Prodigiosin Production and Photoprotective/Antigenotoxic Properties in *Serratia marcescens* Indigenous Strains from Eastern Cordillera of Colombia

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To my parents.

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Resumen

Título: Producción de Prodigiosina y Propiedades Fotoprotectora/Antigenotóxica en Cepas Indígenas de *Serratia marcescens* de la Cordillera Oriental de Colombia[*]

Autor: José Duban Daniel Cediel Becerra[**]

Palabras Clave: Radiación Ultravioleta, Fotoprotección, Antigenotoxicidad, *Serratia marcescens*, Prodigiosina.

Descripción: *Serratia marcescens* es una especie bacteriana productora de un pigmento antibacteriano (Prodigiosina) el cual posee una amplia respuesta adaptativa al estrés ambiental. El estudio tuvo como objetivo investigar la producción de Prodigiosina en las cepas de tipo salvaje de *S. marcescens*, así como su relación con la fotoprotección y la antigenotoxicidad contra la radiación UVB. La producción de Prodigiosina se analizó espectrofotométricamente en los extractos de las cepas bacterianas cultivadas en diferentes medios de cultivo. La eficacia de la fotoprotección *in vitro* se evaluó mediante los índices *in vitro* del factor de protección solar ($FPS_{in\ vitro}$) y la longitud de onda crítica (λc). También se evaluó el porcentaje de estimación de la antigenotoxicidad frente a los rayos UVB (%IG) en el SOS Chromotest. Se utilizó un análisis de correlación para evaluar la relación entre el rendimiento de Prodigiosina, el $FPS_{in\ vitro}$, las estimaciones de %IG y los rasgos ambientales (altitud, temperatura, precipitación e irradiación solar). El rendimiento de Prodigiosina en las cepas de *S. marcescens* varió en función del medio de cultivo utilizado para su crecimiento, y se correlacionó con variables ambientales como la temperatura y la irradiación solar. Las estimaciones de $FPS_{in\ vitro}$ estuvieron bien correlacionadas con la concentración de Prodigiosina y los valores de %IG en las cepas bacterianas estudiadas. La eficacia fotoprotectora UVB de los extractos obtenidos de las cepas de *S. marcescens* depende del rendimiento de Prodigiosina de la cepa y de su potencial antigenotóxico. Los extractos con un rendimiento de Prodigiosina superior a ~17 $\mu g\ mL^{-1}$ podrían utilizarse como fuentes de ingredientes para protectores solares.

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Abstract

Title: Prodigiosin Production and Photoprotective/Antigenotoxic Properties in *Serratia marcescens* Indigenous Strains from Eastern Cordillera of Colombia[*]

Author(s): José Duban Daniel Cediel Becerra[**]

Key Words: Ultraviolet radiation, Photoprotection, Antigenotoxicity, *Serratia marcescens*, Prodigiosin

Description: *Serratia marcescens* is a bacterial species that produces an antibacterial pigment (Prodigiosin) showing a wide adaptive response to environmental stresses. The study aimed to investigate Prodigiosin production in *S. marcescens* wild-type strains, as well as its relation to photoprotection and antigenotoxicity against UVB. Prodigiosin yield was spectrophotometrically assayed in extracts of bacterial strains grown in different culture media. *In vitro* photoprotection efficacy was evaluated using the *in vitro* indices sun protection factor ($SPF_{in\ vitro}$) and critical wavelength (λc). The percentage of UVB- antigenotoxicity estimates (%GI) in the SOS Chromotest was also evaluated. Correlation analysis was used to examine the relationship between Prodigiosin yield, $SPF_{in\ vitro}$, %GI estimates, and environmental traits (altitude, temperature, rainfall, and solar irradiance). Prodigiosin yield in *S. marcescens* strains varied depending on the culture media used for its growth, and it was correlated with environmental variables such as temperature and solar irradiance. $SPF_{in\ vitro}$ estimates were well correlated with Prodigiosin concentration and %GI values in the bacterial strains being studied. UVB photoprotective efficacy of the extracts obtained from *S. marcescens* strains depends on the strain's Prodigiosin yield and its antigenotoxic potential. The extracts with Prodigiosin yield higher than ~17 μg mL^{-1} could be used as sources of sunscreen ingredients.

[*] Degree Work

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Introduction

Skin cancer due to overexposure to sunlight ultraviolet (UV) radiation is considered a global public health problem (1). The sunlight UV radiation comprises long [ultraviolet A (UVA), 320–400 nm], medium [ultraviolet B (UVB), 280–320 nm], and short [ultraviolet C (UVC), 100–280 nm] wavelengths. UVC rays are absorbed by the ozone layer, while UVB (5%) and UVA (95%) rays reach the Earth's surface (2). Both UVA and UVB rays cause DNA damage (e.g., cyclobutane pyrimidine dimers or CPD), which initiates inflammatory processes and skin cancer (3). Since anthropogenic actions have depleted the atmospheric ozone layer increasing solar irradiance at the Earth's surface (4); concern about exposure to sunlight has increased.

Photoprotection is a preventive strategy to protect human skin against cancer and photoaging (5). The topical application of sunscreens is among the most popular strategies used in photoprotection. Sunscreens contain compounds that act like filters either absorbing or refracting the UV radiation (6). Plants have been used as sources of filter and antioxidant ingredients for sunscreens (7,8). Relevant properties of sunscreens used commercially have been recently reviewed (9).

Microorganisms are an underexploited source of natural products with photoprotective potential (10). Pigments produced by different microbial classes have shown effective photoprotective properties (11). *Serratia marcescens* is a bacterial species that produces the red pigment Prodigiosin (12), which exhibits larvicidal, antimalarial, antibacterial, and anti-cancerous activity (13,14). Prodigiosin or related compounds (i.e., undecylprodigiosin) have been associated with UV protection in the *Streptomyces* and *Vibrio* bacterial genera (15,16). When Prodigiosin

was used as an antimicrobial additive on a commercial sunscreen, the UVB photoprotective efficacy of the formulation increased 20 to 65% (17).

This study evaluated Prodigiosin production in *S. marcescens* wild-type strains collected across an altitude gradient in the Eastern Cordillera of Colombia and tested the photoprotective and antigenotoxic activity against UVB of the microbial extracts. Thus, the specific objectives were as follows: *i)* to spectrophotometrically evaluate Prodigiosin production in *S. marcescens* grown in different culture media, *ii)* to determine Prodigiosin production relationships with environmental traits, *iii)* to measure UV-absorption capability of *S. marcescens* extracts by *in vitro* photoprotection indices ($SPF_{in\ vitro}$, and λc) and *iv)* to estimate the antigenotoxicity against UVB of *S. marcescens* extracts using SOS Chromotest and to measure their relationships with photoprotection.

1. Materials and Methods

1.1 Bacterial strains

A total of forty *S. marcescens* strains (Table 1) collected across an altitude gradient in the Eastern Cordillera of Colombia (Fig. 1) were used. The strains were preserved (– 80 °C) at the culture collection "Cepario LMMA-UIS" established by the Universidad Industrial de Santander. A database of this culture collection including taxonomic information, geographical origin, and description of environmental data fields, can be consulted at the Colombian Information System on Biodiversity (https://doi.org/10.15472/uq6pal). The *Serratia marcescens* ATCC®13880™ type strain was included for comparison.

Figure 1

Localities sampled across the Eastern Cordillera of Colombia: Cimitarra (□), Carmen del Chucuri (○), Santa Barbara (△), and Paramo del Almorzadero (◇)

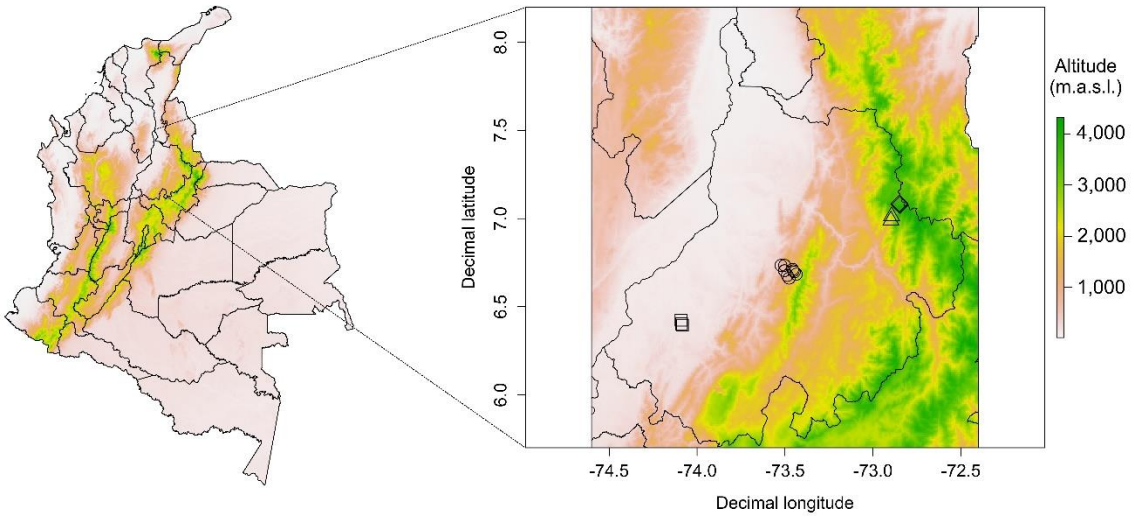


Table 1

List of the studied Serratia marcescens strains and their code numbers in the microbial collection and NCBI database and origin (Localities: C – Cimitarra, CC – Carmen del Chucuri, SB – Santa Barbara, PA – Paramo del Almozadero). Environmental data to collect sites [Alt (masl – meters above sea level): altitude, T (ºC): temperature, SI (kJ m^{-2} day^{-1}): solar irradiance, R (mm day^{-1}): rainfall] are given. Prodigiosin concentration, C (μg mL^{-1}) in each extract was shown. To each extract was also showed the UVB protection efficacy ($SPF_{in\ vitro}$) and critical wavelength (λc) at bacterial extract concentrations. The samples with potential utility in photoprotection for each test appear in bold.

No.	Collection Code (Locality)	NCBI code sequences[†]	Environmental data[‡]				C	$SPF_{in\ vitro}$	λc
			Alt	T	SI	R			
1	UIS0386 (CC)	MT081792.1	807	24.2	17366.8	2071	0.92	2.1 ± 0.1	280 ± 0.0
2	UIS0392 (CC)	MT081803.1	807	24.2	17366.8	2071	0.48	1.6 ± 0.0	280 ± 0.0
3	UIS0417 (CC)	MT081814.1	1369	20.9	17055.7	1969	0.44	1.5 ± 0.0	280 ± 0.0
4	UIS0440 (CC)	MT081825.1	545	25.8	17375.8	2193	12.1	4.3 ± 0.1	337 ± 6.5
5	UIS0447 (CC)	MT081827.1	697	24.6	17403.0	2090	0.81	1.3 ± 0.1	273 ± 6.5
6	UIS0449 (CC)	MT081828.1	697	24.6	17403.0	2090	12.4	7.9 ± 0.3	330 ± 0.0
7	UIS0494 (CC)	MT081829.1	1569	18.8	16968.7	2224	9.51	7.3 ± 0.2	350 ± 0.0
8	UIS0500 (CC)	MT081830.1	1569	18.8	16968.7	2224	4.51	3.9 ± 0.3	330 ± 0.0
9	UIS0502 (CC)	MT081831.1	1569	18.8	16968.7	2224	**17.8**	**20.2 ± 1.0**	350 ± 0.0
10	UIS0526 (CC)	MT081793.1	1569	20.6	17017.2	2018	4.51	3.9 ± 0.3	330 ± 0.0
11	UIS0649 (CC)	MT081794.1	1384	24.5	17336.4	2123	0.49	3.1 ± 0.0	280 ± 0.0
12	UIS0662 (CC)	MT081795.1	705	24.5	17336.4	2123	0.44	2.4 ± 0.0	290 ± 0.0
13	UIS0680 (CC)	MT081796.1	706	25.8	17375.8	2193	2.39	8.7 ± 0.0	360 ± 0.0
14	UIS0684 (CC)	MT081797.1	545	25.8	17375.8	2193	1.99	5.8 ± 0.1	360 ± 0.0
15	UIS0687 (CC)	MT081798.1	545	25.8	17375.8	2193	11.9	9.7 ± 0.0	350 ± 0.0
16	UIS0696 (CC)	MT081799.1	545	24.6	17403.0	2090	**18.7**	**19.1 ± 0.2**	360 ± 0.0
17	UIS0722 (CC)	MT081800.1	684	24.6	17403.0	2090	0.40	1.6 ± 0.0	280 ± 0.0
18	UIS0728 (CC)	MT081801.1	684	24.6	17403.0	2090	0.58	1.2 ± 0.2	310 ± 11
19	UIS0731 (CC)	MT081802.1	684	20.6	16991.9	2019	0.58	1.2 ± 0.2	300 ± 10
20	UIS0766 (CC)	MT081804.1	1365	23.8	17160.4	2023	0.90	3.7 ± 0.0	313 ± 6.5
21	UIS0806 (CC)	MT081805.1	885	23.8	17160.4	2023	0.45	1.4 ± 0.2	283 ± 6.5
22	UIS0811 (CC)	MT081806.1	885	23.8	17160.4	2023	0.54	2.6 ± 0.1	303 ± 6.5
23	UIS0814 (CC)	MT081807.1	885	23.8	17600.1	2747	**40.1**	**30.0 ± 0.0**	**370 ± 0.0**

24	UIS0892 (C)	MT081808.1	148	27.8	17583.8	2738	0.38	1.9 ± 0.0	290 ± 6.0
25	UIS0914 (C)	MT081809.1	118	27.8	17617.5	2740	1.55	**15.3 ± 0.0**	350 ± 0.0
26	UIS0920 (C)	MT081810.1	142	27.8	17617.5	2740	1.13	11 ± 0.0	350 ± 0.0
27	UIS0923 (C)	MT081811.1	142	27.8	17617.5	2740	**19.7**	**30.9 ± 0.2**	360 ± 0.0
28	UIS0940 (C)	MT081812.1	142	27.8	17617.5	2740	**19.6**	**22.7 ± 0.3**	360 ± 0.0
29	UIS0941 (C)	MT081813.1	142	27.8	17600.1	2747	0.95	6.3 ± 0.2	340 ± 0.0
30	UIS0990 (C)	MT081815.1	110	27.8	16804.9	1353	0.58	2.1 ± 0.0	330 ± 0.0
31	UIS1088 (SB)	MT081823.1	1993	15.8	16804.9	1353	0.78	6.2 ± 0.1	330 ± 0.0
32	UIS1093 (SB)	MT081824.1	1993	15.8	17227.5	1415	1.14	**16.8 ± 0.0**	340 ± 0.0
33	UIS1100 (SB)	MT081826.1	1953	15.8	16721.2	1295	0.86	7.7 ± 0.0	340 ± 0.0
34	UIS1132 (PA)	MT081816.1	3463	6.3	16721.2	1295	0.45	3.1 ± 0.0	300 ± 0.0
35	UIS1159 (PA)	MT081817.1	3481	6.3	16721.2	1295	0.69	5.1 ± 0.0	330 ± 0.0
36	UIS1174 (PA)	MT081818.1	3491	6.3	16721.2	1295	1.78	13.8 ± 0.1	350 ± 0.0
37	UIS1182 (PA)	MT081819.1	3491	5.8	16767.6	1330	3.05	**19.9 ± 0.1**	360 ± 0.0
38	UIS1199 (PA)	MT081820.1	3746	5.8	16767.6	1330	2.08	14.7 ± 0.3	360 ± 0.0
39	UIS1200 (PA)	MT081821.1	3746	5.8	16767.6	1330	0.51	4.0 ± 0.3	320 ± 0.0
40	UIS1202 (PA)	MT081822.1	3746	5.8	16767.6	1330	0.52	1.8 ± 0.0	290 ± 0.0
41	ATCC® 13880™	CP041233.1	-	-	-	-	0.68	3.0 ± 0.6	307 ± 13

Nota. †: The best matching to 16S rRNA gene sequences of type species found in the NCBI database ‡: Data obtained from the NASA radar using getData program of the R platform (18). **C**: Prodigiosin concentration was estimated in the extracts as indicated in materials and methods. The $SPF_{in\ vitro}$ values were classified in categories according to the COLIPA guidelines (22) as follows: not protection ($0.0 \leq SPF_{in\ vitro} \leq 5.9$), low protection ($6.0 \leq SPF_{in\ vitro} \leq 14.9$), medium protection ($15.0 \leq SPF_{in\ vitro} \leq 29.9$), high protection ($30.0 \leq SPF_{in\ vitro} \leq 59.9$) and very high protection ($SPF_{in\ vitro} \geq 60.0$). A $\lambda c > 370$ nm defines broad-spectrum protection.

1.2 Chemicals, media, and culture conditions

Prodigiosin hydrochloride pure compound (98 %, HPLC) from *Serratia marcescens* and antibiotics (ampicillin and tetracycline) were obtained from Sigma-Aldrich Co. Inc. (Milwaukee, WI, USA). The substrates for β-galactosidase (*o*-nitrophenyl-β-D-galactopyranoside, ONPG), alkaline phosphatase (*p*-nitrophenylphosphate, PNPP), and the Luria-Bertani (LB) medium were purchased from Amresco (Slon, Ohio, USA). Minimal salt medium M9 (M9), nutrient broth

medium (NB), and reinforced clostridial medium (RCM), as well as, the supplements acetate, agar, casein, ethanol, glucose, glycerol, and yeast extract, were obtained from Oxoid Ltd. (Basingstoke, England). Solvents and other reagents were purchased from Merck (Kenilworth, NJ, USA).

Bacterial strains were grown using different media whose compositions (g L^{-1}) were as follows: LB (10 g tryptone, 5 g yeast extract, 10 g sodium chloride, pH 7.4 ± 0.0); M9 (33.9 g disodium phosphate, 15 g monopotassium phosphate, 2.5 g sodium chloride, 5 g ammonium chloride, 0.018 g phenol red, pH: 7.4 ± 0.2); M9C – (M9 + 10 g casein); M9Y – (M9 + 1 g yeast extract); M9Y.Ac – (M9Y + 10 g sodium acetate); M9Y.Et – (M9Y + 10 mL ethanol); M9Y; Glu – (M9Y + 10 g glucose); M9Y.Gly – (M9Y + 10 mL glycerol); NB (5 g peptone, 3 g beef extract, pH 7.0 ± 0.2); RCM (10 g beef extract, 10 g peptone, 5 g sodium chloride, 5 g dextrose, 3 g yeast extract, 3 g sodium acetate, 1 g soluble starch, 0.5 g L-cysteine HCl, 0.5 g agar, pH 6.8 ± 0.2). Preserved strains were placed in sterile glass canisters containing the different liquid media (20 mL) and the cultures were grown for 4 days at 28 °C and 37 °C under aerobic conditions using a Midi 40^{TM} incubator (Thermo Scientific, Waltham, USA).

1.3 Prodigiosin production in *S. marcescens* strains

To determine the best culture medium for Prodigiosin production, we firstly used a *S. marcescens* strain (UIS0814) that had high pigmentation level. Thus, overnight cultures (0.5 mL) of UIS0814 strain were spread *per* triplicate over Agar media plates (M9C, M9Y.Ac, M9Y.Et, M9Y.Glu, M9Y.Gly, NB, RCM); then incubated for 4 days at 28 °C (producing temperature) and 37º C (non-producing temperature) in an MLW incubator (Wolfsburg, Germany). Sterile distilled water (10 mL) was added to plates, and the bacterial cells detached. Cell suspensions (30 mL) were collected, dispensed into conical tubes, and centrifuged at 7,500 rpm in cool (4 ºC) for 15

min. Cell pellets were dissolved in a similar volume (30 mL) of acidified methanol (1M HCl) and sonicated at 25 °C for 2 h using an E^{+}EP30H ultrasonic thermostatic bath cleaner (Elmasonic, Amazon, USA). Finally, suspensions were centrifuged under similar conditions, supernatants transferred to sterilized conical tubes and, the absorbance spectra of these extracts were measured by using a Multiskan GO type spectrophotometer (Thermo Scientific, MA, USA). The culture medium that produced the bigger area under the spectral curve (see below), was selected to study Prodigiosin production in the remaining strains of *S. marcescens*. The corresponding extracts all were prepared as indicated above.

To quantitative measure Prodigiosin concentration in all the *S. marcescens* extracts, a calibration curve using pure compound Prodigiosin hydrochloride was developed. A correlation analysis between compound concentration and the optical density at 535 nm was carried inside of sensitivity limits to Multiskan GO type spectrophotometer (Thermo Scientific, MA, USA). The fit of the linear regression model allowed us to estimate Prodigiosin concentrations in both rarefaction (interpolation) and prediction (extrapolation) curve zones. All the *S. marcescens* extracts were stored under refrigeration (4º C) until use.

1.4 UV-absorption capability

Aqueous aliquots (1.5 mL) of *S. marcescens* extracts at different Prodigiosin concentrations (0.06 and 80 µg mL^{-1}) were placed in a quartz cuvette (1-cm step length and 1.5 mm glass thickness) and their UV–absorption spectra (wavelengths 200–400 nm) were recorded in triplicate using the Skanlt 3.2 function on a Multiskan GO UV-spectrophotometer (Thermo Scientific, MA, USA). A blank containing distilled water was always used. A minimum of three independent experiments *per* extract dilution were performed. The average absorbance values and

their corresponding standard errors for wavelength intervals of 10 nm were plotted using the ggplot2 library on the R platform (18).

1.5 *In vitro* photoprotection efficacy

We estimated the UVB photoprotection efficacy by using the sun protection factor ($SPF_{in\ vitro}$) described by Sayre *et al*. (19) and further simplified to UV-spectrophotometric Mansur´s method (20): $SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times A(\lambda)$; where $EE(\lambda)$ – erythemal effect spectrum at wavelength λ, $I(\lambda)$ – solar intensity spectrum at wavelength λ, $A(\lambda)$ – absorbance of the extract solution determined by UV spectrophotometry at a wavelength (λ) and, CF – correction factor (CF = 10). The values of EE (λ) x I product function were previously normalized (19) and shown in Table 2.

Table 2

The normalized product function used in the calculation of SPF data. EE = erythemal efficiency spectrum; I = solar simulator intensity spectrum

Wavelength	EE x I (Normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
∑ =	1.0000

The broad-spectrum protection efficacy was determined by calculating the critical wavelength (21): $\lambda c = \int_{290}^{\lambda c} A(\lambda)d\lambda = 0.9 \int_{290}^{400} A(\lambda)d\lambda$; where $A(\lambda)$ is the absorbance at wavelength λ, λc is the critical wavelength (nm) and $d\lambda$ is the wavelength step (5nm). The $SPF_{in\ vitro}$ values were classified in categories according to the COLIPA guidelines (22) as follows: not protection ($0.0 \leq SPF_{in\ vitro} \leq 5.9$), low protection ($6.0 \leq SPF_{in\ vitro} \leq 14.9$), medium protection ($15.0 \leq SPF_{in\ vitro} \leq 29.9$), high protection ($30.0 \leq SPF_{in\ vitro} \leq 59.9$) and very high protection ($SPF_{in\ vitro} \geq 60.0$). A $\lambda c > 370$ nm defines broad-spectrum protection according to FDA guidelines (23).

1.6 Cell irradiation

Cell irradiation was conducted as indicated by Fuentes *et al*. (24). Briefly, cultures (1 mL) with an optical density (OD_{600nm}) of 0.4 ($\sim 2 \times 10^8$ cells mL^{-1}) were distributed into Petri plates with 5 cm diameter for irradiation during assays. Petri plates with the cells were UVB-irradiated in darkness using a UVA/UVB irradiation chamber BS-02 equipped with a radiation controller UV-MAT (Opsytec Dr. Groebel, Ettlingen, Baden-Würtemberg, Germany). This radiation controller continuously measured the irradiance, calculated the irradiation dose, and switched the lamps after reaching the target dose. The radiation dose used for the antigenotoxicity assay was 10 J m^{-2}, which induces adequately SOS functions in *E. coli* cells without significantly affecting their survival (25).

1.7 Genotoxicity of *S. marcescens* extracts

The genotoxicity of the *S. marcescens* extracts was evaluated using the SOS Chromotest as described previously (26). We always used sterile distilled water as the dissolvent and an ultrasonic bath E30H Elmasonic (Elma-Hans Schmidbauer GmbH & Co., Singen, Germany) to

dissolve the *S. marcescens* extracts and Prodigiosin hydrochloride pure compound. Overnight cultures were grown in LB medium to an optical density (OD_{600} nm) of 0.4, diluted 10-fold in fresh LB medium, and distributed (150 µL) into a series of Eppendorf tubes containing 150 µL of the extract or compound to be tested. The bacteria were exposed for 30 min at 4 °C to different *S. marcescens* extracts or pure compound concentrations and then were cultured for 2 h at 37 °C with shaking at 300 rpm in a Thermomixer apparatus (Eppendorf, Sao Paulo, Brazil). Negative (distilled water) and positive (2.34 µM of 4-NQO) controls were always included in each assay. A minimum of four independent experiments *per* treatment with two replicates each were conducted. β-Galactosidase (βG) and alkaline phosphatase (AP) activities were assayed in 96-well plates (Brand GMBH, Germany). For βG activity, cell membranes were disrupted by mixing 135 µL of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 0.1% SDS, and 40 mM β-mercaptoethanol, pH 7.0) with 15 µL of cell culture for 20 min at room temperature. The reaction was started by adding 30 µL of ONPG (4 mg mL^{-1} in 0.1 M phosphate buffer, pH 7.0). After 40 min, the enzymatic reaction was stopped by adding 100 µL of Na_2CO_3 (1 M). For the AP activity, cell membranes were disrupted by adding 135 µL of T buffer (0.1% sodium dodecyl sulfate, 1 M Tris HCl, pH 8.8) to 15 µL of the cell culture. The enzyme reaction was started by adding 30 µL of PNPP solution (4 mg mL^{-1} in T buffer). After 40 min, the reaction was stopped by adding 50 µL of HCl (2.5 M). After 5 min, 50 µL of Tris (2 M) were added to restore the color. The final absorbance of the βG and AP assays were measured at λ = 420 nm, using a microplate reader Multiskan GO (Thermo Scientific, MA, USA). βG and AP activities were calculated using the relationship: enzyme units = $(1000 \times A_{420})/t$, where A_{420} is the optical density at λ = 420 nm and t is the length of incubation (min) with the substrate (ONPG or PNPP). The ratio of βG units to AP units (R = βG/AP) reflects the induction of the *sulA* gene, even when there is some inhibition of

protein synthesis. The genotoxicity criterion used was the SOS induction factor (*I*) representing the normalized induction data of the *sulA* gene in each treatment (extract or pure compound) and was therefore considered an indirect measure of the primary DNA damage (genotoxicity) induced by the treatments. This parameter was calculated as $I = R_t/R_{nt}$, where t and nt were the treated and non-treated cells, respectively. The samples were classified as not genotoxic, if *I* was < 1.5, inconclusive, if *I* was between 1.5 and 2.0, and genotoxic, if *I* was >2.0, and a clear concentration-response relationship was observed.

1.8 Antigenotoxicity against UVB radiation of *S. marcescens* extracts

The antigenotoxicity assay was conducted using a co-incubation procedure (27). The cells were simultaneously treated with *S. marcescens* extracts and 10 J m^{-2} of UVB and cultured for 2 h at 37 °C while shaking at 300 rpm in a Thermomixer apparatus (Eppendorf, Sao Paulo, Brazil). Negative (distilled water) and positive (10 J m^{-2} of UVB) controls were always included in each assay. A minimum of four independent experiments *per* treatment with two replicates were conducted. The βG and AP activities were assayed as indicated above. Antigenotoxicity (the ability of the test extract or pure compound to protect against UV-induced genotoxicity) was measured as a significant reduction in the induction factor (I) in the co-incubation procedure and expressed as a percentage of the genotoxicity inhibition: $\%GI = 1 - (I_{ct} - I_{nt}/I_{UVB} - I_{nt}) \times 100$, where I_{ct} is the SOS induction factor in the co-incubation procedure; I_{nt} is the SOS induction factor in non-treated cells, and I_{UVB} is the SOS induction factor in UVB-treated cells. Negative values of %GI were considered as zero; therefore, this parameter ranged from a minimum of 0% to a maximum of 100%. The minimal concentration that produces a significant ($p \leq 0.05$) genotoxicity inhibition (CGI) in PQ37 cells was used for comparison of the genoprotective potential of the tested samples.

1.9 Statistical analysis

The $SPF_{in\ vitro}$, λc, and %GI values and their corresponding standard errors were calculated. In all cases, the data passed the Kolmogorov–Smirnov and F-maximum tests for normality and variance homogeneity, respectively; therefore, these parametric tests were used in subsequent data analyses. When a significant F-value was obtained in a one-way analysis of variance (ANOVA), the groups were subsequently compared with Tukey's test. Pearson correlation analysis was used to examine the relationship between Prodigiosin concentration, $SPF_{in\ vitro}$, %GI estimates, and environmental traits (altitude, temperature, rainfall, and solar irradiance). Principal Component Analysis (PCA) represented the grouping of *S. marcescens* strains and the relationships between traits. For all statistical analyses, a p-value ≤ 0.05 indicated significance. The R program (18) was used for all analyses.

2. Results

2.1 Prodigiosin production in *S. marcescens* strains

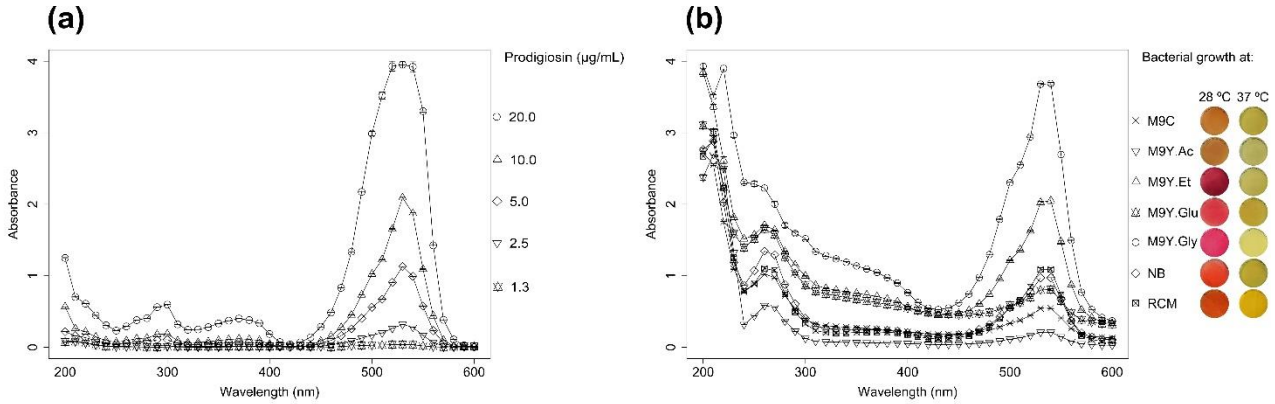
The influence of the culture medium on Prodigiosin production by *S. marcescens* is shown in Fig. 2. First, we showed the UV-VIS absorbance spectrum of the Prodigiosin hydrochloride pure compound for comparison (Fig. 2a). The compound showed the highest absorbance peak at $\lambda = 535$ nm, but other peaks at λ of 370 nm, 300 nm, and 200 nm were also observed. These peaks increased at higher pure compound concentrations to UV-VIS spectrum between 200 and 400 nm (data not shown).

The media pigmentation and UV-VIS absorbance spectrum of the extracts yielded by *S. marcescens* strain UIS0814 varied depending on culture media used (Fig. 2b). The *S. marcescens* extracts were produced in M9Y.Gly medium showed a bigger area under the spectral curve and

the highest absorbance peak at λ = 535 nm, suggesting that this contains more Prodigiosin than extracts produced in other media. Prodigiosin was produced only when the bacterial cells were grown at 28 °C, but not at 37 °C. In Table 1 were showed the Prodigiosin concentrations calculated to each *S. marcescens* extracts as indicated in material and methods. Five *S. marcescens* extracts (UIS0502, UIS0696, UIS0814, UIS0923, and UIS0940) showed the higher Prodigiosin concentration values among studied strains.

Figure 2

UV-VIS absorbance spectral related to Prodigiosin hydrochloride pure compound (a) and the extracts (b) from S. marcescens UIS0814 strain that were obtained using different culture media and temperatures. The colored circles in Figure 2b are pictures of S. marcescens growth over Agar media plates. Error bars indicate the standard error of the mean for at least three independent experiments (n = 3)



2.2 Influence of environmental traits on prodigiosin yield in *S. marcescens*

The Prodigiosin yield of *S. marcescens* strains correlated to the temperature ($R = 0.28$, $p \leq 0.05$) and solar irradiance ($R = 0.30$, $p \leq 0.05$) of the original isolation site, but not to the altitude and rainfall markers. Alternatively, temperature and solar irradiance traits were inversely correlated to the other environmental markers (Table 3). PCA performed on the traits represented the relations among them and grouped studied *S. marcescens* strains (Fig. 3). The first two components of the PCA explained ~94% of the total variability due to the contribution of these variables. PC1 grouped *S. marcescens* strains *per* locality (Cimitarra, Carmen del Chucuri, Santa Barbara, and Paramo del Almorzadero) and related variables. Temperature, solar irradiance, and rainfall traits were grouped separately to altitude, supporting correlation analyses presented in Table 3. Variation in PC2 was majorly represented by variable Prodigiosin concentration. *S. marcescens* strains from Cimitarra and Carmen del Chucuri that showed Prodigiosin yields ≥ 3.05 $\mu g\ mL^{-1}$ were represented separately from the remaining strains in PC2. Both PCA and correlation analysis suggested that additional factors other than environmental traits, influence the Prodigiosin yield in *S. marcescens*.

Figure 3

S. marcescens strains dispersion on the first two principal components in PCA based on Prodigiosin yield and environmental traits data. Ellipses indicate the grouping of strains across sampled localities. Arrows indicate the relationships between traits.

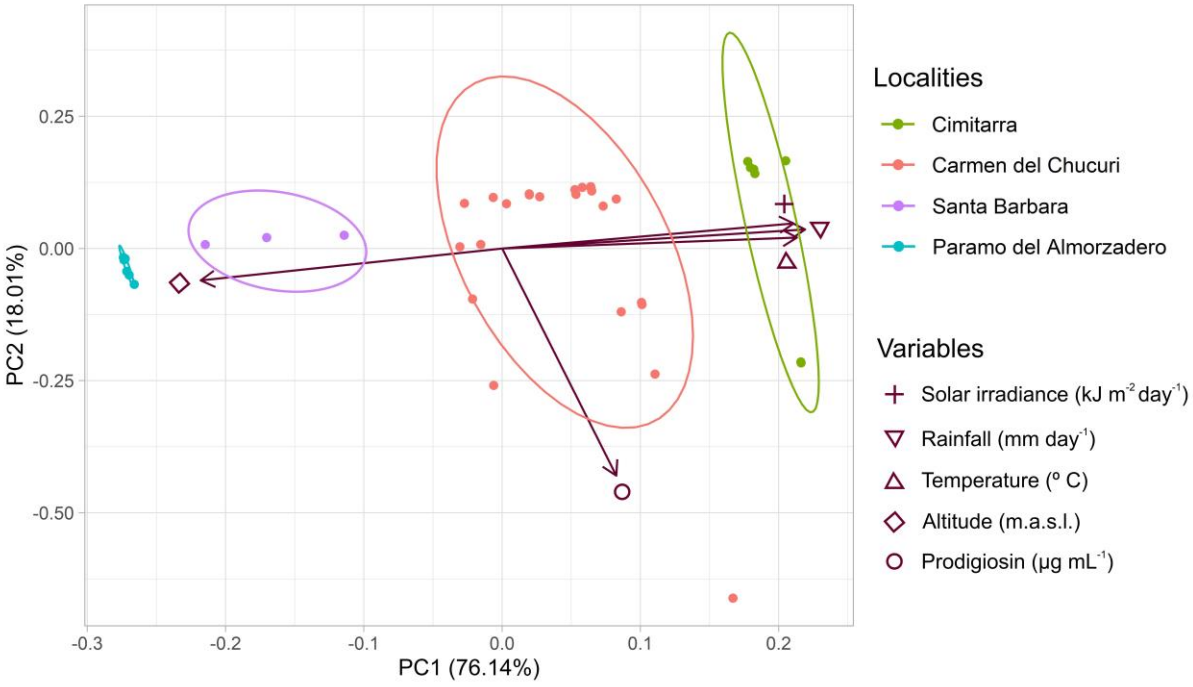


Table 3

Correlation analysis using Prodigiosin concentration (C) and environmental traits (Alt: altitude, T: temperature, SI: solar irradiance, R: rainfall) from sampling sites

Traits	**C**	**Alt**	**T**	**SI**	**R**
C (µg mL^{-1})	–				
Alt (masl)	- 0.24	–			
T (° C)	0.28*	- 0.96*	–		
SI (kJ m^{-2} day^{-1})	0.30*	- 0.89*	0.95*	–	
R (mm day^{-1})	- 0.25	- 0.90*	0.88*	0.85*	–

Nota. *Correlation is significant ($p \leq 0.05$)

2.3 *In vitro* photoprotection efficacy of *S. marcescens* extracts

The $SPF_{in\ vitro}$ values indicated that 18 (~ 45%) of the 40 studied bacterial strains had some degree of UVB photoprotection (Table 1). According to the COLIPA photoprotection efficacy categories (see material and methods), the *S. marcescens* extracts are classified as follows: high (n = 2), median (n = 6), low (n = 10), and not photoprotection (n = 22). Only one *S. marcescens* extract (UIS0814) showed broad-spectrum protection efficacy ($\lambda c \geq 370$ nm) like Prodigiosin hydrochloride pure compound (data not shown), indicating poor UVA protection in the rest of the studied bacterial strains. A Pearson correlation analysis showed a positive correlation ($R = 0.79$, $p \leq 0.05$) between $SPF_{in\ vitro}$ and Prodigiosin concentration values in the *S. marcescens* extracts (Fig. 4a), suggesting that Prodigiosin is the main responsible for the photoprotection properties exhibited by the *S. marcescens* extracts. Unexpectedly, three extracts (UIS0914, UIS1093, and UIS1182) with median photoprotection efficacy showed consistently low Prodigiosin concentrations ($\leq 3.0\ \mu g\ mL^{-1}$).

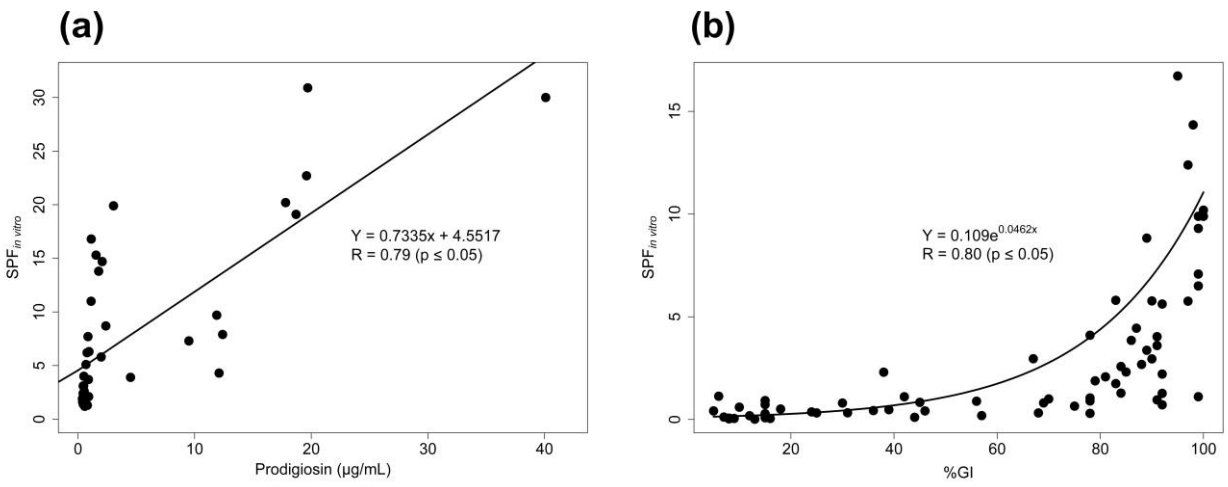
2.4 Relations between $SPF_{in\ vitro}$ and %GI estimates in bacterial extracts

Before antigenotoxic effects of *S. marcescens* extracts were assayed their genotoxicities were investigated using the SOS Chromotest. None of the bacterial extracts increased *I* values at concentrations assayed in this study (data not shown). Therefore, they were considered not genotoxic in *E. coli* PQ37 cells. Randomly selected samples (n = 13) of *S. marcescens* extracts were used to evaluate the relation between UVB photoprotection efficacy ($SPF_{in\ vitro}$) and antigenotoxicity (%GI) estimates. All *S. marcescens* extracts with some degree of UVB photoprotection ($SPF_{in\ vitro} \geq 6.0$) resulted in %GI values higher than 80% (Fig. 4b). The $SPF_{in\ vitro}$

and %GI values in *S. marcescens* extracts were exponentially correlated ($R = 0.80$, $p \leq 0.05$). That is, the greater the UVB photoprotective efficiency, the lower the genetic damage.

Figure 4

Correlation between UVB photoprotection efficacy ($SPF_{in\ vitro}$) and Prodigiosin concentrations in S. marcescens extracts (a) and with their %GI estimates (b). A database containing 71 paired $SPF_{in\ vitro}$ and %GI values, corresponding to 13 bacterial species, was used



3. Discussion

The present work evidenced that wild-type *S. marcescens* strains from the Eastern Cordillera of Colombia contain compounds that could be useful as ingredients for sunscreens. Photoprotective and antigenotoxic capabilities of the *S. marcescens* extracts were mostly related to the metabolite Prodigiosin, supporting physiological functions of this pigment in bacterial photoprotection against sunlight UV radiation. However, three photoprotective extracts (UIS0914,

UIS1093, and UIS1182) showed consistently low Prodigiosin concentrations; suggesting that compounds different to Prodigiosin should be responsible for photoprotection in these three extracts as this has been described for *Streptomyces* specimens (16).

Like previous studies (28–30), we showed the glycerol-supplemented medium (e.g., M9Y.Gly) was the best to reach high-level Prodigiosin synthesis, which occurred at 28 °C, but not at 37 °C (Fig. 2a). Prodigiosin production in *S. marcescens* species has been documented at temperatures between 28 to 36 °C (29–32). In the literature, bacterial Prodigiosin yields in wild-type strains range between 18.4 and 34.7 μg mL^{-1} (15,28). Our results showing prodigiosin yields > 17 μg mL^{-1} were in that range.

The *S. marcescens* strains that yielded high levels of Prodigiosin (> 17 μg mL^{-1}) come from sites, which showed *in situ* average temperatures between 18.8 and 27.8 °C. Romanowski *et al*. (32) have indicated that Prodigiosin synthesis is thermoregulated at the transcriptional level. This could explain why *S. marcescens* strains from localities with low average temperatures (SB = 15.8 °C; PA = 6.0 °C), never produced the red pigment.

We also found correlation ($R = 0.30$, $p \leq 0.05$) between *S. marcescens* pigmentation and *in situ* solar irradiances as it has been empirically proved for other bacterial species from genera *Janthinobacterium*, *Microbacterium*, *Rhodobacter*, and *Shewanella* (33–36). Increased pigmentation and efficient repair of DNA damage are the main ways by which microorganisms acquire resistance to UV radiation (11). Our findings on correlations between Prodigiosin yields with photoprotective ($R = 0.79$, $p \leq 0.05$) and with antigenotoxic ($R = 0.80$, $p \leq 0.05$) properties in *S. marcescens* extracts means that, the photoprotective and antigenotoxic capabilities against UV radiation in *S. marcescens* cells increases with Prodigiosin concentration. Prodigiosin or related compounds (i.e., undecylprodigiosin) have been associated with UV protection in

Streptomyces and *Vibrio* bacterial genera (15,16), suggesting the physiological functions of pigmented compounds in bacterial UV radiation resistance.

The present work evidenced that wild-type *S. marcescens* strains (e.g., UIS0923 and UIS0814) could be useful as sources of ingredients (e.g., Prodigiosin) for sunscreens. A previous study (17), using Prodigiosin as an antimicrobial additive of sunscreen, showed increases photoprotective efficacy of the formulation. Here, we have shown that this compound can act as a filter of UV radiation with a broad-spectrum protection efficacy reducing genotoxicity. However, it would be necessary to test this compound for skin irritability, toxicity, and genotoxicity using mammalian assays for use in photoprotection.

4. Concluding remarks

We demonstrated that Prodigiosin yield in *S. marcescens* strains varied depending on culture media used for its growth, and it was correlated to environmental variables such as temperature and solar irradiance. UVB photoprotective efficacy of the extracts obtained from *S. marcescens* strains depends on the Prodigiosin yield and determined its antigenotoxic potential. The *S. marcescens* strains UIS0923 and UIS0814 showed high photoprotection efficacy and antigenotoxicity against UVB, but only the strain UIS0814 exhibited broad-spectrum (UVA-UVB) photoprotection. The *S. marcescens* extracts with Prodigiosin yield higher than ~17 μg mL^{-1} could be used as sources of sunscreen ingredients that reduce UVB- induced genotoxicity.

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