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STRESS RESPONSES IN PIGMENTED *BACILLUS PUMILUS* SF214

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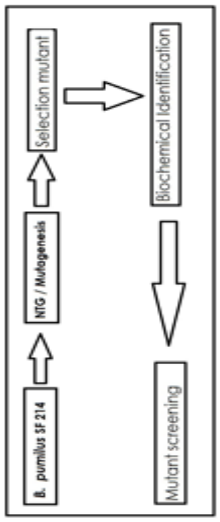
Graphical abstract



Abstract

Microbial producers of carotenoids belongs to a various species of unicellular algae, filamentous fungi and several bacteria. A recent report has shown that up to 15% of aerobic spore-formers identified from soil samples are pigmented and in many cases the pigments are carotenoids. Pigmented spore-forming *Bacillus* was obtained from culture maintained in the microbial culture collection of Department of Structural Functional Biol. University of Naples Federico II and partially characterized their pigments. A classical mutagenesis approach has been used to obtain mutant strains producing altered pigments or no pigments. Our results suggest that pigmentation in spore represent an additional, and may be alternative protection strategy against oxidative stress. A mutants (SF214-M1, SF214-M2, SF214-M3 and SF214-M4) of *Bacillus pumilus strain* SF214 producing a carotenoids water soluble-pigment were obtained after treatment with the mutagenic agent N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Several microbiological and biochemical properties of these 4 strains were analyzed and the results were differences between wild type and other four mutants in producing pigments, color changing, sporulation, cannot produce spores after mutation and sporulation efficiency was constant with color development.

Keywords: *Bacillus pumilus,* sporulation, UV radiation, resistance, mutant

1.0 INTRODUCTION

Spore-forming Bacilli are aerobic, Gram positive organisms sharing a common attribute of being able to differentiate to endospore (spore), a quiescent cell from characterized by several protective layers surrounding a dehydrated cytoplasm [1]. This structural organization makes the spores extremely resistant to external physical and chemical insults and able to survive almost indefinitely in the absence of water and nutrients [1]. It has long been known that some aerobic *Bacillus* are pigmented and examples include strains of *B. megaterium* [2], *B. atrophaeus*[3], *B. indicus* [4], *B. cibi* [5], *B. vedderi* [6], *B. jeotgali* [7], *B. okuhidensis* [8], *B. clarkia* and *B. pseudoformis* [9], and *B. firmus* [10]. More recently, a large number of pigmented Bacilli have been isolated and their pigments identified as carotenoids [11]. Those carotenoids, found associated with either vegetative cells or spores [12], are thought to be resistant to Ultra Violet (UV) and reactive oxygen. For strong antioxidant activity of species, carotenoids of plants, and microbial origin, have been proposed to prevent the onset of chronic diseases [13], and to reduce cancer–risk [3], in human, also for this reason, carotenoids widely are marketed as a dietary supplements. Non- pathogenic bacteria are able to colonize the human gut and able to produce carotenoids, therefore, particularly desirable as food supplements and/ or functional food ingredients.

One important difference that we have found between the laboratory and the field is the nature of the UV radiation encountered in the two places. Most laboratory studies of spore UV resistance were performed using monochromatic 254-nm UV (UV-C). However, sunlight reaching the Earth's surface is not monochromatic 254-nm radiation, but is a mixture of UV, radiation, the UV portion spanning approximately 290 visible, and infrared to 400 nm (the so-called UV-B and UV-A portions of the UV spectrum). Thus, the laboratory model of spore UV resistance has been constructed largely using a wavelength of UV radiation not normally experienced on the Earth's surface reviewed in [14]. We have recently been interested in understanding the mechanisms by which spores resist the physiological stress imposed by the terrestrial solar radiation environmental, using the common spore-forming bacterium *Bacillus pumilus* SF214.

2.0 METHODOLOGY

2.1 Bacterial Strains and Cultivation

Bacillus pumilus SF214 was obtained from microbial culture collection from Department of structural functional Biol. at University of Naples. Federico II. Which isolated originally from water.

B. pumilus was grown either in Luria-Bertani (LB) medium (for 1 L: 10 g Bacto- Tryptone, 5 g Bacto-yeast extract, 10 g NaCl, Ph 7.0) or in Difco Sporulation medium for (DSM) for induction of sporulation (for 1 l: 8 g Bacto-nutrient broth, 10 ml of 10% KCl, 10 ml of 1.2% MgSO4.7H2O , 0.5 ml of 1M of NaOH, the medium was autoclaved and cooled to 50°C before adding the following sterile solutions (1 ml of 1 M Ca(NO3)2, 1 ml of 0,01 M MnCL2, 1 ml of 50 µm FeSO4). Then liquid and solid cultures incubated for 48 hours at 37°C. in aerobic conditions.

2.2 Pigment Production

The production of pigment takes place both on (LB), favoring vegetative growth) and on (DSM), favoring spore formation, plates were incubated at various temperatures at 20, 30, 37, 42 and 50°C up to 48 hours.

2.3 Sonication of Vegetative Cells of B. pumilus SF214.

A single colony was inoculated in 10 ml of (LB) broth for 24 hours, an aliquot of this culture was diluted in 200 ml of (LB) broth to obtain a concentration of 0.1 OD. Then, culture was incubated at 25°C, later, 15 ml of aliquots of cells where harvested at different times of growth and centrifuged to remove supernatant, later they were determined to obtain wet weight of pellets separately via weighing each sample by sensitive balance.

Then pellets were resuspended in 20 ml of Sonication buffer [50 mM Tris (pH 7.6) 1 mM DTT, 1 mM Phenyl methyl sulfonyl fluoride PMSF, 10% Glycerol] and samples were sonicated by setting the following parameters: 10 minutes total for each sample, 15 seconds Time-ON, OFF Time-5 seconds, 40% amplitude.

2.4 Mutagenesis with Nitrosoguanidine (NTG)

B. pumilus SF214 was mutagenized as described by [15] with some modification. A suspension of cells ($3x10^8$ ml^{-1}) in sterile distilled water was transferred to a 1.5 ml microcentrifuge tube, 25 µl NTG (1 mg/ml) was added and cells were incubated at 37°C for 30 min. to obtain a kill percentage of ~ 50%. The cells exposed to NTG were washed twice with 1 ml phosphate buffer (PH 7.1), then centrifuged and suspended in 1 ml of phosphate buffer. Serial dilutions of the suspension were transferred onto (LB) agar plates and incubated at 37°C for 24 hours. The colonies formed were then selected based on pigment formed.

2.5 Determination of Sporulation Efficiency

Single colony was used to inoculate (LB) broth (10 ml). Culture was incubated at 37°C and agitated (200 rpm), until the mid-exponential phase growth was reached (OD600~ 0.6). An aliquot (1 ml) of this culture was then used to inoculate (DSM) broth (50 ml) present in 250 ml baffled flasks. These cultures were then incubated at 37°C (200 rpm) for 2 days. To determine the percentage of sporulation viable counting of vegetative cells and heat-resistant spores was carried out. Data points were collected every 20 hours from inoculation. At each time point a culture aliquot (0.5 ml) was taken and 0.1 ml was used for serial dilution in phosphate buffer [0.85% (w/v) PH 7]. Each dilution was plated onto DSM agar after 20, 40, 60, 80 hours respectively and after incubation at 37°C, colonies were counted. The remaining 0.4 ml of culture was heated at 65°C for 1 hour to eliminate vegetative cells and retain heat-resistant spores. Serial dilutions of the heat-treated solution were then carried out. Data were expressed as colony forming unit (cfu) per milliliter and the sporulation efficiency was provided as a percentage of heat- resistant spores divide by the number of vegetative cells. To routinely ascertain the sporulation cultures were examined by phase-contrast microscopy.

2.6 UV Irradiation Experiment

Spores in aqueous suspension (10^7 spores /ml) were exposed to UV-C radiation from a mercury low-pressure lamp, and to defined spectral ranges of UV-(A + B) or UV-A radiation. During irradiation the spore suspension was stirred continuously to ensure homogeneous exposure. The spectral irradiance was

measured by use of a double mono-chromator After UV radiation at defined fluencies, 100 µl from the aqueous suspension was taken for further analysis. Survival was determined from appropriate dilutions in distilled water as colony forming unit (cfu) after growth overnight on (LB) agar at 37°C. The surviving fraction was determined from the quotient N/N0, with N = the number of colony formers of the irradiated sample and N0 that of the non-irradiated control. Plotting the logarithm of N/N0 as a function of flounce, survival curves were obtained.

3.0 RESULTS AND DISCUSSIONS

B. pumilus SF214 produce an orange-red pigment. As can be seen in Figure 1, the production of pigment takes place both on the (LB) and on (DSM) media, although it appears to be greater at first time. In order to define an optimal temperature of growth of *B. pumilus* SF214 this determined before range of temperature within which bacteria grew. As a results showed the growth of *B. pumilus* SF214 was in range between 20 to 50°C the plates of (LB) and (DSM) were incubated at various temperatures and observed that the pigment was very intense at 20°C, then progressively less intense with increasing temperature as shown in Figure 2. The presence of an intense pigmentation was at 20°C when growth of bacteria is slow, therefor we have suggest the production of pigment may be was a cellular adaptation to unfavorable environmental conditions.

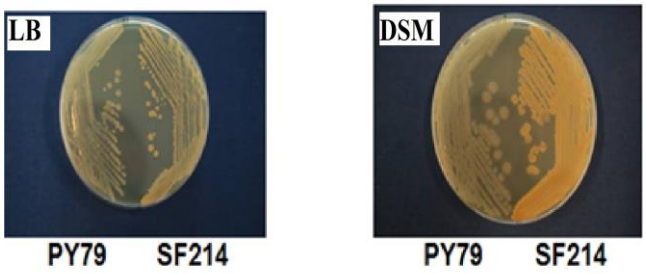


Figure 1 *B. pumilus* SF214 on DSM agar (right) and LB agar (left), PY79 (*B. subtilis*) is a non-pigmented *Bacillus* as control

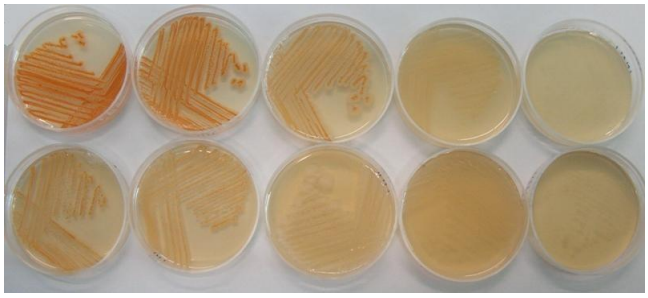


Figure 2 Pigment production in *B. pumilus* SF214 strain on LB agar (in upper) and DSM agar (in lower), plates were incubated at 20, 30, 37, 42 and 50 °C respectively for 48 hours

Aliquots of cells were harvested at different times of growth, centrifuged and used to determine the wet weight of the obtained pellets. The cells were then suspended in Sonication buffer (methodology) and sonicated to break cell membranes. Sonication allowed the extraction of pigmented proteins. The concentration of pigment extracts was determined by spectrophotometer reading at 490 nm and the protein concentration was determined. As reported in Table 1, we observed a clear increase in pigment production with the progression of the stationary phase of growth. In fact, in advanced stationary phase of growth (OD 600nm = 4.22) the amount of pigment product is more than 10 folds higher than in exponential phase (OD 600nm = 0.86). This observation is consistent and confirm that the pigment is produced primarily in conditions of cellular stress.

Table 1 Production of pigment by stage of growth

O.D. increase of 600 nm	Humid Weight (mg)	Concentration of protean (γ/λ)	O. D. (λ=490 nm)	O. D. (λ=490 nm) Concentration of protean
0.86	100	1.5	0.088	0.087
1.48	130	2.2	0.163	0.111
1.98	190	2.5	0.359	0.218
2.3	200	2.5	0.666	0.406
2.9	200	3.2	0.899	0.434
3	140	3.1	1.51	0.759
4	300	3.8	1.72	0.750
4.22	300	3	2.29	1.19

A schematic of the method used for the mutagenesis of pigment producing strain *B. pumilus* SF214 is shown in Figure 3. The parent strain, was treated with NTG and spread on (LB) and (DSM) agar. Based on colony size, pigment formation we recognize four mutants depend on color formation in comparison with parent strain as shown in Figure 4.

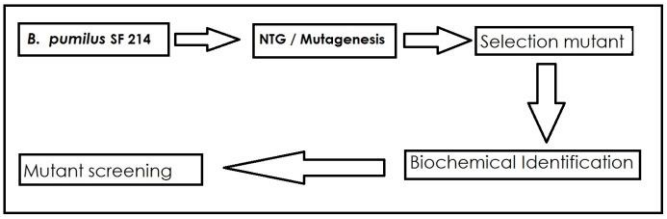


Figure 3 Schematic of random mutagenesis, selection and screening scheme

This study demonstrated a method for the rapid mutation of bacteria using random mutagenesis with (NTG) and growth selection of mutants with the variation in color formation, we did not rely on known information of gene targets. There have been many studies regarding producing mutants strains by random mutagenesis [16].

To further characterize of *B. pumilus* SF214 and to determine efficiency of sporulation, expressed as a percentage of cells resistant to heat (65°C for 1 h) present on (DSM) agar after 20, 40, 60 and 80 hours after entry into stationary phase (T0) compared to total cells. The ability of *B. pumilus* SF214 to form spores during development has been reported previously [11].

The ability of *B. pumilus* SF214 and other yellow / orange colored mutants was observed to form spores during development along the period of the experiment with simultaneous color change from yellow to orange to red (mutant 2 does not form spore after mutation).

Cultivation in (DSM) media revealed the presence of spores at 20 hours with the same levels, reach to 50% by the mutant 4 at day 4 (Table 2) with the variation in sporulation efficiency and was constant with the color development Figure 5.

Table 2 Efficiency of Sporulation of *B. pumilus* SF214 and the mutants

B.pumilus		20 after T.Z	40 after T.Z	60 after T.Z	80 after T.Z
Wt	cell	$128x10^7$	$148x10^7$	$141x10^7$	$129x10^7$
	spore	$13.0x10^7$ 10.1%	$28.8x10^7$ 18%	$38.0x10^7$ 26%	$48.0x10^7$ 37%
Mut 1	cell	$100x10^7$	$104x10^7$	$75x10^7$	$73.0x10^7$
	spore	$10.1x10^7$ 10.1%	$17.1x10^7$ 16.44%	$18.6x10^7$ 22.8%	$20.0x10^7$ 27.4%
Mut 3	cell	$131x10^7$	$159x10^7$	$157x10^7$	$148.0x10^7$
	spore	$15.0x10^7$ 11.45%	$25.9x10^7$ 16.28%	$45.0x10^7$ 28.66%	$62.0x10^7$ 41%
Mut 4	cell	$130x10^7$	$15x10^7$	$89.0x10^7$	$10x10^7$
	spore	$11.0x10^7$ 8.46%	$3.3x10^7$ 22%	$30.0x10^7$ 33%	$4.8x10^7$ 50%

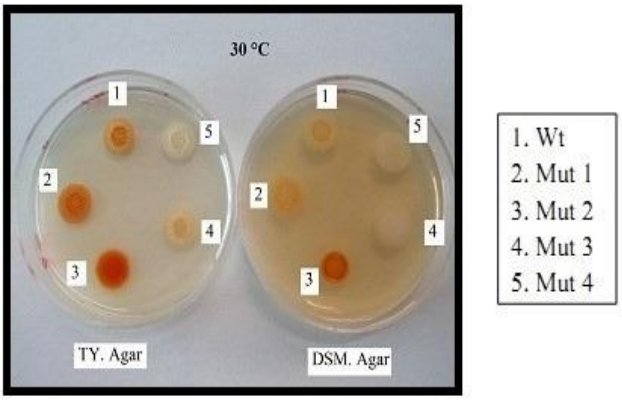


Figure 4 Growth of *B. pumilus wild* type and the others mutants on LB and DSM agar at 30°C

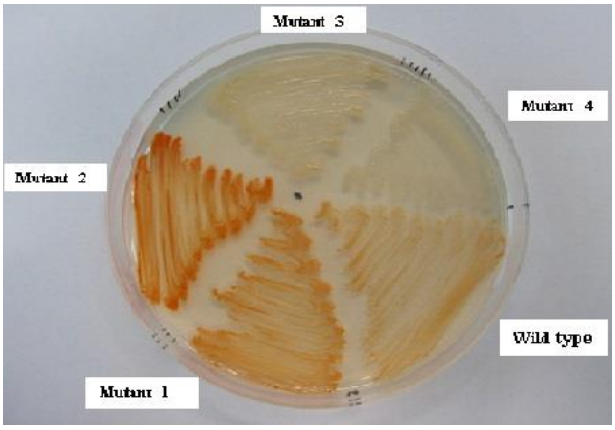


Figure 5 *B. pumilus* SF214 Wild type, mutant 1 yellow to orange, mutant 2 red color, mutant 3 white yellow and mutant 4 white color

An important first line of defence against environmental UV which is manifested by many types of spores, both fungal and bacterial, is production of UV- absorbing pigments. *Bacillus spp.* spores are colored by a brown melanin- like pigment whose production is due in part to the activity of a spore coat protein called CotA, which was recently shown to be a copper dependent lacase [17-18]. Mutant *B. subtilis* strains containing a deletion of the CotA gene produce spores which are significantly more sensitive to artificial UV-B, UV-A, and simulated solar light (Figure 6).

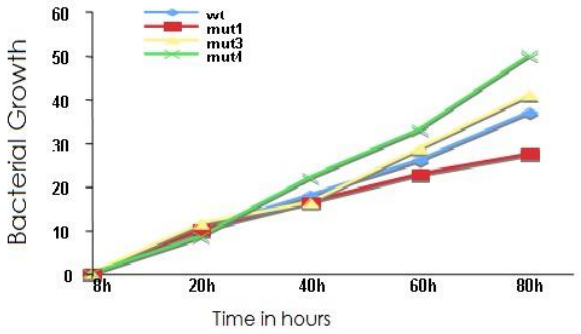


Figure 6 UV resistance of *Bacillus* spore. Spore of Mutant 4 (white pigment) are more resistant after 80 hours treatment

In the laboratory, exposure of spores to physiologically-relevant doses of 254-nm UV-C produces almost exclusively SP which can be efficiently repaired by either NER or SP lyase acting alone [14]. In stark contrast, upon exposure to sunlight, spore DNA accumulates various damages including SP, CPDs, and double- and single strand breaks [19]. Therefore, because of the greater diversity of spore DNA damage induced by solar UV exposure, this damage must necessarily be repaired during germination by a wider variety of DNA repair

systems in addition to SP lyase and NER. In particular, combinational repair is likely to be important in the repair of UV-A damage as the complex structure of DNA at the site of strand breaks often precludes their repair by simple ligation [20]. In conclusion, bacterial spores rely on a broad range of protective and repair mechanisms in order to maintain the integrity of their genetic material in the face of solar UV radiation exposure.

4.0 CONCLUSION

Using the laboratory spore UV resistance model as a starting point and the recent studies described above, we are beginning to build a detailed model describing the mechanisms of bacterial spore resistance to solar radiation. For *B. pumilus* spores, the main lethal factor present in sunlight is the direct UV radiation component, rather than the indirect effects of heating or desiccation. Bacterial spores rely on a broad range of protective and repair mechanisms in order to maintain the integrity of their genetic material in the face of solar UV radiation exposure.

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