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ORIGINAL PAPER

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Preliminary report on the biological effects of space flight on the producing strain of a new immunosuppressant, Kanglemycin C

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Abstract Kanglemycin C (K-C) is a new immunosuppressant isolated from the culture broth of *Nocardia mediterranei* var. *kanglensis* 1747-64. To improve the productivity of K-C and to study the biological effects of space flight on its producing strain, spores from five K-C producing strains (U-10, U-15, U-7, M-13, γ-33) mutated from the wild strain *N. mediterranei* var. *kanglensis* 1747-64 were carried into space by an unmanned spaceship, "Shenzhou III" (Divine Vessel III) on March 25, 2002. Comparatively, the strain U-7 was the highest K-C producing strain among the above five starting strains when cultivated in 500-ml Erlenmeyer flasks. After a 6 day and 18 h flight, the treated spores went through serial screening processes to screen for high-yield K-C mutant strains, using thin layer chromatography and high performance liquid chromatography (HPLC). The K-C yield produced by one mutant strain, designated as F-16, derived from the starting strain U-7 was increased by up to 200% when compared to that produced by the starting strain U-7 in 500-ml Erlenmeyer flasks after careful postflight HPLC analysis. Another mutant strain, designated as F-210, derived from the starting strain M-13 showed reduced productivity of K-C as well as exhibited changes in some morphological and physiological characteristics. For example, the broth color of the strain F-210 changed from yellow to purple after 96 h of culture, but that of the ground control strain M-13 remained yellow. Similarly, the mycelium morphological change from filamentous to coccoid of F-210 occurred later than that of ground control M-13. Examination of the survivability of postflight spores indicated that exposure to radiation, during the 162 h of space flight, plays a critical role in the survival rates of spores such that spores exposed to strong radiation exhibited lower survival rates than spores exposed to weak radiation.

Keywords Space flight · Kanglemycin C · *Nocardia mediterranei* var. *kanglensis* 1747-64 · *Amycolatopsis mediterranei* subsp. *kanglensis* 1747-64 · Sporulation · Secondary metabolites · Fermentation · Survival rate

Introduction

A biological response of microorganisms to space conditions, such as microgravity, cosmic radiation and vacuum, untapped a new spectrum of application for strain improvement. It has been reported that space culturing can improve the production of secondary metabolites such as Actinomycin D and Monorden [2, 3]. Kanglemycin C [1, 9] (K-C, as shown in Fig. 1) is a novel angucycline antibiotic with yellow fluorescence, excited in UV 360 nm. It is produced by *Nocardia mediterranei* var. *kanglensis* 1747-64, which was isolated from soil in the Kang-Le area, Guangdong province, People's Republic of China [6]. Pharmacodynamic tests have demonstrated that K-C strongly suppresses T- and B-lymphocyte proliferation induced by mitogens and alloantigen. K-C also inhibits mouse splenocyte proliferation in a different manner than cyclosporine, and shows no toxicity to the splenocytes at the treated doses [5]. Therefore, K-C is a promising immunosuppressant for clinical use in the future.

Initial fermentation studies indicated that the yield of K-C produced by the wild-type *N. mediterranei* var. *kanglensis* 1747-64 is approximately 0.1 μg/ml, which is extremely low for industrial scale production. To

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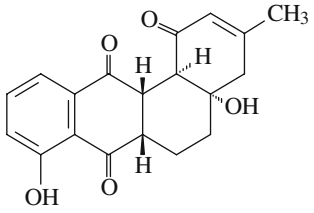

Fig. 1 The chemical structure of Kanglemycin C (K-C)

improve the yield of K-C, several methods including classic random spontaneous mutation, chemically induced mutagenesis and physically induced mutagenesis (UV radiation, γ-radiation and microwave treatment) of the wild-type strain were studied. Physically induced mutagenesis proved to be the most effective method in terms of obtaining high-yield K-C producing mutant strains [10, 11]. After exposure to UV-C radiation at 254 nm for 3 min at a distance of 36 cm, a mutant strain designated as U-7 was obtained and its K-C productivity proved to be 3.9 μg/ml when cultivated in 500-ml Erlenmeyer flasks [10, 11]. Encouraged by the results, we hypothesized that the multiple physical factors present in a space environment may have a significant effect on the K-C producing strain, *N. mediterranei* var. *kanglensis* 1747-64, since microorganisms in the space environment are not exposed to single, isolated stresses as studied in the laboratory, but rather to the integration of various stresses. Spores from five K-C producing strains—U-10, U-15, U-7, M-13 and γ-33—as starting strains were carried into space by the unmanned spaceship, "Shenzhou III" in March 2002. Paired space flight and ground control spores from the five K-C producing strains (U-10, U-15, U-7, M-13 and γ-33) in hardened plastic tubes were similarly prepared in the laboratory.

In this paper, we report the survival rate of onboard spores and two postflight mutants, mutant F-16 and mutant F-210. Mutant F-16 was derived from the starting strain U-7 in the weak radiation group, while mutant F-210 was derived from the starting strain M-13 in the weak radiation group.

Materials and methods

Microorganisms

The K-C producing strain, *N. mediterranei* var. *kanglensis* 1747-64, was isolated from the Kang-Le area, Guangdong province, People's Republic of China, and maintained at the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences. Five mutant strains, U-10, U-15, U-7, γ-33 and M-13, with a relative high productivity of K-C were selected as starting strains. Their origins are listed in Fig. 2. Among the five

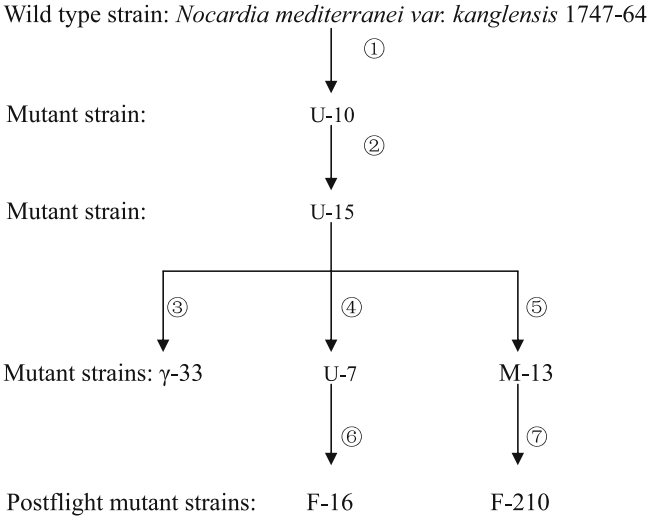

Fig. 2 Screening pedigree of K-C producing strains.

① Exposure to UV-C radiation at 254nm for 100 seconds at a distance of 36 centimeters.
② Exposure to UV-C radiation at 254nm for 100 seconds at a distance of 36 centimeters.
③ Exposure to 1kGy γ-ray for 36mins.
④ Exposure to UV-C radiation at 254nm for 3 minutes at a distance of 36 centimetres.
⑤ Exposure to 2450MHz , 1250W microwave for 90 seconds.
⑥ Exposure to space factor for 162 hours in the weak radiation group.
⑦ Exposure to space factor for 162 hours in the weak radiation group.

starting strains, the strain U-7 was the highest K-C producing strain when cultivated in 500-ml Erlenmeyer flasks. Spores obtained from agar slants of the five starting strains were each mixed with 1 g of sterile sand to be around 10^7 colony-forming unit (CFU) per gram and were placed into a sterile plastic tube (14 mm inner diameter × 40 mm) and screwed tightly. Three identical sample sets were prepared, each composed of five tubes containing spores from the five different starting strains mixed with sterile sand as described above. One sample set was maintained at 4°C as ground control samples, while the other two identical sample sets were stored at 4°C in the space center to be used as space flight samples.

Culture media, growth conditions and strain isolation

Postflight spores and ground controls were plated on Gause no. 1 agar slants (KNO_3 0.1%, NaCl 0.05%, K_2HPO_4 0.05%, $FeSO_4 7H_2O$ 0.001%, $MgSO_4 7H_2O$ 0.05%, soluble starch 2.0%, agar 1.5%, pH 7.0) and were allowed to incubate at 28°C for 7 days. Spores were washed with sterile water and spore suspensions were prepared to contain around 10^7 CFU/ml. Fifty milliliter of the seed medium (glucose 3%, yeast meal 0.5%, $(NH_4)_2SO_4$ 0.5%, $CaCO_3$ 0.5%, pH 6.5) contained in 250-ml Erlenmeyer flasks were inoculated with 1 ml spore suspensions and incubated at 28°C for 48 h with shaking. Five milliliter of the seed culture was then transferred to 500-ml Erlenmeyer flasks containing 100 ml of the production medium (glucose 4%, yeast meal 1%, peanut meal 0.5%, peptone 0.5%, $CaCO_3$ 0.1%, pH 6.5). The fermentations were carried out at 28°C for 90 h with an agitation rate of 220 rpm.

Space flight

Two sets of space flight samples were packed into two cloth packages. One sample set, labeled as the weak radiation group, was put inside a protective box made of aluminum foil to impair cosmic radiation, while another sample set, labeled as the strong radiation group, was not wrapped with aluminum foil. Both sample sets were placed in the returning module of the unmanned spaceship, "Shenzhou III". "Shenzhou III" was launched at 10:15 p.m. Beijing time on March 25, 2002 from the Jiuquan Satellite Launching Center in Gansu Province, People's Republic of China. Ten minutes after blast-off, the spaceship entered its preset orbit and successfully orbited the earth 108 times in a period of 162 h. The orbit inclination angle was 42.2°, altitude of the apogee was 335 km and altitude of the perigee was 332–338 km. Physical conditions of the samples were: sample temperature: 23 °C ± 1, microgravity: 10^{-3}–10^{-6} g, irradiation: 0.095–0.197 mGY. After flying over more than 4 million km in outer space, the returning module landed safely in the central Inner Mongolia Autonomous Region at 4:51 pm Beijing time on April 1, 2002. The returning module of "Shenzhou III" was opened in Beijing on April 4, 2002. The package containing the spore samples of the K-C producing strains was in good condition when removed from the returning module. The samples were transported to the laboratory and stored at 4 °C.

Postflight TLC and HPLC analysis

Ten milliliter samples of fermentation broth from either postflight or ground control spores were centrifuged at 6,000 rpm for 20 min. The supernatant was extracted with 10 ml ethyl acetate and evaporated under reduced pressure and the residue was dissolved in 1 ml methanol. One hundred microliters of methanol containing K-C was spotted onto a thin layer chromatography plate (TLC, Merck silica gel 60F254). The TLC plate was developed with methanol:chloroform (1:9) and the productivity of K-C was evaluated according to the yellow fluorescent strength emitted by K-C when excited at UV 360 nm. The sample for high performance liquid chromatography (HPLC) analysis was prepared in the same manner. The 1 ml methanol solution was filtered through a 0.22 μm membrane. The quantity of K-C in each sample was determined using a Shimadzu LC-10Avp instrument equipped with a diode array detector, SPD-M10AVP. HPLC analytical conditions were as follows: HPLC column: Zorbax extend-C18 (4.6 × 250 mm, 5 μm, Agilent technologies); mobile phase: 20–40% acetonitrile in distilled water, linear gradient wash for 40 min; flow rate: 1 ml/min; sample inject volume: 10 μl.

Results

Comparison of survival rates

To compare the survival rates, equal weights of soil containing spores from postflight samples and ground controls were each dissolved in 10 ml of a 0.9% NaCl

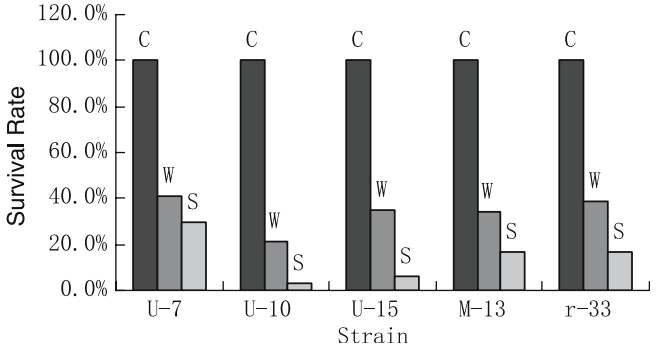

Fig. 3 Survival rate of postflight strains in the weak radiation group (*W*), strong radiation group (*S*) and ground control group (*C*)

sterile water solution and 1:10 serial dilutions were performed. One hundred microliters of the various concentrations were spread on Gause no. 1 agar plates and incubated at 28°C for 7 days. Assuming the CFU/ml of each ground control to be 100%, the survival rate of each strain in the strong and the weak radiation group is shown in Fig. 3.

Data for the postflight spores showed that survival rates of the spores in the strong radiation group (U-7: 29.5%; U-10: 2.7%; U-15: 5.9%; M-13: 16.6%; γ-33: 16.6%) are much lower than those in the weak radiation group (U-7: 41.0%; U-10: 21.5%; U-15: 34.8%; M-13: 34.3%; γ-33: 38.9%). The weak radiation group spores and the strong radiation group spores had experienced identical space conditions including temperature and microgravity; however, the former was protected against some solar UV radiation and the components of cosmic radiation in space by the aluminum foil.

Kanglemycin C production

Four hundred and forty six colonies generated from both the strong and weak radiation groups were picked from the Gause no. 1 agar plates and tested for their K-C productivities. The production of K-C by ground control strain U-7 and by the postflight mutant strains was first analyzed by comparing the intensities of the K-C fluorescent spots developed on the TLC plates. Based on the TLC analysis, yields of K-C in 11 strains from 446 postflight strains were increased, while the yields of K-C in the other strains proved to be unchanged, reduced or completely abolished as showed in Fig. 4.

Extracts from the mutant strains which emitted a more brilliant yellow fluorescence under UV 360 nm wavelength in TLC than the ground control strain U-7 were further analyzed by HPLC. The online UV spectra (characteristic absorption wavelength at 232 nm) and the retention time at 31 min confirmed the presence of K-C in both postflight and ground control samples. K-C was quantified by comparing the peak areas with a standard K-C calibration curve. HPLC analysis of the 11 improved mutant strains showed that the postflight mutant strain F-16 gave the highest K-C yield,

Fig. 4 The *bright spot* in *lane 8* K-C produced by ground control strain U-7; mutant strain in *lanes 6 and 9* failed to produce K-C; mutant strain in *lane 2* produced little of K-C; productivity of K-C of the mutant strain in *lane 1* was reduced; mutant strain in *lane 5* produced about the same amount of K-C; mutant strain in *lanes 3, 4* and *7* appeared to exhibit improved productivity of K-C

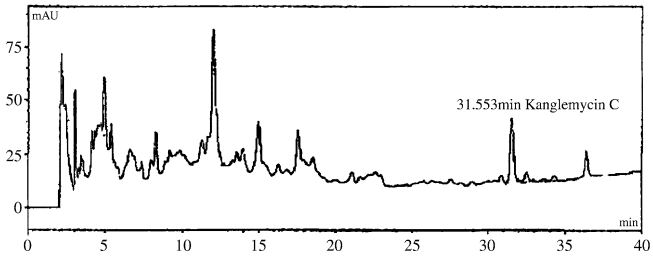

Fig. 5 High performance liquid chromatography (HPLC) chromatogram of ground control strain U-7 at UV 232 nm

12.5 ± 0.2 μg/ml (average of three replicates). The HPLC chart is shown in Figs. 5 and 6. The mutant strain F-16 was generated from the starting strain U-7 in the weak radiation group. Interestingly, the starting strain U-7 produced the highest amount of K-C before the space flight.

Studies on the mutant F-210

During the process of screening for K-C high-yield mutant strains, it was found that the postflight mutant strain F-210, which came from the starting strain M-13 in the weak radiation group, was not only reduced in its productivity of K-C, but also had a changed color of its fermentation broth. After 72 h of incubation of F-210 in a K-C producing medium with shaking at 28 °C, the color of the broth changed from yellow to gray. After continuous culture for 96 h, the color of the entire broth had completely changed to purple, whereas the broth of the ground control M-13 still remained yellow, as shown in Fig. 7.

Moreover, the mycelium morphology of the ground control M-13 changed from filamentous to coccoid at 72 h, while the mycelium morphology of the postflight mutant strain F-210 remained filamentous until 120 h before finally changing to coccoid at 144 h. Meanwhile, cell volume in the 10 ml culture of the strain F-210 increased when compared to that of the ground control of M-13 at 96 h harvesting time. Comparison of fermentation cultivation features between the postflight mutant strain F-210 and the ground control of M-13 is shown in Table 1.

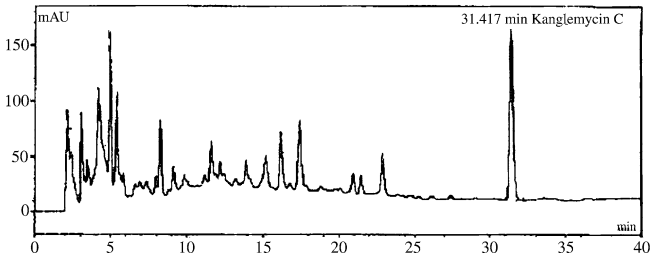

Fig. 6 High performance liquid chromatography chromatogram of the postflight mutant strain F-16 at UV 232 nm

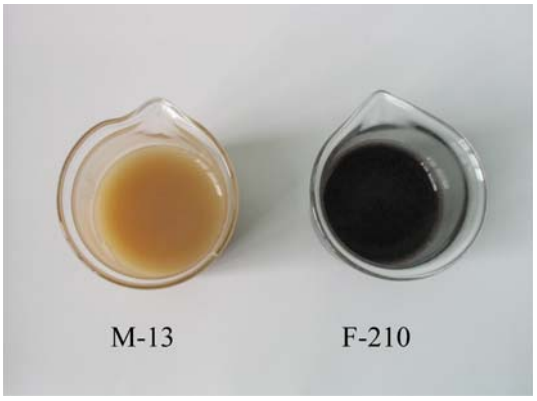

Fig. 7 Color of the fermentation broths of ground control strain M-13 and postflight mutant strain F-210 at 96 h

To identify certain individual species of actinomycetes, it is essential to recognize some of their characteristic properties. Pigment production is one of the most important and most easily recognizable characteristics [8]. The culture characteristics of the postflight mutant strain F-210 and the ground control M-13 in six different agar media are shown in Table 2. Culture characteristics of F-210 demonstrated that F-210 still retained the main characteristics of *N. mediterranei* var. *kanglensis* 1747-64, which are rose, erect aerial mycelium and wrinkly, fission, yellow-brownish substrate mycelium [5]. The difference between F-210 and M-13 is the soluble pigment in glucose nitrate agar, glycerol–asparagine agar and glucose–asparagine agar. F-210 produces a violet soluble pigment. In contrast with F-210, the pigment produced by M-13 in the same three agar media is slightly yellow or colorless. In another three agar media (inorganic salts-starch agar, Gause's synthetic medium and sucrose nitrate agar), the pigment of F-210 and M-13 is the same. It is known that Nocardias can form violet pigment [8]. Studies on the morphology and the culture characteristics of F-210 and ground control M-13 in six different agar media including the appearance of K-C indicated that F-210 was a mutant of *N. mediterranei* var. *kanglensis* 1747-64.

Conclusion and discussion

Several facts such as the K-C yield of the postflight mutant strain F-16, which increased up to 12.5 ± 0.2 μg/ml, the K-C yield increase, decrease and absence in 446 postflight colonies, and the changes that occurred with regard to morphology and culture characteristics of the postflight mutant strain F-210 demonstrate that space conditions can cause mutations of microorganisms and that *N. mediterranei* var. *kanglensis* 1747-64 is sensitive to these conditions. Comparison of survival rates between ground controls and onboard samples indicated that integrated space conditions, including temperature, microgravity, cosmic radiation, etc., are responsible for the differences observed in survivability, since the sample tubes were tightly closed and the air composition (pressure and humidity) was the same between these two groups. Also, the trend for the survival rate of each strain in the weak radiation group to be higher than that in the strong radiation group supports the conclusion that cosmic radiation should be the main cause effecting the survivability of spores considering the only difference between these two groups was the use of the aluminum foil to impair the intensity of cosmic radiation in the weak radiation group. Even though the amount of radiation is as low as 0.095–0.197 mGY, the exposure time is as long as 162 h and that might mutate or kill the spores in the space vacuum condition [7].

Amycolatopsis mediterranei was originally classified as '*Streptomyces mediterranei*', later as *N. mediterranei* and, finally, was transferred to the novel genus *Amyco-*

Table 1 Comparison of fermentation cultivation features between F-210 and M-13 at harvest time (96 h)

	Fermentation broth	Supernatant	Mycelium	Mycelium volume (ml/10 ml whole broth)	Mycelium morphology at 96 h	Fermentation cycle (h)
M-13	Light yellow	Light brown	Light yellow	1.8	Coccoid	90
F-210	Violet	Red	Violet	2.3	Filament	144

Table 2 Comparison of culture characteristics of ground control strain M-13 and postflight mutant strain F-210

Agar medium	Aerial mycelium		Substrate mycelium		Soluble pigment	
	M-13	F-210	M-13	F-210	M-13	F-210
Inorganic salts–starch agar	None	Mealy white	Wrinkly light brownish	Wrinkly light brownish	None	None
Gause's synthetic medium	Mealy white-rose	Mealy white-rose	Fission yellow-brownish	Fission yellow-brownish	Yellow	Yellow
Glucose nitrate agar	Short hair white	Short hair rose	Fission red-brownish	Fission yellow-brownish	Yellow	Violet
Sucrose nitrate agar	Mealy white-rose	Short hair white-green	Fission red-brownish	Fission pink-green	Yellow	Yellow
Glycerol–asparagine agar	None	Short hair rose	Wrinkly yellow-orange	Wrinkly red-brownish	Yellow	Violet
Glucose–asparagine agar	Mealy soft cream	Short hair rose	Wrinkly soft cream	Wrinkly red-brownish	None	Violet

latopsis by Lechevalier et al. [4]. To keep a consistent relationship between the papers published about K-C, we in this paper use the name *N. mediterranei* var. *kanglensis* 1747-64.

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