by liquid assay (Table 3). In liquid assay, three different concentrations of rapamycin were used and methanol was also tested as a control since rapamycin is dissolved in methanol. 100% methanol did not inhibit growth of *B. brevis* or *S. cerevisiae*. 10 μg/ml and higher concentrations of rapamycin were active against growth from the spore suspension of *B. brevis* Nagano.

Since rapamycin inhibited germination of *B. brevis* Nagano spore suspensions but did not inhibit growth from a vegetative cell stock culture, we also tested the effect of rapamycin on growth of vegetative cells at various time points up to the stationary phase. To prepare such vegetative cells, we inoculated *B. brevis* Nagano spores into LB medium and harvested cells at various times from 16 to 42 hours. These cells were used to seed agar in Petri dishes to which discs of rapamycin were added after solidification.

Table 1. Antimicrobial activity of rapamycin (10 mg/ml) against different microorganisms as measured by agar-diffusion assay.

Microorganisms	Diameter of inhibition zone (mm) Average of 2 discs
Bacillus brevis Nagano (spores)	15.2
Bacillus subtilis JH642[a] (vegetative cells)	< 6.3[b]
Escherichia coli ESS	< 6.3
Escherichia coli ZK4	< 6.3
Micrococcus luteus	< 6.3
Proteus vulgaris	< 6.3
Pseudomonas aeruginosa ATCC 27853	< 6.3
Staphylococcus epidermidis	< 6.3
Zoogloea ramigera 115	< 6.3
Candida albicans	24.7
Saccharomyces cerevisiae	33.8
Aspergillus niger	27.8

[a] Non-sporulating mutant.

[b] Growth was not inhibited by rapamycin; the disc diameter is 6.3 mm.

Table 2. Activity of rapamycin against growth from spore and vegetative cell suspensions of *Bacillus* strains and vegetative cells of yeast, as measured by agar-diffusion assay.

Strains	Diameter of inhibition zones (mm)			
	Rapamycin (10 μg/ml)		Rapamycin (1 mg/ml)	
	Vegetative cells	Spores	Vegetative cells	Spores
Bacillus subtilis K	< 6.3[a]	< 6.3	< 6.3	< 6.3
Bacillus subtilis Marburg	< 6.3	< 6.3	< 6.3	< 6.3
Bacillus subtilis 168	< 6.3	< 6.3	< 6.3	< 6.3
Bacillus subtilis JH64	< 6.3	< 6.3	< 6.3	< 6.3
Bacillus licheniformis 634	< 6.3	< 6.3	< 6.3	< 6.3
Bacillus cereus 9139	< 6.3	< 6.3	< 6.3	< 6.3
Bacillus brevis ATCC 9999	< 6.3	< 6.3	< 6.3	< 6.3
Bacillus brevis Nagano	< 6.3	7.2	< 6.3	10.3
Bacillus brevis BI-7	< 6.3	< 6.3	< 6.3	< 6.3
Saccharomyces cerevisiae	20.2	ND[b]	39.8	ND

[a] Growth was not inhibited by rapamycin.

[b] ND = not done.

Table 3. Activity of rapamycin against growth from a spore suspension of *B. brevis* Nagano and vegetative suspensions of *S. cerevisiae* as measured in liquid medium by Klett units and 660 nm absorbance.

Organisms	Rapamycin (μg/ml)	Klett Units (%)	OD_{660} (%)
Bacillus brevis	0[a]	157 (100)	6.78 (100)
Nagano spores	1	138 (88)	3.84 (57)
	10	9 (5.7)	0.45 (6.6)
	100	9 (5.7)	0.59 (8.7)
Saccharomyces cerevisiae vegetative cells	0[a]	103 (100)	7.35 (100)
	1	9.5 (9.2)	0.65 (8.8)

[a] 100% Methanol

Table 4. Lack of effect of rapamycin on *B. brevis* Nagano vegetative cells of various ages used as inocula for agar plates.

Microorganism used as inocula	Inhibition zones (mm)[a]			
	Culture times for inocula			
	16 h	24 h	36 h	42 h
Bacillus brevis Nagano vegetative cells	< 6.3	< 6.3	< 6.3	< 6.3
Controls:				
Saccharomyces cerevisiae vegetative cells	40.1			
Bacillus brevis Nagano spores	9.5			

[a] 20 μl of rapamycin at 1 mg/ml added to each disc.

Table 4 shows the activity of rapamycin against such cell populations of *B. brevis* Nagano. It can be seen that there was no effect of the age of the *B. brevis* Nagano population as long as spores were not present, *i.e.* stationary phase cells were not inhibited.

The minimum inhibitory concentration was calculated as the concentration of rapamycin giving a 7.0 mm inhibition zone when 20 μl of the rapamycin is added per disc. We chose 7.0 mm since the diameter of the disk is 6.33 mm. We found the minimum inhibition concentration of rapamycin against *B. brevis* Nagano spores to be 7.5 μg/ml and for *S. cerevisiae* 0.42 μg/ml.

Discussion

Rapamycin is known to possess antifungal, antitumor, and immunosuppressive activities[3~8,10~12]. It was approved as an immunosuppressant by the Food and Drug Administration in 1999. To our knowledge, there are no reports that rapamycin has growth-inhibitory activity against bacteria despite the fact that this had been examined previously[3,4].

In this study, we have tested rapamycin's antimicrobial activity against Gram-positive and Gram-negative bacteria

and in all but one case, found that rapamycin indeed does not inhibit growth of bacteria. The one exceptional case is the growth of *B. brevis* Nagano when inoculated with spore suspensions. Vegetative growth of *B. brevis* Nagano was not inhibited and neither was growth from vegetative cells or spores of two other *B. brevis* strains, one a gramicidin S-producer (ATCC 9999) and the other a non-producing mutant of *B. brevis* Nagano (BI-7). This extreme specificity of rapamycin action is unexplained at the present time. We expect that this intriguing inhibition effect will teach us more about unknown mechanisms of rapamycin action. One might propose the existence of a protein interacting with rapamycin in *B. brevis* Nagano spores which is lost during germination but why would it be missing in spores of the non-producing mutant? If one postulates that it is required for gramicidin S production, why would it be absent in the Russian strain ATCC 9999[15~17] and present in the Japanese Nagano strain?

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