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Full Length Research Paper

Bacteriocinogenicity and production of pyocins from *Pseudomonas* species isolated in Lagos, Nigeria

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A total of 52 strains of *Pseudomonas* identified as *P. aeruginosa* (27), *P. putida* (9), *P. fluorescens* (13) and *P. stutzeri* (3) were isolated from Nigerian patients with burns, wound and skin infections, UTI, diarrhoea and eye infections. 81.5, 55.6, 76.9 and 33.3% of these species produced pyocins at a range of 410 – 670 □g/ml. High yields of pyocins at 35 – 37°C, which declined sharply at temperatures above 37°C were obtained from all the producing strains. The strains also displayed significant pyocin expression (P < 0.05) under UV irradiation. Pyocins from a total of 25 producing strains; *P. aeruginosa* (14/27), *P. putida* (4/9), *P. fluorescens* (7/13) were inhibitory to the growth of *P. putida* indicator strain with four strains: PA02, PA20, PP03 and PF01 producing inhibition zone diameter >3 mm. These pyocins also displayed growth inhibitory activity against some Nigerian isolates of Gram-positive and Gram-negative bacteria when undiluted and at 1:2 - 1:16 dilutions. The susceptible organisms include *Bacillus cereus, Listeria monocytogenes, Klebsiella* spp., *Staphylococcus aureus, S. epidermidis, Proteus* spp. and *Vibrio parahaemolyticus*. The results of this study have provided evidence for broad-spectrum antibacterial activity of pyocins elicited by *Pseudomonas* species from Nigeria.

Key words: Pyocins, antibacterial activity, *Pseudomonas* species, Nigeria.

INTRODUCTION

Human *Pseudomonas* species, with preference for moist body sites have been recognized to cause infections of high morbidity and mortality by virtue of several virulence factors that they express (Pollack, 1994). These factors include endotoxins, which mediate the pathogenesis of bacteremia and sepsis, exotoxin A, which arrests protein synthesis in the host cell (Klinger et al., 1978) and pyocins, which are pigments of many uses (Vanloon et al., 1998; Padilla et al., 1992). Parret and De Mot (2001) demonstrated the pyocins of non-fluorescent Pseudomonads as plant growth promoting substances and as agents of importance with respect to the population dynamics of isolates colonizing the rhizospheres of crops. Furthermore, pyocin-based coliform detecting systems have been developed as an assessor of microbial quality of water samples and foods (Betts et al., 1993), while the antibacterial property of these pigments is highly varied and seems to know no bounds (Hassan and Fridovitch, 1980; Duncan et al., 1997). It has been reported that 25 out of the over 140 species of *Pseudomonas* infect man and the pre-eminence of these species as etiological agents of bacteremia and sepsis as well as other opportunistic infections is grossly heterogeneous (Woods and Iglewski, 1983). In Nigeria, little work has been done on *Pseudomonas spp*. To the best of our knowledge, there is virtually no report on species production of pyocins as documented elsewhere and the potential of these pigments as bacteriocides has not been studied.

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Scientific opinions on a global scale are yet to succumb to the need of refining pyocins for commercial antibacterial use. However, to strengthen the consideration of an industrial pyocin for use as antibacterial agents, it is essential that reports concerning antibacterial properties of pyocins and factors influencing pigment yields by isolates from Africa, other developing and underdeveloped countries are furnished and updated. In the present study, clinical isolates of *Pseudomonas* species were recruited and the antibacterial activity of pyocins from producing strains against Gram-positive and Gram-negative bacteria was studied. We also investigated the roles of ultraviolet radiation and temperature in pyocin production.

MATERIALS AND METHODS

Pseudomonas species

A total of fifty-two *Pseudomonas* isolates were obtained from thirty-three (33) in-patients and nineteen (19) outpatients at the General and Teaching hospitals in Lagos, Nigeria. The patients were diagnosed and being treated for urinary tract infections, wound infections, septicaemia, ear infection and eye infections. The isolates were identified based on growth on Pseudomonas agar supplemented with cetrimide and sodium nalidixate (Oxoid, England) and further biochemical differentiation according to Weyant et al. (1995). The speciated isolates were studied for antibacterial activity of the pyocin that they expressed. The effects of temperature and ultraviolet radiation on pyocin production in these isolates were also investigated. The pure isolates were used immediately for pyocin analysis.

Pyocin yield and selection of producing strains

Five distinct colonies of each *Pseudomonas* strain on Pseudomonas agar were subcultured in 5 ml of tryptic soy broth. The inoculated broths were then incubated aerobically at 37°C for 24 h. Pyocin producing strains were selected as those that yielded yellow, yellowish brown, green or greenish blue pigmentation after incubation. The method of Rogers (1973) was used for pyocin quantitation. Briefly, pyocin producing strains were grown aerobically overnight at 37°C with shaking (120 rpm) in Tris-minimal succinate solution without iron and glucose but containing $MgCl_2$ (500 µM), $CaCl_2$ (100 µM) and methionine (700 µM). The resulting cultures were then centrifuged at 4000 rpm for 10 min and supernatant acidified with ethyl acetate in 5:2 volume ratio. The acidified pyocin fraction was then concentrated under reduced pressure using a rotary evaporator at 50°C. The crude pyocin preparation obtained was then dissolved in 400 µl sterile water, sterilized by passage through a 0.45 µM filtration unit and the yield measured in µg/ml. A non-pyocin strain of *Pseudomonas putida* PUC 34 was used as control.

Effect of temperature on pyocin production

The pyocins producing *Pseudomonas* strains studied were selected and further incubated aerobically in tryptic soy broth at 20, 25, 30, 37, 40 and 42°C. The yields of pyocins produced were determined as described earlier. A sterile broth solution was used as a negative control. The turbidity of the broth culture was first adjusted to 0.5 McFarland standard (10^8 cfu/ml) and finally to an inoculum size of 5 x 10^5 cfu/ml using sterile broth.

Effect of ultra violet radiation

Four young cultures of each of the pyocin producing species were grown for 16 h at 35°C under ultra violet light. As a measure of comparison of pyocin yield, cultures were also grown in tryptic soy broth for 16 h but in the absence of ultraviolet irradiation. Cultures were irradiated at a distance of 30 cm from the UV transilluminator (Memmer, Germany). The amounts of pyocin expressed were determined and compared.

Preliminary antibacterial testing of pyocins

The initial antibacterial testing of pyocin was carried out using the double agar diffusion method as described by Parret and De Mot (2000). An inoculum of size 10^8 cfu/ml of each of the pyocin producing strains was used to inoculate nutrient agar followed by incubation at 35°C for 8 h. The resultant colony was then killed by flooding with 10 ml of chloroform for 30 min. Thereafter, chloroform was removed by plate inversion and evaporation in open air. One ml of *P. putida* indicator strain 15070 culture (10^8 cfu/ml) in tryptic soy broth was mixed with 12 ml of molten soft agar (0.8%) and used to overlay the plate. The plate was further incubated aerobically at 35°C for 24 h. The bacteriocinogenicity of the pyocin tested was defined as the appearance of clear zone of no bacterial growth around the colonies. A non-pyocin producing strain of *P. putida* was used a negative control.

Measurement of bacteriocinogenicity of Pseudomonas pyocins

On species basis, each pyocin producing strain was subcultured into tryptic soy broth in quadruple and pyocin was extracted after 24 h of incubation. The extracted pyocins were pooled together and each pool was diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 with tryptic soy broth. The antibacterial activity of these dilutions was subsequently tested on the following isolates: *Bacillus cereus, B. licheniformis, B. subtilis, Listeria monocytogenes, Clostridium difficile, Staphylococcus aureus, S. epidermidis, Esherichia coli, Klebsiella* spp., *Salmonella typhi, Proteus* spp., *Vibrio parahaemolyticus, Shigella flexneri* and *S. dysenteriae.* The organisms were local isolates recovered from diarrhoeal stools, urine, swabs and food samples at the Genetics and Microbiology laboratory of the National Institute for Medical Research, (N.I.M.R.) Nigeria. Minimum inhibitory dilution of pyocin produced by Pseudomonas against the local strains tested was defined as the maximum dilution at which no growth occurred.

Statistically analysis

The parameters obtained as pyocin yields were expressed as mean ± standard deviation from mean. The difference between values was analyzed using the Student's t - test and at 95% confidence limits. Probability value less than 0.05 was considered significant.

RESULTS

The origins of fifty-two *Pseudomonas* species studied for pyocin production and bacteriocinogenicity are summarized in Table 1. Four species of *Pseudomonas* were identified biochemically from patients with eye

Table 1. Sources of the *Pseudomonas strains* tested for pyocin production and antibacterial activity against microorganisms including an indicator strain of *Pseudomonas putida*.

Infection	Isolates (N)
Skin infection	*Pseudomonas aeruginosa* (8)
Skin infection	*Pseudomonas fluorescens* (4)
Skin infection	*Pseudomonas putida* (2)
Wound infection	*Pseudomonas aeruginosa* (7)
Wound infection	*Pseudomonas putida* (4)
Wound infection	*Pseudomonas fluorescens* (2)
Burns	*Pseudomonas aeruginosa* (3)
Burns	*Pseudomonas fluorescens* (7)
Burns	*Pseudomonas putida* (2)
Burns	*Pseudomonas stutzeri* (2)
Ear infection	*Pseudomonas aeruginosa* (2)
Ear infection	*Pseudomonas putida* (1)
Ear infection	*Pseudomonas stutzeri* (1)
Urinary tract infection (UTI)	*Pseudomonas aeruginosa* (4)
Urinary tract infection (UTI)	*Pseudomonas putida* (2)
Eye infection	*Pseudomonas aeruginosa* (2)
Septicaemia	*Pseudomonas aeruginosa* (2)

N = Number of isolates.

Table 2. Pyocin production among the *Pseudomonas* isolates.

***Pseudomonas* species (N)**	**Pyocin producers n (%)**	**Pyocinyield (□g/ml)**
Pseudomonas aeruginosa)	22 (81.5)	600
P. putida (9)	5 (55.6)	670
P. fluorescens (13)	10 (76.9)	640
P.stutzeri (3)	1 (33.3)	410

N = Total number of isolates; n = number of pyocin producing isolates.

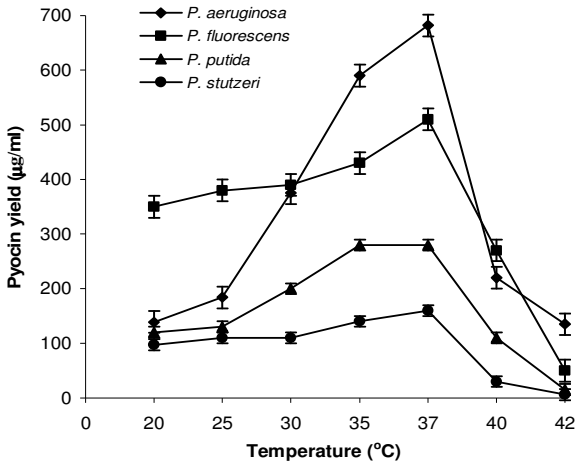

Figure 1. Effect of temperature on pyocin production. Projections on data points represent standard deviations of mean pyocin yield.

infection, diarrhoea, ear infection, burns, wound and skin infections. The isolates were *P. aeruginosa* (27 strains), *P. putida* (9 strains), *P. fluorescens* (13 strains) and *P. stutzeri* (3 strains) (Table 1). 22 of the 27 *P. aeruginosa* strains, 5 of 9 *P. putida*, 10 of 13 *P. fluorescens* and 1 of 3 *P. stutzeri* strains expressed pyocins at a yield range of 410 – 670 µg/ml. The 81.5 and 33.3% of *P. aeruginosa* and *P. stutzeri* corresponded to the highest and lowest producers of pyocin s among the strains tested (Table 2). Pyocin production increased with temperature up to 37°C in all the pyocin producers tested. However, at above 37°C, pyocin production declined sharply in all the strains. Only *P. aeruginosa* produced little pyocin at 42°C (Figure 1). The higher pyocin production in *P. aeruginosa* (1400 vs. 1025 µg/ml), *P. putida* (1225 vs. 862.5 µg/ml) and *P. fluorescens* (1375 vs. 1012.5 µg/ml) were expressed under UV irradiation. These increases were found to be significant ($P < 0.05$) when compared with

Table 3. A comparative study of the effect of ultraviolet radiation on pyocin production among the *Pseudomonas* isolates.

	Pyocin yield ± SD µg/ml)		
	P. aeruginosa	***P. putida***	***P. fluorescens***
+UV Light	1400 ± 182.6[a]	1225 ± 170. 6[a]	1375 ± 179.2[a]
-UV Light	1025 ± 64.5	862.5 ± 149.3	1012.5 ± 131.5

Data are expressed as mean ± standard deviation (SD); [a]P < 0.05 (+UV light vs. –UV Light) (Student's t- test).

Table 4. Bacteriocinogenicity of *Pseudomonas* isolates against indicator strains.

Inhibition zone diameter (IZD) in millimeters (mm) of the tested strains.		
+	++	+++
PA01	PA05	PF01
PP02	PP05	PA02
PP04	PF08	PP03
PF04	PF09	PA20
PF05	PA11	
PA07	PA17	
PA08		
PA10		
PF11		
PA12		
PF12		
PA15		
PA18		
PA19		
PA22		

Symbols: +, Inhibition zone diameter (IZD) ≤ 1mm; ++, IZD 1 - 3mm; +++, IZD > 3mm. Strains: PA, *Pseudomonas aeruginosa*; PP, *Pseudomonas putida*; PF, *Pseudomonas fluorescens.*

pyocin expression in the absence of UV light in these strains (Table 3). The data presented in Table 4 shows that, 14 of the 22 pyocin strains of *P. aeruginosa* and 7 of 10 *P. fluorescens* were inhibitory to the growth of the indicator strain (*P. putida* 15070), producing inhibition zone diameter (IZD) mostly in the range of 1 – 3 mm. Four of the *P. putida* pyocin strains gave similar result. IZD > 3 mm was produced by pyocins of four strains of *Pseudomonas* tested; PA02, PA20, PP03 and PF01. The pyocins also elicited antibacterial activity against some Nigerian isolates of Gram-positive and Gram-negative bacteria at different dilutions. For instance, the growth of *B. cereus*, *Clostridium difficile* and *Klebsiella spp* was inhibited by undiluted pyocins from *P. aeruginosa*, *P. putida* and *P. fluorescens*, while *B. subtilis*, *Staphylococcus aureus*, *E. coli* and *Salmonella typhi* were inhibited at 1:2 – 1:16 dilutions (Table 5).

DISCUSSION

Pseudomonas species have on many occasions been reported as major etiological agents of nosocomial infections and as pathogens predominantly isolated in burns and wounds in Nigeria (Atoyeba et al., 1982; Egri-Okwaji et al., 1988). The prevalence of pyocin producing *P. aeruginosa* as obtained in this study may not be unconnected with the virulence of potential of pyocin in causing diseases (Wood and Iglewski, 1983). Some of the *Pseudomonas* strains were observed not to produce these pigments and this indicates the co-existence of pyocin and non-pyocin strains in bacterial population of Pseudomonads. Our observation supports the work of Parret and De Mot (2001) where important functional roles were ascribed to the non-pyocin producing strains of *P. putida* in the ecology of microbes colonizing the rhizospheres of crops. We also observed that these pyocins were differently expressed among the producing strains and this implies variation in pyocin expression among the strains. Differences in promoter-mediated transcription of pyocin gene or whether expression is inducible or constitutive cannot be ruled out. This is because for instance, expression of three different types of pyocins: S, F, and R have been demonstrated in *P. aeruginosa* and in a separate study these pyocins were shown to bear genetic relatedness to different phages (Keisuke et al., 2001). Matsui et al. (1993) had earlier noted that the regulation of bacteriocins in bacteria is under SOS control. The functional domains of most pyocins could also be the basis for their varied expressions as these functions may determine the mechanism of virulence amongst *Pseudomonas (*Sano et al., 1993).

The expression of pyocins by the producing strains was also observed to increase with temperature up to 37°C and declined sharply at temperatures above 37°C. Maximum colonial growth of all the organisms was observed at 35-37°C (results not shown). This may mean that pyocin production is linked to the growth of the organisms. However, only *P. aeruginosa* strains elaborated little pyocins at 42°C and this may be because the organism readily demonstrates growth at this temperature (Pollack, 1994). The role played by ultraviolet light in the expression of bacteriocins by microorganisms has also been well documented. Braun

Table 5. The determination of minimum inhibitory dilution of pyocins of *Pseudomonas* species against test organisms.

Test organisms	Minimum inhibitory dilution (MID) of pyocins		
	P. aeruginosa	***P. putida***	***P. fluorescens***
Bacillus cereus	1:2	Undiluted	Undiluted
B. subtilis	1:2	1:2	1:2
Clostridium difficile	Undiluted	ND	Undiluted
Listeria monocytogenes	Undiluted	ND	1:2
Staphylococcus aureus	1:4	1:2	Undiluted
S. epidermidis	1:8	1:2	Undiluted
Escherichia coli	1:8	1:8	1:4
Salmonella typhi.	1:16	Undiluted	1:8
Klebsiella spp.	*1:4*	*1:2*	Undiluted
Proteus spp.	Undiluted	ND	1:2
Vibrio parahaemolyticus	1:4	1:2	1:8
Shigella flexneri	Undiluted	Undiluted	ND
Shigella dysenteriae	ND	ND	ND

ND = Not determined.

et al. (1994) demonstrated the induction of bacteriocins production in *E. coli* by UV irradiation, while in the works of Sano et al. (1993) and Duport et al. (1995), s-type pyocin production in *P. aeruginosa* strains under UV stimulation was clearly shown. In this study, a significant increase ($P < 0.05$) in pyocin expression was observed in all the production strains when grown in the presence of ultraviolet light. This result connotes the inducibility of pyocin genes in Pseudomonads and further attests to previous findings (Sano et al., 1993; Duport et al., 1995). Although, molecular weight characterization of pyocins was not carried out in this study, it will be of interest with regards to pyocin induction to note that a bacteriocins-like protein with a molecular weight of approximately 14 kDa has been isolated from *Pseudomonas* spp. BMW11M1 strain under UV stress (Parret and De Mot, 2001). Pyocins have also been found to be active against closely related species or strains (Ryder and McClure, 1997). That some of the pyocins isolated in this study inhibited *P. putida* 15070 indicator strain is supportive of the work of Parret and De Mot (2001) where crop colonized pyocin-producing *Pseudomonas* strains inhibited *P. fluorescence* indicator strains and *P. putida* 15070. However, the inability of some of the isolated pyocins to inhibit *P. putida* 15070 further reflects the variations inherent in the functions of pyocins. Pyocins have been found to function as tRNase, pore formers and DNase (Parret and De Mot, 2000; Sano, 1993). This study also reveals the broad-spectrum antibacterial activity of the pyocins of *Pseudomonas* species from Nigeria. Soil bacteria such as *Bacillus subtilis* and *B. cereus* were found to be susceptible to pyocins (Parret and De Mot, 2001; Petrocheilou-Malleiru et al., 1987). In addition some organisms of epidemiological and clinical importance in Nigeria were also inhibited. These organisms include such as *Shigella flexneri* (Iwalokun et al., 2001), *Staphylococcus aureus* (Olukoya et al., 1995) and *Salmonella typhi* (Akinyemi et al., 2000).

It can be concluded that *Pseudomonas* isolates from Nigeria produce pyocins with broad-spectrum antibacterial activity and whose production is accelerated under UV stress. Further work on molecular characterization of pyocins will be carried out and new microorganisms will be tested for bacteriocinogenicity in future studies.

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