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Multiple antibiotics resistant among environmental isolates of *Stenotrophomonas maltophilia*

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ABSTRACT: In this study we assessed the functionality of integrons, melanin-like pigment and biofilm formation on multidrug resistance among environmental isolates of *Stenotrophomonas maltophilia*. Marked resistances were noted against aztreonam (60%), cefepime (68%), ceftazidime (77%), ciprofloxacin (72%), gentamicin (65%), meropenem (75%), piperacillin/tazobactam (65%) in *S. maltophilia*. Ticarcillin/clavulanic acid (66%) and trimethoprim/sulfamethoxazole (75%) were the active antibiotics against *S. maltophilia.* Class 1 intergron was significantly detected in 56.3% (54/96) of *S. maltophilia* strains. Integron-positive strains were significantly resistant to cefepime (69%), ceftazidime (78%), ciprofloxacin (74%), gentamicin (65%), and meropenem (72%). Gene cassettes arrays within integrons were identified as aminoglycoside resistance genes *aacA4*, *aadA2*, *aadB*, *aacC4*, and *aacA6'-Ib*; β-lactams resistance genes bla_{IMP}, bla_{OXA}, and bla_{CARB}; chloramphenicol resistance genes *cmlA* and *catB2*; quaternary ammonium compound (QAC) resistance genes *smr* and *qac*; and multi-gene cassettes: *smr/aacA4* and bla_{IMP}*/aac6-II/aadA5*. High-pigment-producing *S. maltophilia* strains revealed significant correlation with resistance to cefepime, ceftazidime, ciprofloxacin, levofloxacin and piperacillin/ tazobactam. Biofilm formation was not significant with resistance to ciprofloxacin, levofloxacin, meropenem, ticarcillin/clavulanic and trimethoprim /sulfamethoxazole. Our findings characterize the significant roles of integrons, melanin-like pigment and biofilm formation in the multidrug resistance of *S. maltophilia.* The range of antibiotics resistance genes and mobile genetic elements found suggests that the organism could potentially act as a reservoir of drug resistance determinants in environmental and clinical settings, which is an issue of public health concern.© JASEM

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Introduction: *Stenotrophomonas maltophilia* (formerly known as *Pseudomonas maltophilia*, *Xanthomonas maltophilia*) is a ubiquitous species from the gamma sub-division of Proteobacteria (Hayward *et al.,* 2010). *S. maltophilia* is a Gram-negative opportunistic pathogen, and increasing incidence of nosocomial and community-acquired *S. maltophilia* infections is of particular concern for immunocompromised individuals, as this bacterial pathogen is associated with a significant fatality rate (Jumaa *et al.,* 2006; Brooke, 2012). *S. maltophilia* is an environmental bacterium found in aqueous habitats, plant rhizospheres, animals, foods, contaminated medical care fluids, and water sources (Ryan *et al.,* 2009). Infections of *S. maltophilia* can occur in a range of organs and tissues; the organism is commonly found in respiratory tract infections (Brooke, 2012).

S. maltophilia has emerged as one of the most frequently found bacteria in cystic fibrosis (CF) patients (Waters *et al*., 2007). *S. maltophilia* has been associated with infections of the eyes (Penland and Wilhelmus, 1996), urinary and respiratory tracts infections (Vartivarian *et al.,* 1996). *S. maltophilia* possess endogenous β-lactamase production and low outer membrane permeabilit resulting to its resistant to many broad-spectrum of antibiotics including penicillins, carbapenems, and aminoglycosides (Gilligan and Whittier, 1999). The molecular mechanisms underlying pathogenicity of *S. maltophilia* are mainly unknown. Although its high capacity to adhere to various surfaces with biofilm formation or its intrinsic resistance to majority of commonly used antibiotics are factors that positively contribute to the infection process (Looney *et al.,* 2009).

S. maltophilia exhibits variety of mechanisms that singly or collectively contribute to its multidrug resistance (MDR) status. Intrinsic resistance includes inducible efflux pumps (Li and Nikaido, 2004; Liaw *et al.,* 2010) and multiple β-lactamase expression (Avison *et al.,* 2002) but not mutations in the quinolone resistance-determining region (Valdezate *et al.,* 2005). In addition, *S. maltophilia* can acquire resistance through integrons, transposons, and plasmids (Barbolla *et al.,* 2004; Liaw *et al.,* 2010). Class 1 integrons have been characterized from *S. maltophilia* strains isolated in Argentina and Taiwan, which indicates that they contribute to trimethoprim /sulfamethoxazole (TMP/SMX) resistance through the *sul1* gene carried as part of the 3′ end of the class 1 integron (Barbolla *et al.,* 2004). Integrons are located on transposons or plasmids that facilitate the rapid spread of integrons to other strains and bacterial species (Jones *et al.,* 1997). In this

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study we examine the functionality of integrons, melanin-like pigment and biofilm formation on multidrug resistance (MDR) among environmental isolates of *S. maltophilia*.

MATERIALS AND METHODS

Collection of samples: Samples were collected from different environmental settings in Benin City, Nigeria which includes:- abattoir (runoff water and effluent); hospital environment (wastewater outlets, pollutant dumpsite and reservoir water) and animal farms (feeds, water and effluent discharge).

Isolation of S. maltophilia isolates:
Stenotrophomonas maltophilia strains were isolated from environmental samples; samples were process and incubated overnight using Nutrient broth (Merck, South Africa). Aliquots of the broth cultures were spread on Mueller-Hinton agar (Merck, South Africa) and imipenem disks (Mast Diagnostics, Merseyside, United Kingdom) were applied on the bacterial lawn. Tiny colonies observed in the inhibition zone after 18 h of growth at 30^{o}C were carefully picked and purified (Bollet *et al.*, 1995). All pure isolates were identified by phenotypic characteristics and standard biochemical reaction using API 20 NE test kit (bioMerieux, France). Isolate inoculation into API 20 NE strips was performed as recommended by the manufacturer (bioMerieux, France). After incubation for 48 h at 30^{o}C, results were read and analysed using the Analytical Profile Index (API) database (V4.1) with the apiweb^{TM} identification software. Only confidence levels expressed as 'excellent identification' or 'very good identification' was considered in this study. All candidate isolates were confirmed by polymerase chain reaction with *S. maltophilia*-specific primers SM1f/SM4 as describe by Adamek *et al.* (2011).

Antimicrobial susceptibility testing: Antimicrobial susceptibilities were determined by the agar dilution method as described by the Clinical and Laboratory Standards Institute (CLSI, 2005, 2008). The following antibiotics were used included: aztreonam (ATM); cefepime (FEP); ceftazidime (CAZ); ciprofloxacin (CIP); gentamicin (GEN); levofloxacin (LEV); meropenem (MER); piperacillin/tazobactam (TZP); ticarcillin/clavulanic acid (TIM) and trimethoprim/sulfamethoxazole (SXT). Control strains for susceptibility testing included *Pseudomonas aeruginosa* ATCC 19582, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739.

Genomic DNA preparation: S. maltophilia isolates from an overnight culture were resuspended in 200µl of sterile deionized water, boiled for 15 min and centrifuged for 5 min at 12 000 ×*g*. The supernatant was stored at -20^{o}C for further use as genomic DNA template for PCR.
Detection of Integron by polymerase chain reaction (PCR): Class 1 integrons were detected with class I integrase specific primers (5'-ACATGTG ATGGCGA CGCACGA-3' and 5'-ATTTCTGTCC CTGGC TGGCGA-3'). Gene cassettes within integrons were amplified with primers specific for the integron 5'CS (5'-GGCATC CAAGCA GCAAG-3') and 3'CS (5'-AAGCAGACTTGACCTGA-3') (Ploy *et al.*, 2000). The amplicons were sequenced and sequence comparisons were made using the BLAST program.
Pigment bioassay

A melanin-like pigment bioassay was carried out as described by Wang *et al.* (2000). *S. maltophilia* were cultured in L-tyrosine-containing agar to facilitate the observation of brown pigment production. Pigment formation were recorded as 0, 1+, 2+ or 3+, with increased intensity and intensity equal to or greater than 2+ designated as high pigment expression.

Biofilm bioassay: The biofilm bioassay was carried out as described by Di Bonaventura *et al.* (2004). *S. maltophilia* were cultured overnight in Luria-Bertani medium, cultures were aseptically transferred to the wells of polystyrene microtitre plates. The plates were incubated at 37^{o}C for 10 h and subsequently stained with crystal violet.

RESULTS

The results of susceptibility testing (Table 1) showed that most of the *S. maltophilia* strains were resistant to aztreonam (60%), cefepime (68%), ceftazidime (77%), ciprofloxacin (72%), gentamicin (65%), levofloxacin (58%), meropenem (75%), piperacillin/tazobactam (65%). Ticarcillin/ clavulanic acid (66%) and trimethoprim/sulfamethoxazole (75%) were the active antibiotics against *S. maltophilia* (Table 1).

Fifty-five environmental isolates of multiple antibiotic resistance (MAR) [(multiple antibiotic resistance, defined as resistant to four or more of six categories of antibiotics tested, including cephalosporins, β-lactams, β-lactamase inhibitors, carbapenems, monobactams, aminoglycosides, quinolones and trimethoprim/sulfamethoxazole (SXT)] and 41 non-MAR (i.e. resistant to three or less of the six categories of antibiotics tested). Among the antibiotics tested, MAR and non-MAR isolates reveal slight differences in resistance to aztreonam (91% vs. 76%), ciprofloxacin (100% vs. 75%), gentamicin (93% vs. 73%), meropenem (89% vs. 73%), and piperacillin/tazobactam (84 vs. 80%) respectively (Table 2).

Fifty-four strains 56.3% (54/96) were significantly detected with class 1 integrase (Table 1). Integron-positive strains were significantly resistant to cefepime (69%), ceftazidime (78%), ciprofloxacin (74%), gentamicin (65%), and meropenem (72%) (Table 1). Also the MAR strains 71% (39/55) possessed class 1 integron than the non-MAR strains 15/41 (37%) (Table 3).

The identified gene cassettes within class 1 integrons included aminoglycoside resistance genes *aacA4*, *aadA2*, *aadB*, *aacC4*, and *aacA6'-Ib*; β-lactams resistance genes bla_{IMP}, bla_{OXA}, and bla_{CARB}; chloramphenicol resistance genes *cmlA* and *catB2*; quaternary ammonium compound (QAC) resistance genes *smr* and *qac*; and multi-gene cassettes: *smr/aacA4* and bla_{IMP}*/aac6-II/aadA5*.

A good number of MAR isolates produced a higher level of pigment than non-MAR isolates (82% vs. 15%) (Table 3). High-pigment-producing *S. maltophilia* strains revealed significant correlation with resistance to cefepime, ceftazidime, ciprofloxacin, levofloxacin and piperacillin/tazobactam (Table 4). Aztreonam, gentamicin and meropenem were not significant with pigment producing strains (Table 4). MAR isolates had an average OD_{540nm} value of 0.85 ± 0.21 compared with a value of 0.25 ± 0.01 for non-MAR isolates ($P < 0.002$) (Table 3). Biofilm formation was not significant with resistance to ciprofloxacin, levofloxacin, meropenem, ticarcillin/clavulanic and trimethoprim /sulfamethoxazole (Table 4).

DISCUSSION

The rapid spread of antibiotic resistance genes among bacterial isolates is an increasing problem in infectious disease control. In the study, we examined the roles of integrons, melanin and biofilm formation in relation to multidrug resistance in *S. maltophilia*. A significant correlation was established between the mechanisms studied and multidrug resistance. Studies have revealed that conserved DNA sequence, integron, may be carried on these episomal genetic structures (Stokes and Hall, 1989; Rowe-Magnus and Mazel, 1999). Integrons possess two conserved segments separated by a variable region that includes integrated cassettes, which often include antibiotic resistance genes (Recchia and Hall, 1995). Many resistance genes are present as gene cassettes within integrons, which may themselves be located on transmissible plasmids and transposons (Recchia and Hall, 1995).

Antimicrobial Surveillance Program isolates indicated that the newer fluoroquinolones demonstrated good efficacy; the most active were levofloxacin (6.5% resistance) and gatifloxacin (14.1%) (Sader and Jones, 2005). The resistance to levofloxacin was higher in our study (58% resistance) compare to previous the study of Sader and Jones (2005). A recent study encompassing data from Europe, Latin America and North America indicates that the level of resistance to TMP/SMX is 3.8%; however, previous studies indicate that the level is higher in Latin America than North America (Gales *et al.*, 2001; Fedler *et al.*, 2006). The current drug of choice for treating *S. maltophilia* infections is trimethoprim-sulphamethoxazole, but resistance is seen in *S. maltophilia* isolates due to a mobile determinant (Toleman, *et al.*, 2007; Nicodemo and Paez, 2007). Other drugs with reasonable activity against *S. maltophilia* are minocycline and newer fluoroquinolones (Nicodemo and Paez, 2007). However, mutants resistant to these last resort drugs are readily selected *in vitro*. One mutation may be sufficient to cause resistance to these drugs, and worryingly, this mutation can be selected for in the presence of a front-line antimicrobial such as amikacin (Gould and Avison, 2006). Although surveillance studies are few, resistance to TMP/SMX appears to be emerging, and recent *in vitro* modeling studies have shown that combination therapies of TMP/SMX plus ciprofloxacin and TMP/SMX plus tobramycin exhibit a greater killing capacity then TMP/SMX alone (Zelenitsky *et al.*, 2005; Al-Jasser, 2006).

The class 1integron (56.3%) positive isolates obtained in this study is slightly similar to that of Liaw et al. (60%) (2010) but higher than that reported by Chang et al. (22%) (2004). Such variation could be attributed to the different origins of the samples collected. The finding that the most common gene cassette carried by class 1 integron was comprised of aminoglycoside resistance determinants may account for the significant difference in susceptibility to gentamicin between the integron-positive and integron-negative isolates (strains). Integrons were significantly associated with resistance to certain antibiotics, including aminoglycosides (gentamicin), quinolones (levofloxacin and ciprofloxacin), and beta-lactam agents. This is not surprising, since many antibiotic resistance gene cassettes encoding resistance to a wide range of antibiotics have been reported previously (Sallen *et al.*, 1995; Rowe-Magnus and Mazel, 1999).

Integrons capture genes as part of a genetic element known as a gene cassette. Most cassettes within integrons with known functions confer antibiotic or quaternary ammonium compound (QAC) resistance. Most of the integron arrays contained more than one resistance gene cassette, which can mediate resistance to multiple antibiotics. Increasing proportions of isolates containing class 1 integrons were detected in *S. maltophilia* (Barbolla *et al.*, 2004; Toleman *et al.*, 2007). Among the 17 SXT-resistant isolates, 15 (88%) carried class 1and 2 integrons of them possessed

*sul1*genes. This report confirms the increased incidence of class 1 integron in SXT-resistant clinical isolates of *S. maltophilia* (Barbolla *et al.,* 2004; Toleman *et al.,* 2007). Toleman *et al.* (2006) indicates that resistance genes are linked to insertion sequence common region (ISCR) elements, which are DNA sequences found beyond and close to the 3' conserved sequences of class 1 integrons. These ISCR elements have been identified in numerous Gram-negative bacteria and a few Gram-positive bacteria, and are responsible for the mobility and dissemination of many antibiotic resistance genes, including extended spectrum β-lactamase, carbapenemase genes, aminoglycoside, chloramphenicol, quinolone as well as trimethoprim resistance genes (Toleman *et al.,* 2006). The use of quaternary ammonium compounds (QAC) in the natural environment has the potential to select for antibiotics resistance (Gaze *et al.,* 2005). The frequent occurrence of the *qac* gene cassette within class 1integron in the present study implies that multidrug resistance may result from the introduction of biocides into hospital settings.

Melanin-like pigment has been shown to protect cells from environmental attack in bacteria and fungi (Coyne and al-Harthi, 1992; Gomez and Nosanchuk, 2003). In this study, it was revealed that pigment production in *S. maltophilia* was associated with antibiotic resistance. Our findings show that cell with and without pigment formation indicates that pigment-bearing cells were more resistant to antibiotics. The MAR phenotype and biofilm formation of *S. maltophilia* observed gives a clearer insight why the bacterium is persistent, and difficult to eradicate.

In conclusion this study revealed that all resistance determinants contributed to the multidrug resistant phenotype of *S. maltophilia*. Along with the presence of pigment production, biofilm formation and class 1 integron may possibly play important roles leading to multidrug resistance of *S. maltophilia*. The varieties of antibiotics resistance genes and mobile genetic elements found suggests that the organism could potentially act as a reservoir of drug resistance determinants in a environmental and clinical settings, which is an issue of public health concern.

Table 1: The relationship between antibiotic susceptibility profile and integrons in *S. maltophilia*

Antibiotic	Antibiotic profile (*n* = 96)		Integron-positive isolates (*n* = 54)		Integron-negative isolates (*n* = 42)		*P*-value
	R *n* (%)	S *n* (%)	R *n* (%)	S *n* (%)	R *n* (%)	S *n* (%)	
ATM	58 (60)	38 (40)	32 (59)	22 (41)	26 (62)	16 (38)	0.002
FEP	65 (68)	31(32)	37 (69)	17 (31)	28 (67)	14 (33)	0.001
CAZ	74 (77)	22 (23)	42 (78)	12 (22)	32 (76)	10 (24)	0.003
CIP	69 (72)	27 (28)	40 (74)	14 (26)	29 (69)	13 (31)	0.005
GEN	62 (65)	34 (35)	35 (65)	19 (35)	27 (64)	15 (36)	< 0.001
LEV	56 (58)	40 (42)	31 (57)	23 (43)	25 (60)	17 (40)	< 0.001
MER	72 (75)	24 (25)	39 (72)	15 (28)	33 (79)	9 (21)	< 0.001
TZP	62 (65)	34 (35)	30 (56)	24 (44)	32 (76)	10 (24)	< 0.001
TIM	33 (34)	63 (66)	14 (26)	40 (74)	19 (45)	23 (55)	0.021
SXT	24 (25)	72(75)	12 (22)	42 (78)	12 (29)	30 (71)	<0.001

Value in parenthesis represents percentage profile

Legend: ATM-aztreonam; FEP-cefepime; CAZ-ceftazidime; CIP-ciprofloxacin; GEN-gentamicin; LEV-levofloxacin; MER-meropenem; TZP-piperacillin/tazobactam; TIM-ticarcillin/clavulanic acid and SXT-trimethoprim/sulfamethoxazole; R-resistant; S-susceptible

Table 2: Susceptibility profile of multiple antibiotic resistant (MAR) and non-MAR *S. maltophilia* strains

Antibiotic	MAR (*n* =55)				Non-MAR (*n* =41)			*P*-value
	Range[a]	MIC_{90} (µg/ml)	R *n* (%)	S *n* (%)	MIC_{90} (µg/ml)	R *n* (%)	S *n* (%)	
ATM	0.5 - >256	> 256	50 (91)	5 (9)	256	31(76)	10 (24)	<0.001
FEP	0.5 - >256	256	52 (95)	3 (5)	64	8 (20)	33 (80)	<0.001
CAZ	0.5 - >256	> 256	54 (98)	1(2)	>256	12 (29)	29 (71)	0.002
CIP	4 - 128	128	55 (100)	0 (0)	8	31 (75)	10 (24)	0.005
GEN	0.5 - >256	> 256	51 (93)	4 (7)	64	30 (73)	11 (27)	0.001
LEV	4 - 128	64	45 (82)	10 (18)	8	5 (12)	36 (88)	0.001
MER	0.5 - >256	> 256	49 (89)	6 (11)	256	30 (73)	11 (27)	<0.001
TZP	0.5 - >256	> 256	46 (84)	9 (16)	>256	33 (80)	8 (20)	<0.001
TIM	4 - >128	>128	21 (38)	34 (61)	16	1 (2)	40 (98)	<0.001
SXT	0.0625/1.1875 - >16/304	16/304	20 (36)	35 (64)	2/38	1 (2)	40 (98)	0.001

Value in parenthesis represents percentage profile

Legend: MIC_{90}, minmum inhibitory concentration for 90% of the organisms; ATM-aztreonam; FEP-cefepime; CAZ-ceftazidime; CIP-ciprofloxacin; GEN-gentamicin; LEV-levofloxacin; MER-meropenem; TZP-piperacillin/tazobactam; TIM-ticarcillin/clavulanic acid and SXT-trimethoprim/sulfamethoxazole; R-resistant; S-susceptible, [a] Breakpoints from CLSI

Table 3: Correlation profile of multiple antibiotic resistant with class 1 integron, formation of pigment and biofilm in environmental strains of *S. maltophilia*

Variables	Class 1 integron		Pigment		Biofilm
	+ve (*n* = 54)	-ve (*n* = 42)	H (*n* = 51)	L (*n* = (45)	Mean (S.D) OD_{540nm}
MAR (*n* = 55)	39 (71)	16 (29)	45 (82)	10 (18)	0.85 ± 0.21
Non-MAR (*n* = 41)	15 (37	26 (63)	6 (15)	35 (85)	0.25 ± 0.01
***P* - value**	0.001	0.001	<0.001	<0.001	<0.002

Values in parentheses indicate MAR and non-MAR percentage profile

Legend: +ve, presence of integrons; -ve, absence of integrons; H, high expression; L, low and no expression; S.D. standard deviation; OD_{540nm}, optical density at 540nm

Table 4: Effect of pigment production and biofilm formation on antibiotic susceptibility profile of environmental strains of *S. maltophilia*

Antibiotic	Pigment high (or +ve (*n* = 51)		Pigment low (or –ve) (*n* = 45)		*P* -value	Biofilm high[a] (*n* = 42)		Biofilm low[a] (*n* = 54)		*P* -value
	R	S	R	S		R	S	R	S	
	n (%)	*n* (%)	*n* (%)	*n* (%)		*n* (%)	*n* (%)	*n* (%)	*n* (%)	
ATM	44 (86)	7 (14)	35 (78)	10 (22)	ns	40 (95)	2 (5)	45 (83)	9 (17)	<0.001
FEP	45 (88)	6 (12)	25 (56)	20 (44)	0.002	38 (90)	4 (10)	19 (35)	35 (65)	<0.002
CAZ	47 (92)	4 (8)	14 (31)	31 (69)	0.001	35 (83)	7 (17)	16 (30)	38 (70)	0.001
CIP	42 (82)	9 (18)	21 (47)	24 (53)	0.005	22 (52)	20 (48)	39 (72)	15 (27)	ns
GEN	40 (78)	11(22)	37 (82)	8 (18)	ns	39 (93)	3 (7)	42 (78)	12 (22)	<0.005
LEV	31 (61)	20 (39)	15 (33)	30 (67)	0.001	28 (67)	14 (33)	16 (30)	38 (70)	ns
MER	44 (86)	7 (14)	37 (82)	8 (18)	ns	36 (86)	6 (14)	46 (85)	8 (15)	ns
TZP	41 (80)	10 (20)	11 (24)	34 (76)	0.002	33 (79)	9 (21)	15 (28)	39 (72)	<0.001
TIM	12 (24)	39 (76)	5 (11)	40 (89)	0.001	19 (45)	23 (56)	5 (9)	49 (91)	ns
SXT	9 (18)	42 (82)	8 (18)	37 (82)	ns	10 (24)	32 (76)	12 (22)	42 (78)	ns

Value in parenthesis represents percentage profile

[a] High, optical density at 540 nm ($OD540_{nm}$) ≥0.4; low, OD_{540nm} ≤0.1

Legend: ATM-aztreonam; FEP-cefepime; CAZ-ceftazidime; CIP-ciprofloxacin; GEN-gentamicin; LEV-levofloxacin; MER-meropenem; TZP-piperacillin/tazobactam; TIM-ticarcillin/ clavulanic acid and SXT-trimethoprim/sulfamethoxazole; R-resistant; S-susceptible; ns- not significant

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