JJBS

Volume 13, Supplementary Issue, December 2020
ISSN 1995-6673
Pages 725 - 729

Jordan Journal of Biological Sciences **Short Communication 2**

Cyanoxanthomycin, a Bacterial Antimicrobial Compound Extracted from Thermophilic *Geobacillus* sp. Iso5

Dummi M. Gurumurthy[1*], T. P. Charanraj[2], Basheerabegum Faniband[3], Preeti N. Tallur[4], Zabin K. Bagewadi[5], Shivayogeeswar E. Neelagund[6] and Sikandar I. Mulla[2,*]

[1]Department of Biotechnology, GM Institute of Technology, Davangere-577 006, [2]Department of Biochemistry, [3]Department of Physics, School of Applied Sciences, REVA University, Bangalore-560064, [4]Government Arts & Science College, Karwar. [5]Department of Biotechnology, KLE Technological University, Hubballi-580 031, [6]Department of Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shimoga-577 451, Karnataka, India.

Received: January 14, 2020; Revised: February 13, 2020; Accepted: February 22, 2020

Abstract

The simple and reliable method was developed for the extraction of a novel class of cyanoxanthomycin (3-(3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2,6-dioxo-2,6-dihydro-1H-benzo[cd]pyren-4-yl) propanenitrile) type antibiotic from thermophilic *Geobacillus* sp. Iso5. The molecule was a typical cytoplasmic pigment, and can be readily identified (both in extraction and thin layer chromatography) by yellow-orange fluorescence under long UV/blue intensities. GC-MS analysis revealed that the molecular structure of the isolated pure extract was cyanoxanthomycin type antibiotic pigment composed of a benzopyrene with five fused rings in a planar delocalized structure. FT-IR spectra confirms the presence of hydroxyl, carbonyls, methyl and cyano group in the pigment. Furthermore, the *in-vitro* antibacterial cyanoxanthomycin was determined to have selective growth inhibition against various bacterial strains (Gram +ve/-ve). Further studies on various physiological and genetic level effects of this antimicrobial compound will provide the possibility for the future therapeutic and industrial applications.

Keywords: Thermophiles, *Geobacillus*, cyanoxanthomycin, fluorescence, pyrene

1. Introduction

Recent advancement in structural, molecular and genetics of thermophiles which were adopted to extreme temperature provided us with significant knowledge for their utilization in industrial and pharma biotechnology (McMullan et al. 2004; Stetter 1999). These thermophiles produce unique proteins, enzymes, cellular components and other biochemicals that function under conditions in which mesophilic counterparts cannot be sustained (Bull et al. 2000). Because of their unusual structural integrity of the molecules allow them to withstand harsh conditions typically associated with industrial processes, which has consequently resulted in them being used in several novel applications (Dufossé et al. 2005; Morozkina et al. 2010).

Thermophiles, as a result, is driven by the novel bioactive molecules found to be convenient in many applications. Despite, certain groups of hyperthermophilic bacteria can able to produce simple to more complex compounds in their existing environment. Especially, the production of fluorescent pigment is predominant in a member of the mesophilic group unless it is found less in thermophiles and hyperthermophilic bacteria. Although, a variety of plants, animals and microorganisms produce fluorescent pigments. Few bacterial groups produce blue, blue-green, yellow-orange and green-red coloured pigments, which are having different functions and chemical properties. On the other hand, the pigment productions on selective media are important taxonomic tools for the differentiation and classification of bacteria (Jordan 1899; Nazina et al. 2001). Most of the industrially viable fluorescent pigments used currently are extracted from plants and other mesophilic bacteria. It could be an advantage to produce natural fluorescent pigments from thermophilic bacteria due to their stability and other novel applicabilities.

The pentacyclic derived compounds from bacteria also exhibit fluorescent properties, which are functionally similar to other pentacyclic pigments from plants and other sources. Xanthomycin is one such pentacyclic compound containing cyano group which is absent or less predominant in hyperthermophiles. Some pigments are of cytoplasmic origin and produce characteristic yellow-orange colour in long UV radiation. The fluorescent pigment is less predominant in many bacterial genera, which exhibit the characteristic yellow-orange coloured fluorescent property. There are very few reports on cyano group containing crude/pure drug extracted from bacteria, especially *Bacillus* genus (Nair et al. 2019).

On the basis of its occurrence and distribution, there is no evidence of pentacyclic derived fluorescent pigment in thermophilic and hyperthermophilic groups especially in the members of bacterial group 5, *Geobacillus*. Here we have described a simple, rapid and novel method in isolation and characterization of yellow-orange fluorescent pigment from hyperthermophilic *Geobacillus* sp Iso5.

* Corresponding author e-mail: sikandar.mulla@gmail.com; drgurumurthydm@gmit.ac.in.

2. Materials and Methods

2.1. Bacterial cultivation and culture media

Thermophilic aerobic *Geobacillus* sp. Iso-5 was isolated previously from alkaline thermal spring of Karnataka, India (Mahadevan 2012). To be precise, the strain was isolated from Bendruthirtha Hot water springs of Karnataka. The bacterium, *Geobacillus* sp. Iso5 is a gram-positive, non-motile, rod shaped with 1.3-1.5 µm in width and 3-6 µm in length. Its growth range was observed between 65–90 ºC, with an optimum at 70 ºC; the pH range for optimal growth is between 6 – 9. The mineral enrichment of LB cultured cells was aerobically transferred to SPYM media containing (w/v) 1 g of Starch; 0.5 g of Peptone; 0.2 g of yeast extract, 0.05 g of Sodium chloride, 1% vitamin mixture and 0.5% mineral salt solution. The medium was prepared pH 8.0 by adding either 1 N HCl or 1 N NaOH and sterilized. The culture was inoculated at 65 °C and kept for 48 hours under constant agitation. Tryptic Soy Agar (TSA) (HiMedia, Mumbai), Nutrient Agar (NA) (HiMedia, Mumbai) were also used to perform antimicrobial properties of the pigment extract.

2.2. Extraction of Fluorescent pigment

A desired seed culture of the bacterium was hygienically transferred to 2.5 liters of SPYM and incubated for 48 hours at 65 °C. The cells were then harvested by centrifugation at 10,000 × *g* in 4 °C for 25 min. The bacterial pellets were collected and washed with a minimum amount (~25 ml) of 50 mM Phosphate buffer (pH 7.0) and approximately twenty-five grams biomass was then freeze-dried. The freeze-dried sample was washed twice with of 80% ice-cold acetone and agitated for the two-minute cold condition. Finally, the agitated mixture was filtered using Whatman No 1 filter paper (Whatman, USA) under cold condition and air-dried. The filtrate was then extracted three times using under reflux for one hour by chloroform/methanol (2:1) (HiMedia, Mumbai) solvent mixture. After each subsequent reflux step, the extracts were combined and evaporated to dryness under room temperature.

2.3. Thin Layer Chromatography

The purity of the fluorescent pigment was analyzed by thin layer chromatography (TLC) using Silica Gel 120. The dried extract was dissolved in minimum amount of chloroform/methanol (2:1) and purity was determined under 0.5-mm silica gel sheets (Merck 60F-254) using chloroform-ethyl acetate (3:1) solvent system. For the extraction from silica gel, the preparative TLC was performed under thin Silica Gel plates (0.3 mm, Merck, Mumbai). The fluorescent compound was irradiated and visualized with a long-wavelength (340- 365 nm) UV lamp (Philips, USA) (Magyarosy et al. 2002). The fluorescent bands were scraped off from the plate, dissolved in minimum amount of ice-cold acetone, and filtered through Whatman No1 filter paper.

2.4. Analytical Methods

The GC-MS profile of the fluorescent pigment was determined by the VF-5ms 30 m x 0.25mm (ID) x 0.25µm quartz capillary column with helium as the carrier gas, injector temperature was 280 °C, split ratio 15:1.0 and sample size 1.0 µl for GC condition. The ionization mode was EI, electron bombarding energy was 70 eV, charging multiplier tube voltage at 500 V, scan range from m/z 40 to 650 at 3 scan/sec was used, and solvent delay at 3 min was maintained for mass determination. The infrared spectrum of the pigment was recorded on Nicolet Magna-IR 380 FTIR spectrometer (Thermo Electron, USA) spectrophotometer using KBr pellets at 32 average scans rate.

2.5. Antibacterial assay

The antibacterial property of the cyanoxanthomycin pigment was determined by agar well diffusion method using Muller-Hinton (MHA) agar plates. The concentration of 50µM (1 µM= 429µg/ml) was prepared by dissolving the appropriate amount of pure extract (cyanoxanthomycin) in sterile distilled water. The equal amount of standard ciproflaxin using distilled water was also prepared. The overnight grown individual bacterial strains were uniformly swabbed on to the surface of MHA plates by sterile cotton swabs. The cell concentration of *Bacillus subtilis* (MTCC 3053), *Escherichia coli* (MTCC 1698), *Pseudomonas aeruginoasa* (MTCC 6458), *Staphylococcus aureus* (MTCC 6908) and *Streptococcus* sp. (MTCC 9724) were 2.1×10^6, 2.6×10^6, 2.5×10^6, 2.9×10^6, and 3.1×10^6 cfu/mL, respectively (Mulla et al., 2016 a and b). Each 50µL of sample were aseptically loaded in well and the plates were incubated in an upright position for 1 day at 37 °C. The clear zone of inhibition around colonies indicated the antibacterial property of the pigment.

3. Results and Discussion

Extremophiles, isolated from the extreme ecological niches are well adapted to unfavourable environmental factors and have huge biotechnological potential (Dufossé et al. 2005; Fujiwara 2002; Singh and Gabani 2011). Especially, the biologically active substances synthesized by extremophiles, currently seeking to be important for their ability to survive and proliferate. These compounds are generally nonessential for growth of the organism and are synthesized with the aid of genes involved in intermediary metabolism. Among bioactive compounds of microbial origin have been characterized, a limited number of compounds is used for biomedical, agricultural and industrial applications. One such natural bioactive compound is fluorescent pigment with potent antibiotic properties.

3.1. Extraction and characterization of the Pigment

Herein, a simple procedure was developed for isolation of water-insoluble fluorescent pigment from *Geobacillus* sp. Iso5. It was characterized as yellow-orange colour producing pigment under long wavelength of UV intensity (Figure 1). The chloroform/methanol extraction (2:1) of pigment under reflux condition yielded a bright yellow-orange coloured spot on silica gel plate (data not shown). The purity of the extraction protocol was further analyzed by preparative thin layer chromatography, also revealed the presence of single yellow-orange colored spot (R_f value, 0.53) on the thin silica gel plates. Further fluorescent properties in different solvents such as acetone, dimethyl sulfoxide, methanol and pyridine also revealed the presence of same yellow-orange fluorescent colour.

The molecular mass of the pigment analyzed by using GC-MS revealed the presence of one major peak at *m/z* 429.14 with 90% intensity at 12 minutes (Figure 2). Additionally, from FT-IR, the CN stretching frequency is observed for the molecule between 2200-2300 cm^{-1}. From these results it was identified as cyanoxanthomycin of *Geobacillus* sp. Iso5 is a conjugated pigment. The production of fluorescent pigments by many bacterial groups was previously reported (Jordan 1899; Seleen and Stark 1943). Indeed, particularly abundant with the fluorescent *pseudomonads* adjacent to the atmosphere, spermosphere and rhizosphere in the soil (Elliott 1958; Huge 1964; Turfreijer et al. 1938). The *Pseudomonas fluorescens* produces yellow-green fluorescent pigment (YGFP) from brewery waste yeast with and without mineral salts in the medium (Silva and Almeida 2006). Elliott (1958) and Meyer (2000) described some of the properties of the water-soluble fluorescent pigment of the *Pseudomonads* called Pyoverdine. There are only a few reports available on the fluorescent pigment production from *Bacillus* sp. (Kolev 1991; Sneath 1986). Although, the influence of some natural and synthetic medium for the production of blue, blue-green, yellow-orange and green-red fluorescent coloured pigments from *B. fluorescens albus, B. fluorescens tennis, B. fluorescens mesentericus, B. fluorescens putridus. B. viridians and, B. fluorescens liquefaciens* was also reported (Flugge 1886; Jordan 1899; Meyer 1978; Seleen and Stark 1943). Further, Turfitt (1937) employed ordinary nutrient media for *B. pyocyaneus*, *B. fluorescens liquefaciens*, and *B. fluorescens non-liquefaciens* accompanied by the diffusion of a green or greenish blue fluorescence throughout the culture. However, there are only few *Bacillus* spp. produces yellow-green diffusible pigments were reported.

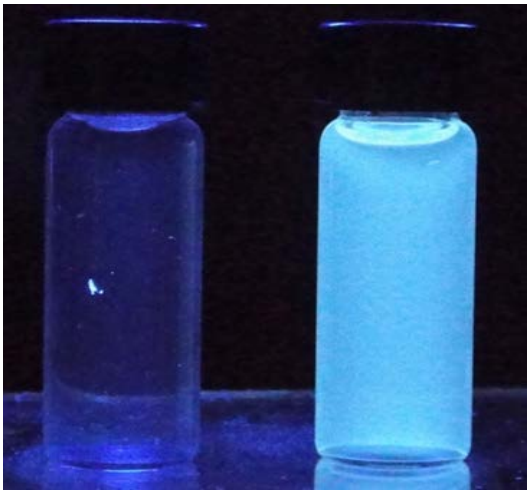

Figure 1 Flourescent property of chloroform/methanol without (A) and with extract (B). Dried extract was suspened in chloroform/methanol (1:1), and visualized by long-wavelength UV intensity (350 nm). "A" indicates the control system containing only solvent without extract did not show any folurescent property.

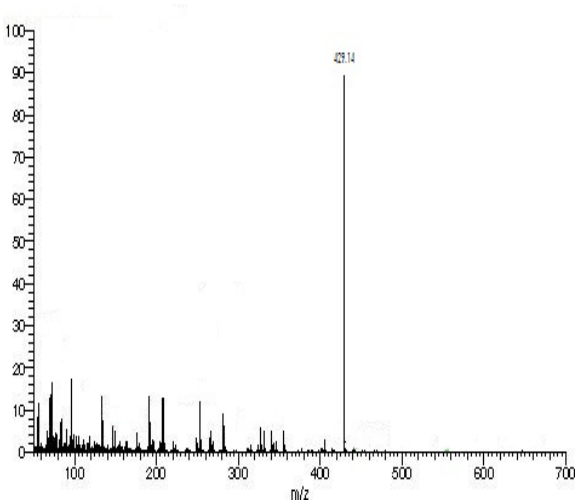

Figure 2 GCMS spectum of the purified chloroxanthomycine showing an single peak at 429.14 with 90% average intensity.

Description

Based on the spectral assignment, the cyanoxanthomycin (3-(3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2,6-dioxo-2,6-dihydro-1H-benzo[cd]pyren-4-yl) propanenitrile) composed of a benzopyrene with five fused rings in a planar delocalized structure. There are four hydroxyl groups, two carbonyls, three methyl group and propanenitrile containing cyano group (Figure 3).

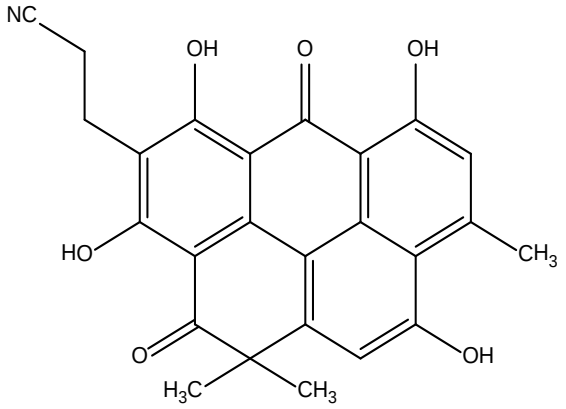

Figure 3 Structure of Cyanoxanthomycin.

3.2. Antimicrobial activities

The extract, cyanoxanthomycin was shown potent antibacterial properties against selected bacterial (Gram +ve/-ve) strains (Table 1). At the concentrations of 50 µM, it was observed between 11.6 mm zone to 19.2 mm zone of inhibition for a given bacterial cultures, compared to the standard antibiotic which shows between 7.5 mm zone to 11.2 mm zone of inhibition. For *B. subtilis*, it was inhibited by showing 11.6±0.1 mm zone of inhibition around the well which is the lowest inhibition whereas at same concentration of pure extract (cyanoxanthomycin) shows maximum inhibition 19.2 for *P. aeruginoasa.* The detailed inhibitory activity of cyanoxanthomycin on different gram-positive and negative bacteria was presented in Table 1**.**

The reported pigment has a conjugated ring system with strong intramolecular hydrogen bonds between the hydroxyl and carbonyl groups.

The polycyclic aromatic ring system belongs to the benzo [cd] pyrene structural class and is responsible for the strong fluorescence activity. The cyanoxanthomycin is likely to that of resistomycin, resistoflavin and itamycin pigments which exhibit similar structure (Kozhevina 1982). However, Resistomycin is a fluorescent pentacyclic, highly conjugated pigment and having potent antibacterial, antiviral and anti-cancer properties (Eckardt et al. 1972; Kozhevina 1982). There was no evidence of pentacyclic, cyano group fluorescent pigments from thermophiles and hyperthermophiles. Like Resistomycin, cyanoxanthomycin of *Geobacillus*, it produces yellow-orange fluorescent intensity and has selective antibacterial properties.

Table 1. Antibacterial activity of Cyanoxanthomycin.

	Organisms				
Concentration (50 µM)	*B. subtilis* (MTCC 3053) in mm	*E. coli* (MTCC 1698)	*P. aeruginoasa* (MTCC 6458)	*S. aureus* (MTCC 6908)	*Streptococcus* sp. (MTCC 9724)
Cyanoxanthomycin	11.6±0.1	16.9±0.1	19.2±0.1	18.4±0.1	17.9±0.1
Ciprofloxacin	08.2±0.1	09.1±0.1	09.6±0.1	11.2±0.1	7.5±0.1

*The values of each constituent consisted of Mean ± SE of three replicates. The concentration of 50 µM (1 µM = 429 µg/ml) was prepared by dissolving the appropriate weight of cyanoxanthomycin in sterile double distilled water (sterilized).

4. Conclusion

This is the first report of a fluorescent, pentacyclic pyrene having cyano group, a natural compound produced by a member thermophiles. We have employed a simple and easy procedure for the extraction of the fluorescent pigment from *Geobacillus* sp. Iso5. It also has characteristic antibacterial property on different bacteria. Due to lack of description on pentacyclic-derived pigments from hyperthermophiles, we believe that it has wider industrial and therapeutic applications.

Acknowledgement

The authors would like to thank all the staff and members of the Department of Biochemistry, Kuvempu University.

References

Bull AT, Ward AC and Goodfellow M. 2000. Search and discovery strategies for biotechnology: the paradigm shift. *Microbiol Mol Biol Rev*, 64: 573-606.

Dufossé L, Galaup P, Yaron A, Arad SM, Blanc P, Chidambara Murthy KN and Ravishankar GA. 2005. Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends Food Sci Tech*, 16: 389-406.

Eckardt K, Bradler G, Tresselt D and Fritzsche H. 1972. Resistoflavin, a new antibiotic of the resistomycin family. *Adv Antimicrobial Antineoplastic Chemo*, 1:1025–1027.

Elliott RP. 1958. Some Properties of Pyoverdine, the Water-soluble Fluorescent Pigment of the *Pseudomonads*. *Appl Microbiol*, 6: 241-246.

Flugge C. 1886. Die Mikroorganismen : mit besonderer Berücksichtigung der Ätiologie der Infektionskrankheiten. In: Flügge Carl FW (ed). Zweite völlig umgearbeitete Auflage der "Fermente und Mikroparasiten" edn, Leipzig, Germany

Fujiwara S. 2002. Extremophiles: developments of their special functions and potential resources. *J Biosci Bioeng*, 94: 518-25.

Gell C, Brockwell D and Smith A. 2006. Handbook of single molecule fluorescence spectroscopy. Oxford University Press, Oxford, USA

Goldys EM. 2009. Fluorescence Applications in Biotechnology and Life Sciences. John Wiley and Sons Ltd, Hoboken, United States

Huge R, Guarraia L and Hatt H. 1964. The proposed neotype strains of *Pseudomonas fluorescens* (trevisan) migula 1895. *Int J Syst Evol Microbiol*, 14: 145-155.

Jordan EO. 1899. The production of fluorescent pigment by bacteria. *Bot Gaz*, 27: 19-36.

Kolev D, Antonova TN, Nikoevska TA and Petrova KP. 1991. Syhthesis of pigments by dissociative forms of Bacillus substiles 76. *Acta Microbiol Bulg*, 28: 27-33.

Kozhevina LS, Vinogradova, K.A and Struve ME. 1982. Morphological and cytochemical differentiation of colonies of actinomycetes geliomycin producers in the developmental process on solid media. *Biologicheski Nauki*, 8: 88-91.

Magyarosy A, Ho JZ, Rapoport H, Dawson S, Hancock J and Keasling JD. 2002. Chlorxanthomycin, a fluorescent, chlorinated, pentacyclic pyrene from a *Bacillus* sp. *Appl Environ Microbiol*, 68: 4095-4101.

Mahadevan GD and Neelagund ES. 2012. Molecular characterization of industrially viable hyperthermostable α-amylase from thermoalklophilic *Geobacillus* sp. Iso5 isolated from geothermal spring. *J Pure Appl Microbiol*, 6: 1759-1773.

McMullan G, Christie JM, Rahman TJ, Banat IM, Ternan NG and Marchant R. 2004. Habitat, applications and genomics of the aerobic, thermophilic genus *Geobacillus*. *Biochem Soc Trans*, 32: 214-217.

Meyer JM. 2000. Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. *Arch Microbiol*, 174: 135-142.

Meyer JM and Abdallah MA. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J Gen Microbiol*, 107: 319-328.

Morozkina EV, Slutskaia ES, Fedorova TV, Tugai TI, Golubeva LI and Koroleva OV. 2010. Extremophilic microorganisms: biochemical adaptation and biotechnological application (review). *Prikl Biokhim Mikrobiol*, 46: 5-20.

Mulla SI, Hu A, Wang Y, Sun Q, Huang S-L, Wang H and Yu CP. 2015. Degradation of triclocarban by a triclosan degrading *Sphingomonas* strain YL-JM2C. *Chemosphere*, 144: 292-296.

Mulla SI, Sun Q, Hu A, Wang Y, Ashfaq M, Eqani SAMAS and Yu CP. 2016. Evaluation of sulfadiazine degradation in three newly isolated pure bacterial cultures. *PLoS ONE*, 11: e0165013.

Nair NM, Kanthasamy R, Mahesh R, Iruthaya Kalai Selvam S, Ramalakshmi S. 2019. Identification of antibacterial compound from *Bacillus horikoshii* isolated from rhizosphere region of alfalfa plant. *J Appl Sci*, 19: 140-147.

Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV (2001) Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. *nov*., sp. *nov*. and *Geobacillus uzenensis* sp. *nov*. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, G. th. *Int J Syst Evol Microbiol* 51(Pt 2):433-46.

Sander KMJ, Hunter, K.B (1993) Modern NMR spectroscopy: A guide for chemists., 2 edn. Oxford University press.

Seleen WA, Stark CN (1943) Some characteristics of green-fluorescent pigment-producing bacteria. *J Bacteriol* 46(6):491-500.

Silva GA, Almeida EA (2006) Production of yellow-green fluorescent pigment by Pseudomonas fluorescens. *Brazilian Arc Biol Technol* 49(3):411-419.

Singh OV and Gabani P. 2011. Extremophiles: radiation resistance microbial reserves and therapeutic implications. *J Appl Microbiol*, 110: 851-861.

Sneath PHA (1986) Endospore-forming Gram-positive rods and cocci. In: P. H. A. Sneath NSM, M. E. Sharpe, and J. G. Holt (ed) Bergey's manual of systematic bacteriology. vol 2. The Williams & Wilkins Co, Baltimore, MD, USA,, pp 1104–1139.

Stetter KO. 1999. Extremophiles and their adaptation to hot environments. *FEBS Lett*, 452: 22-25.

Turfitt GE. 1937. Bacteriological and biochemical relationships in the *Pyocyaneus-fluorescens* group: Investigations on the green fluorescent pigment. *Biochem J*, 31: 212-218.

Turfreijer A, Wibaut JP and Kingma Boltjes TY. 1938. The green fluorescent pigment of *Pseudomonas fluorescens*. *Recueil des Travaux Chimiques des Pays-Bas*, 57: 1397-1404.