Multidrug resistance in clinical isolates of *Stenotrophomonas maltophilia*: roles of integrons, efflux pumps, phosphoglucosyltransferase (SpgM), and melanin and biofilm formation

S.-J. Liaw^{a,b}, Y.-L. Lee^a, P.-R. Hsueh^{b,c,*}

^a Department and Graduate Institute of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, Taipei, Taiwan

^b Department of Laboratory Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan

^c Department of Internal Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan

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ABSTRACT

Integrons, efflux pumps, phosphoglucosyltransferase (SpgM), and melanin and biofilm formation were investigated in 40 multidrug-resistant (MDR) and 30 non-MDR *Stenotrophomonas maltophilia* isolates recovered from patients treated at National Taiwan University Hospital (Taipei, Taiwan). Class 1 integrons were clearly associated with multidrug resistance. Sequencing data revealed that aminoglycoside-modifying genes occurred most frequently in the integron and disclosed a new *bla*_{IMP-8}/*aac6-II/aadA5* gene cassette. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed to assess the expression of *Sme* efflux pumps and SpgM in *S. maltophilia*. MDR isolates exhibited hyperexpression of *SmeABC*, and *SmeDEF* and SpgM were more frequently found in MDR isolates than non-MDR isolates. In addition, the ability of MDR isolates to form melanin-like pigment and biofilm was also greater than that of the non-MDR isolates. The *SmeABC* or *SmeDEF* pump was shown to be associated with resistance to all agents tested. The presence of an integron as well as production of pigment and biofilm was also responsible for resistance against eight, six and six of the tested agents, respectively. High SpgM expression was associated with resistance to only three of the tested agents. These findings define the important roles of integrons, efflux pumps, and melanin-like pigment and biofilm formation in the multidrug resistance of *S. maltophilia*. MDR isolates possessed more resistance mechanisms than susceptible strains.

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1. Introduction

Stenotrophomonas maltophilia, a non-fermentative Gram-negative bacillus, is one of the multiresistant opportunistic nosocomial pathogens especially affecting immunocompromised patients and is being isolated worldwide with increasing frequency [1–4]. Clinical isolates of *S. maltophilia* are often highly resistant to most of the currently used antimicrobial agents, including carbapenems, and therapy of *S. maltophilia* infection presents a significant challenge both for clinicians and microbiologists [2–4]. A variety of antimicrobial resistance determinants have been reported in *S. maltophilia*, including tripartite efflux pumps (*SmeDEF* and *SmeABC*), integrons and phosphoglucosyltransferase (SpgM) [1,5–7]. A role in multidrug resistance has been demonstrated for *SmeDEF*, and other efflux pumps are also known to be involved in resistance but only in some *S. maltophilia* isolates [8,9]. Integrons comprise an integrase-encoding gene that allows

for site-specific insertion of resistance gene cassettes between two highly conserved adjacent nucleotide sequences [3' conserved segment (3'CS) and 5' conserved segment (5'CS)]. Integrons are located on transposons or plasmids that facilitate the rapid spread of integrons to other strains and bacterial species [10–12]. SpgM, a homologue of AlgC in *Pseudomonas aeruginosa* associated with lipopolysaccharide (LPS) and alginate biosynthesis, was shown to play a role in virulence and antibiotic resistance of *S. maltophilia* [7]. Melanin-like pigment and biofilm formation were generally implicated as common causes of antimicrobial resistance [13–15].

In the present study, we assessed the roles of integrons, efflux pumps, SpgM, melanin and biofilm on multidrug resistance among clinical isolates of *S. maltophilia*.

2. Materials and methods

2.1. Bacterial isolates

Forty clinical multidrug-resistant (MDR) [i.e. resistant to five or more of the six categories of drugs tested, including cephalosporins and β -lactams plus β -lactamase inhibitors, carbapenems,

* Corresponding author. Tel.: +886 2 2312 3456x65355; fax: +886 2 2322 4263.
E-mail address: hsporen@ntu.edu.tw (P.-R. Hsueh).

monobactams, aminoglycosides, quinolones and trimethoprim/sulfamethoxazole (SXT)] and 30 non-MDR (i.e. resistant to four or less of the six categories of drugs tested) *S. maltophilia* isolates were collected at National Taiwan University Hospital (Taipei, Taiwan) during 2002–2003. These isolates were obtained from sputum ($n=23$), wounds ($n=2$), central venous catheter ($n=1$), urine ($n=8$), blood ($n=34$), cerebrospinal fluid ($n=1$) and eye ($n=1$).

All of the isolates were identified by conventional biochemical identification methods and were confirmed by the API 20E (bioMérieux, La-Balme-les-Grottes, France) and Vitek automated systems (bioMérieux Vitek, Hazelwood, MO). Random amplified polymorphic DNA (RAPD) analysis (Operon Technologies, Inc., Alameda, CA) was used to exclude repeat isolates [1].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibilities were determined by the agar dilution method as described by the Clinical and Laboratory Standards Institute [16,17]. The following agents were included: gentamicin (GEN); ciprofloxacin (CIP) and levofloxacin (LEV); SXT; ceftazidime (CAZ) and cefepime (FEP); ticarcillin/clavulanic acid (TIM) and piperacillin/tazobactam (TZP); aztreonam (ATM); and meropenem (MER). Control strains for susceptibility testing included *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213.

2.3. Integron polymerase chain reaction (PCR)

Class 1 integrons were detected with class I integrase-specific primers (5'-ACATGTG ATGGCGACGCACGA-3' and 5'-ATTTCTGTCCCTGGCTGGCGA-3'). Gene cassettes within integrons were amplified with primers specific for the integron 5'CS (5'-GGCATCAAGCAGCAAG-3') and 3'CS (5'-AAGCAGACTTGACCTGA-3') [18]. Template DNA for PCR was prepared as described by Ploy et al. [18]. The amplicons were then sequenced and sequence com-

parisons were made using the BLAST program. Plasmid DNA was extracted by the method of Kado and Liu [19].

2.4. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Cells were prepared and inoculated onto a Mueller–Hinton agar plate as in the agar dilution method [16,17,20]. Following overnight culture, cells were collected to make a 1.5 mL suspension of optical density at 550 nm (OD_{550nm}) = 1.0. RNA was prepared using an RNA-Bee™ Kit (Tel-Test Inc., Friendswood, TX) and cDNA was obtained with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corp., CA) using random hexamers. Primer pairs 5'-GTCGACCTG GTACAGCA-3'/5'-ACCTTAACCTGTGCCTTG-3', 5'-CCAAGAGCTTTC CGTCAT-3'/5'-TCACGCTGAAGTCCGAGA-3' and 5'-GTGACTTCGACC GTTGCTTC-3'/5'-ATCTTTTCCTTGATGAACGC-3' were used for PCR to detect the expression of *smeA*, *smeD* and *spgM*, respectively, using cDNA of 16S rRNA as an internal control. Four micrograms of total RNA was reverse transcribed to cDNA. The RNA was then denatured by the addition of NaOH and incubation at 65 °C for 10 min and 1 μ L of cDNA solution was used as the template for PCR. PCR was performed with a 10 min denaturation step at 95 °C followed by 25 cycles (cDNA of 16S rRNA), 30 cycles (*smeD*) or 35 cycles (*smeA* and *spgM*) of 1 min at 95 °C and 1 min at 55 °C (for *smeD*) or 65 °C (for cDNA of 16S rRNA, *smeA* and *spgM*) for annealing and extension. Gel images were digitally captured with a CCD camera and band intensity was analysed using ImageJ software (<http://rsb.info.nih.gov/ij/>). The relative expression quantity was evaluated by the ratios of band intensity to cDNA of 16S rRNA for three independent experiments. Isolates with a ratio greater than the average ratio of all isolates were designated as high expression, and those with equal to or less than the average ratio were designated as low expression. A number of different cycle numbers and template concentrations were used to confirm linearity within the experiment.

Table 1

Antibiotic susceptibility profiles of multidrug-resistant (MDR) and non-MDR *Stenotrophomonas maltophilia* isolates.

Antimicrobial agent	MDR ($n=40$)			Non-MDR ($n=30$)		
	MIC ₉₀ (μ g/mL)	%S (n)	%R (n)	MIC ₉₀ (μ g/mL)	%S (n)	%R (n)
CAZ	>256	5 (2)	95 (38)	>256	83 (25)	17 (5)
FEP	256	2.5 (1)	98 (39)	64	73 (22)	27 (8)
TIM	128/2	63 (25)	38 (15)	16/2	100 (30)	0 (0)
TZP	>256/4	18 (7)	83 (33)	>256	80 (24)	20 (6)
MER	>256	2.5 (1)	98 (39)	256	20 (6)	80 (24)
ATM	>256	2.5 (1)	98 (39)	256	23 (7)	77 (23)
GEN	>256	10 (4)	90 (36)	64	30 (9)	70 (21)
CIP	128	0 (0)	100 (40)	8	83 (25)	17 (5)
LEV	64	33 (13)	68 (27)	8	90 (27)	10 (3)
SXT	16/304	58 (23)	43 (17)	2/38	100 (30)	0 (0)

MIC₉₀, minimum inhibitory concentration for 90% of the organisms; %S, percent susceptible; %R, percent resistant; CAZ, ceftazidime; FEP, cefepime; TIM, ticarcillin/clavulanic acid; TZP, piperacillin/tazobactam; MER, meropenem; ATM, aztreonam; GEN, gentamicin; CIP, ciprofloxacin; LEV, levofloxacin; SXT, trimethoprim/sulfamethoxazole.

Table 2

Correlation of multidrug resistance with the class I integron, expression of *smeA*, *smeD* and *spgM*, and the formation of pigment and biofilm in clinical isolates of *Stenotrophomonas maltophilia*.

	Class I integron		SmeA		SmeD		SpgM		Pigment		Biofilm	
	+ve ($n=42$)	-ve ($n=28$)	H ($n=29$)	L ($n=41$)	H ($n=44$)	L ($n=26$)	H ($n=33$)	L ($n=37$)	H ($n=38$)	L ($n=32$)	Mean (S.D.)	OD _{540nm}
MDR ($n=40$)	33 (83) ^a	7 (18)	24 (60)	16 (40)	34 (85)	6 (15)	23 (58)	17 (43)	30 (75)	10 (25)	0.52	(0.34)
Non-MDR ($n=30$)	9 (30)	21 (710)	5 (17)	25 (83)	10 (33)	20 (67)	10 (33)	20 (67)	8 (27)	22 (73)	0.15	(0.13)
	$P<0.01^b$		$P<0.01^b$		$P<0.01^b$		$P<0.05^b$		$P<0.01^b$			$P<0.01^c$

MDR, multidrug-resistant (as defined in Section 2.1); +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 540 nm.

^a Percentage of MDR or non-MDR isolates in parentheses.

^b Comparison between MDR and non-MDR isolates by Fisher's exact test.

^c Comparison between MDR and non-MDR isolates by Student's *t*-test.

2.5. Biofilm assay

The biofilm assay was performed as described previously [14]. Overnight Luria–Bertani cultures were transferred to the wells of polystyrene microtitre plates. The plates were incubated at 37 °C for 10 h and then stained with crystal violet. Solubilised crystal violet was determined.

2.6. Pigment assay

A melanin-like pigment assay was performed as described previously [21]. Bacteria were cultured in L-tyrosine-containing agar to facilitate the observation of brown pigment production. Pigment formation was recorded as 0, 1+, 2+ or 3+, with increased intensity and intensity equal to or greater than 2+ designated as high pigment expression.

3. Results

3.1. Bacterial isolates and antimicrobial susceptibility

The RAPD patterns of the 70 isolates were not identical, indicating the diversity of the isolates (i.e. not clonally related). The results of susceptibility testing (Table 1) showed that most of the *S. maltophilia* isolates were resistant to MER (90%), ATM (89%), GEN (81%), FEP (67%), CIP (64%) and CAZ (61%). Among the agents tested, MDR and non-MDR isolates exhibited minor differences in resistance to MER, ATM and GEN (98% vs. 80%, 98% vs. 77% and 90% vs. 70%, respectively). TIM and SXT (79% and 76% susceptible, respectively) were the most active drugs against *S. maltophilia*.

3.2. Integrons

Forty-two isolates (60%) carried detectable class 1 integrons (integron I). More MDR isolates (83%) possessed class 1 integrons than non-MDR isolates (30%) (Table 2). Integron-positive isolates were significantly more likely to be resistant to CIP, CAZ, FEP, TIM, TZP, SXT, MER and GEN (Table 3). Gene cassettes within integrons included *aacA4*, *aadB*, *aacC4*, *aacA6'-Ib*, *smr*, *smr/aacA4*, *qac*, *cmlA*, *catB2* and *bla_{IMP-8}/aac6-II/aadA5*. Two isolates harboured plasmid DNA, but Southern blot assay demonstrated that neither of these two isolates carried a plasmid-mediated integron.

3.3. Efflux pumps

The rate of high *smeD* expression (63%) was greater than the rate of high *smeA* expression (41%) in the *S. maltophilia* isolates studied. MDR isolates expressed high *SmeD* and high *SmeA* more frequently than non-MDR isolates (85% vs. 33% for *SmeD* and 60% vs. 17% for *SmeA*) (Table 2). High *smeA* or *smeD* expression was correlated with resistance to all of the drugs tested (Table 3).

3.4. SpgM

The difference in *spgM* expression between MDR and non-MDR isolates was less significant compared with the other determinants tested ($P < 0.05$) (Table 2). High *SpgM* expression was correlated with three lactams (CAZ, TIM and TZP) only (Table 3).

3.5. Melanin-like pigment

Most MDR isolates produced a higher level of pigment than non-MDR isolates (75% vs. 27%) (Table 2). High-pigment-producing isolates exhibited significant correlation with resistance to CIP, LEV, CAZ, FEP, TIM and TZP (Table 3).

Table 3
Effect of the presence of integrons, expression of *SmeA*, *SmeD* and *SpgM*, and the production of pigment and biofilm on antibiotic susceptibility profiles of clinical isolates of *Srenotrophomonas maltophilia*.

Antibacterial agent	Integron +ve (n=42)		Integron -ve (n=28)		P-value ^a	SmeA or SmeD high (n=45)		SmeA and SmeD low or negative (n=25)		P-value ^a	Pigment high (n=38)		Pigment low or negative (n=32)		P-value ^a	SpgM high (n=33)		SpgM low or negative (n=37)		P-value ^a	Biofilm high ^b (n=25)		Biofilm low ^b (n=37)		P-value ^a
	R	S	R	S		R	S	R	S		R	S	R	S		R	S	R	S		R	S	R	S	
CAZ	32	10	11	17	<10 ⁻²	40	5	3	22	<10 ⁻³	36	2	7	25	<10 ⁻³	28	5	15	22	<10 ⁻³	20	5	15	22	<10 ⁻²
FEP	34	8	13	15	<10 ⁻²	36	9	11	14	<10 ⁻²	31	7	16	16	<10 ⁻²	20	13	27	10	N.S.	23	2	17	20	<0.005
TIM	14	28	1	27	<10 ⁻³	15	30	0	25	<0.005	14	24	1	31	<10 ⁻³	12	21	3	34	<10 ⁻²	14	11	1	36	<10 ⁻³
TZP	32	10	7	21	<10 ⁻³	31	14	8	17	<10 ⁻²	29	9	10	22	<10 ⁻³	25	8	14	23	<10 ⁻²	19	6	15	22	<10 ⁻²
MER	42	0	21	7	<10 ⁻³	45	0	18	7	<10 ⁻³	34	4	29	3	N.S.	30	3	33	4	N.S.	21	4	34	3	N.S.
ATM	37	5	25	3	N.S.	44	1	18	7	<10 ⁻²	34	4	28	4	N.S.	30	3	33	4	N.S.	25	0	29	8	10 ⁻²
GEN	42	0	15	13	<10 ⁻³	43	2	14	11	<10 ⁻³	28	10	29	3	N.S.	27	6	30	7	N.S.	24	1	25	12	<10 ⁻²
CIP	32	10	13	15	<10 ⁻³	35	10	10	15	<10 ⁻²	30	8	15	17	<10 ⁻²	24	9	21	16	N.S.	13	12	25	12	N.S.
LEV	20	22	10	18	N.S.	25	20	5	20	<10 ⁻²	23	15	7	25	<10 ⁻²	17	16	13	24	N.S.	14	11	14	23	N.S.
SXT	15	27	2	26	<10 ⁻²	16	29	1	24	<10 ⁻²	8	30	9	23	N.S.	8	25	9	28	N.S.	9	16	8	29	N.S.

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

^a By Fisher's exact test.

^b High, optical density at 540 nm (OD_{540nm}) ≥ 0.4; low, OD_{540nm} ≤ 0.1.

Fig. 1. Comparison of numbers of resistance mechanisms between multidrug-resistant (MDR) and non-MDR *Stenotrophomonas maltophilia* isolates.

3.6. Biofilm formation

MDR isolates had an average OD_{540nm} value of 0.52 compared with a value of 0.15 for non-MDR isolates ($P < 0.01$) (Table 2). Biofilm formation was not correlated with resistance to CIP, LEV (both quinolones), SXT or MER (Table 3).

The data obtained in this study imply that all resistance determinants contribute to the MDR phenotype of *S. maltophilia*. Among them, the presence of efflux pumps and integron I may play important roles leading to multidrug resistance of *S. maltophilia*. Fig. 1 shows that 28 MDR isolates possessed over four mechanisms of resistance, whereas most non-MDR isolates possessed less than three mechanisms.

4. Discussion

Both combined specific resistance genes and multidrug resistance determinants have been described as conferring multidrug resistance in other Gram-negative bacteria [22–25]. It is expected that such mechanisms are likely to be present in *S. maltophilia*. In this study, we assessed the contribution of integrons, efflux pumps, SpgM, and melanin and biofilm formation to multidrug resistance of *S. maltophilia*. A partial correlation was found between the mechanisms studied and multidrug resistance—some were strong (i.e. integron I) and some were only weak (i.e. SpgM). Further experiments such as genetic knockouts should be performed to determine whether they are playing a role in multidrug resistance.

It has been shown that class 1 integrons in Gram-negative isolates from different hospitals are associated with decreased susceptibility to multiple antibiotic agents [10,11,26]. In this study, we found that integron I contributed to resistance against eight of the drugs tested. The spread of metallo- β -lactamases (MBLs) in Enterobacteriaceae is becoming a clinical concern in Taiwan and consistent and constant surveillance of MBL-producers should be the prime measure to prevent their further dissemination [27,28]. A kind of MBL, IMP-8, was previously identified from clinical isolates of *Klebsiella pneumoniae* and *Enterobacter cloacae*, and these *bla*_{IMP-8}-positive (usually carried on transferrable plasmids) isolates were all MDR [27,28]. The present study is the first to identify a *bla*_{IMP-8}-containing isolate of *S. maltophilia*.

The origin of *bla*_{IMP-8} remains to be determined by cloning its flanking sequences. None of the integron-positive isolates carried plasmids. The ability of *S. maltophilia* to be involved in genetic transfer and the acquisition of resistance determinants from other bacteria remains to be clarified. The integron I carriage rate in this study is higher than that reported by Chang et al. (60% vs. 22%) [1],

which may due to the different origins of the samples collected. The finding that the most common gene cassette carried by integron I was comprised of aminoglycoside resistance determinants may account for the significant difference in susceptibility to GEN between the integron-positive and -negative isolates. The presence of an integron I was found to make a significant contribution to SXT resistance. Among the 17 SXT-resistant isolates, 15 (88%) carried an integron I and 11 of them possessed *sul1* genes. This finding confirms the increased incidence of integron I in SXT-resistant clinical isolates of *S. maltophilia* [29,30]. Both the use of quaternary ammonium compounds and triclosan in the natural environment have the potential to select for resistance to other antibiotics [31,32]. The frequent occurrence of the *qac* gene cassette within integron I in the present study implies that multidrug resistance may result from the introduction of biocides into hospital settings to prevent bacterial infections.

Because of its association with LPS synthesis, SpgM expression may affect the structure of the bacterial envelope and alter susceptibility to antimicrobial agents belonging to the β -lactam class that act on penicillin-binding proteins located in the periplasm. In this study, we found that high expression of SpgM in *S. maltophilia* was associated with resistance to CAZ, TZP and TIM but not to MER, ATM and FEP. We also found that most isolates that exhibited no significant correlation between high SpgM expression and resistance to MER, ATM and FEP expressed L1 or L2 β -lactamases. This implies that resistance to MER, ATM and FEP in SpgM high-expressed *S. maltophilia* might be due to expression of L1 or L2 β -lactamases.

The finding that *SmeD* expression was more important than *SmeA* expression in the MDR isolates studied here is contrary to the report of Chang et al. [1]. Different sample populations and the use of different gene targets (*smeD* vs. *smeF* and *smeA* vs. *smeB*) may account for this discrepancy [1].

Melanin-like pigment has been shown to protect cells from environmental insult in bacteria and fungi [15,33]. In this study, we found that pigment production in *S. maltophilia* was associated with antibiotic resistance. Our preliminary data on drug susceptibility testing using cells with and without pigment formation indicated that pigment-bearing cells were more resistant to antibiotics.

It is clear that biofilm-grown cells express properties distinct from those of planktonic cells, one of which is increased resistance to antimicrobial agents. The nature of the resistance generated by biofilms, despite extensive research, remains an enigma. We found that resistance to CIP and LEV was not associated with high biofilm-forming ability. This finding is consistent with a study by Di Bonaventura et al. [14], which disclosed that quinolones reduce adherence of *S. maltophilia* to plastic surfaces and can reduce pre-formed biofilms of *S. maltophilia*.

In conclusion, our findings define the important roles of integrons, efflux pumps, melanin-like pigment and biofilm formation in the multidrug resistance of *S. maltophilia*. MDR isolates possessed more resistance mechanisms than susceptible strains. Integron PCR or semi-quantitative RT-PCR for either *SmeABC* or *SmeDEF* might be a useful method of screening for MDR *S. maltophilia*.

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