Universiti Putra Malaysia

Could clinical *Stenotrophomonas maltophilia* be a potential pathogen in clinical setting?

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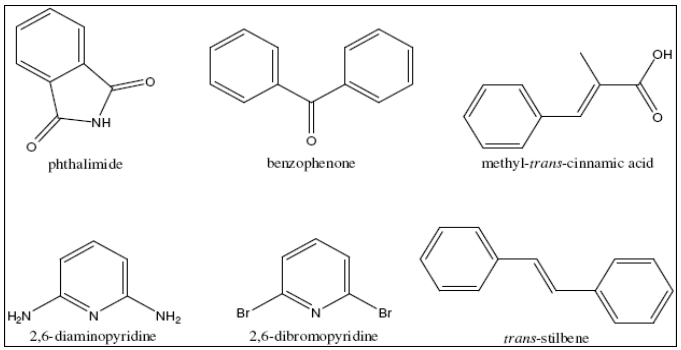
- *Stenotrophomonas maltophilia,* previously known as *Pseudomonas maltophilia* or *Xanthomonas maltophilia,* is ubiquitously found in nature

- Well known environmental microbe with several biotechnological applications.

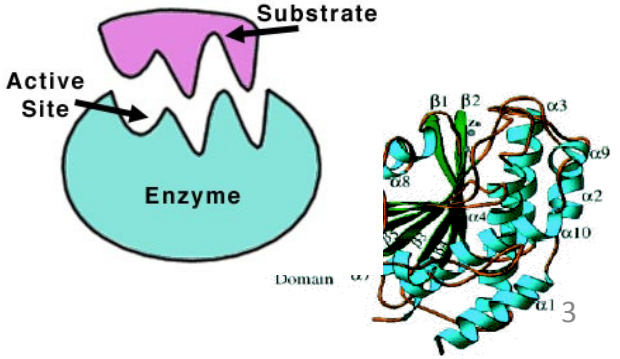
Biocontrol and growth enhancer

BIOTECHNOLOGICAL APPLICATIONS

Bioremediation and phytoremediation

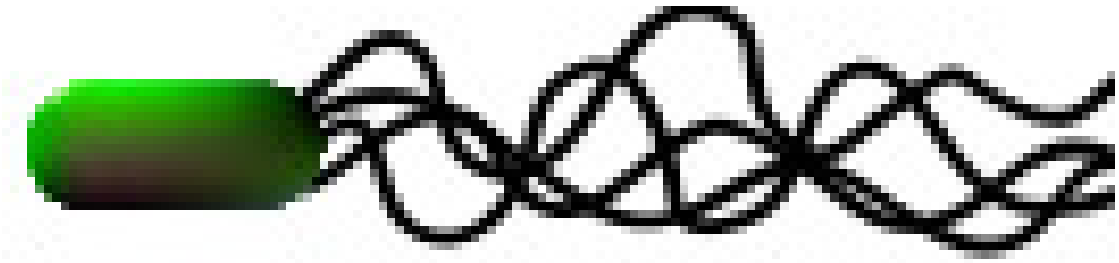


Secondary metabolite production



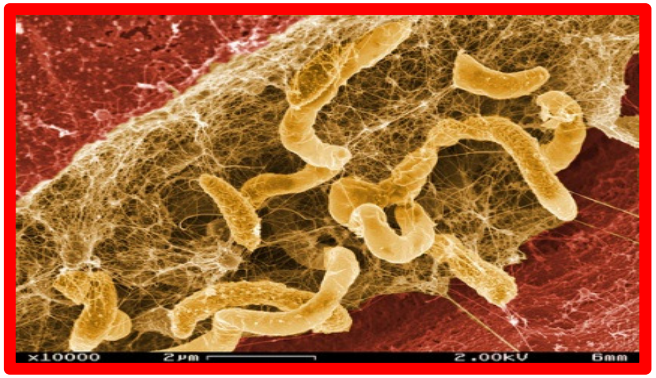
STENOTROPHOMONAS MALTOPHILIA

Most worrisome threat among unusual non-fermentative gram negative bacteria in hospitalized patients



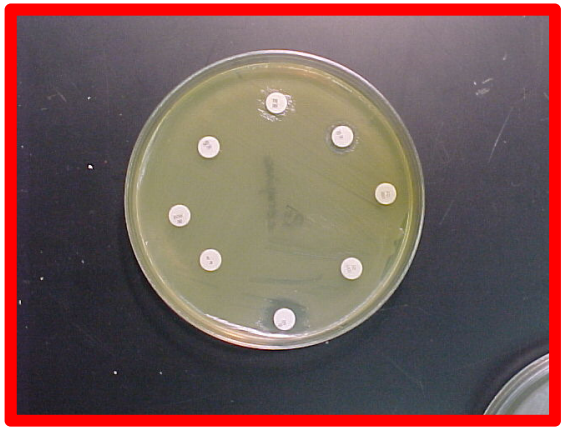
Colonizes human and medical devices

Pathogenic determinants and biofilm



SUCCESSFUL NOSOCOMIAL PATHOGEN

Antibiotic resistance



Highly diverse clones

HISTORY

- 1943: First isolated from pleural fluid in 1943 by J. L. Edwards, named as *Bacterium bookeri*

- 1961: Classified as *Pseudomonas maltophilia* by Hugh and Ryschenko when similar strain was isolated in 1958 from an oropharyngeal swab from a patient with an oral carcinoma.

- 1981: Reclassified as *Xanthomonas maltophilia* by Swings and group based on the rRNA cistron homology generated through the DNA-rRNA hybridization techniques.

- 1993: Finalized by Palleroni and Bradbury as *S. maltophilia* since *X. maltophilia* did not match well including the specific 16SrRNA gene

- At present : 8 species
  S. maltophilia, S. nitritireducens , S. rhizophila , S. acidaminiphila , S. koreensis, S. chelatiphaga , S. terrae , S. humi and *S. africana*.

CHALLENGES IN COMBATING S. MALTOPHILIA INFECTIONS

Management of *S. maltophilia* infections represents a great challenge to clinicians

- in vitro susceptibility testing
- lack of clinical trials to determine optimal therapy
- intrinsic resistance to a plethora of antimicrobial agents
- Opportunistic pathogen targets immunocompromised population, prolonged hospitalization, malignancy, immune suppression, and breakdown of muco-cutaneous defense barriers (e.g., following catheterization, artificial implantation, tracheotomy, or peritoneal dialysis
- Different strains behave differently
- Ubiquitously present in the environment
- Source tracing is difficult
- No clear information on virulence factors or pathogenicity
- Debate on colonizer or pathogen

Epidemiology

In Malaysia, highest number of *S. maltophilia* infections was observed among Tracheal Aspirate of about 39%.

S.maltophilia Infection

Hospital Acquired (60%)

- Respiratory Tract
- Bacteremia
- Bloodstream
- Urinary Tract
- Wound
- Gastro-Intestinal
- Neural

Community Acquired (45.8%)

Infections that occurred 48 or 72 h prior hospitalization

- Bacteremia
- Ocular infection
- Respiratory tract infection
- Wound / soft tissue infections
- Urinary tract infection

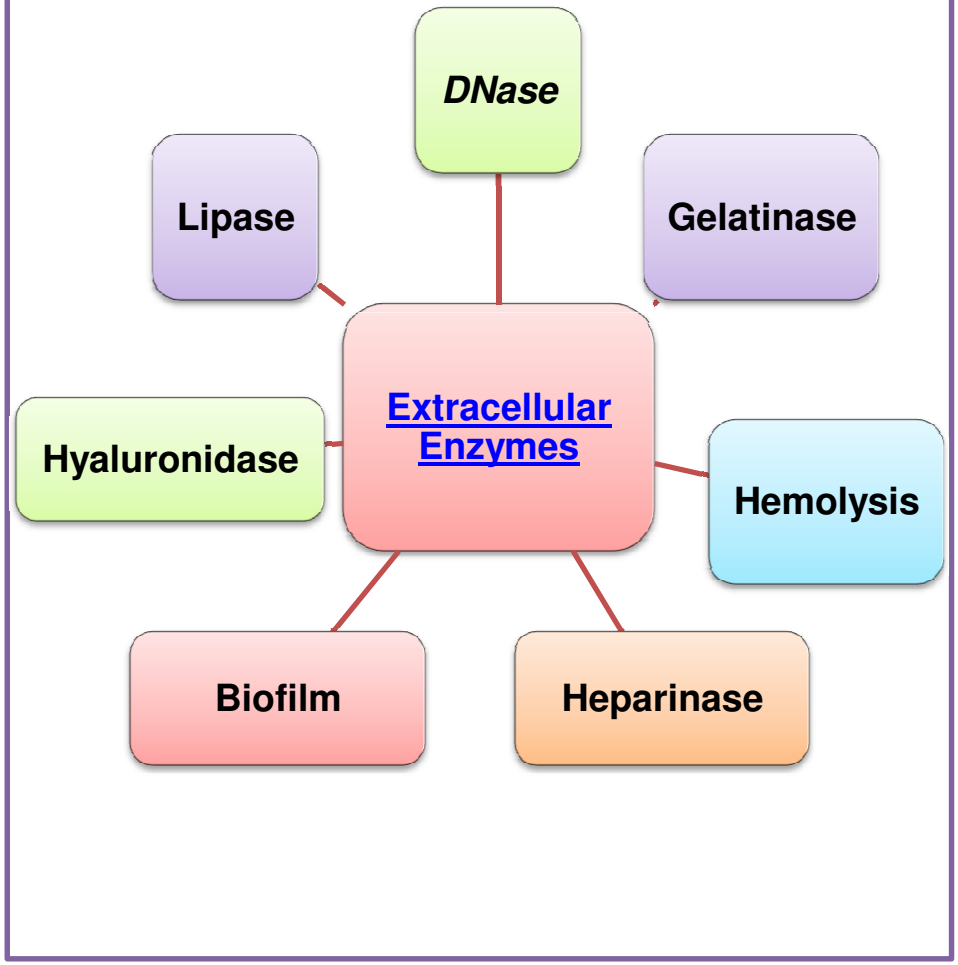
Radical increase over the past decades (2-4 fold increase)

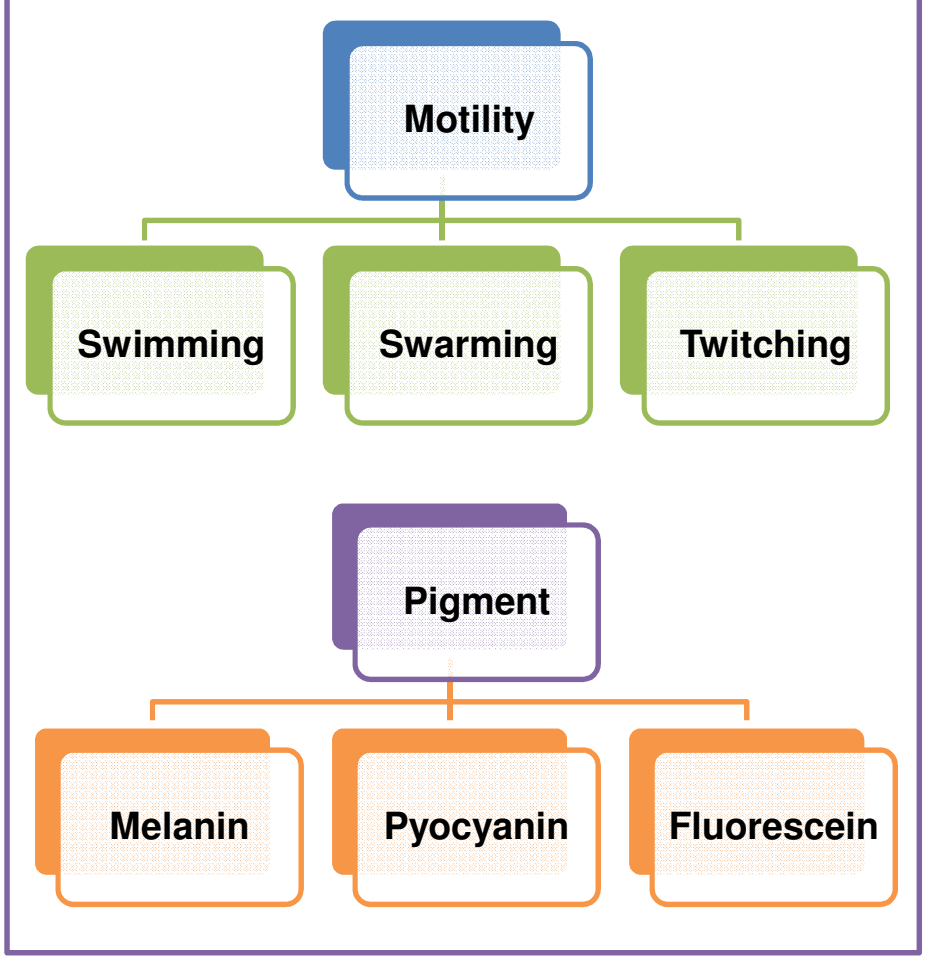
[Chang *et al. J Microbiol Immuno and Infect* . 2014]
[Neela *et al. Int j of Infect Dis.* 2012]

S. MALTOPHILIA COLONIZATION OR PATHOGEN ?

- The failure to distinguish between colonization and infection has led to the belief that *S. maltophilia* is an organism of limited pathogenic potential that is rarely capable of causing disease in healthy individuals.
- Reports indicate that infection with this organism is associated with significant morbidity and mortality rates particularly in severely compromised patients.
- Its mechanism of pathogenesis is poorly understood

Study1: Extracellular enzyme profile of *S.maltophilia*

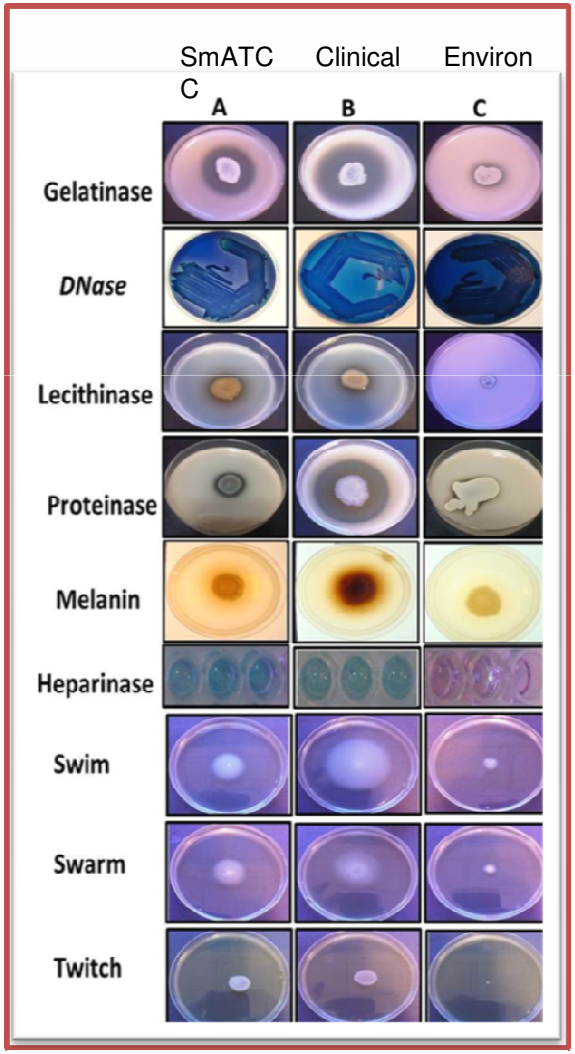




Different hydrolytic enzyme assay using plate method

Enzyme	Method	Result	Reference
DNase	1. DNase agar test - 0.01% toluidine blue was used to determine DNase production after 72 h of growth at 37°C. 2. Modified DNase tube test was also employed to evaluate the DNase production as described elsewhere.	1. DNase activity was indicated by the formation of a large pink halo around an inoculum spot . 2. Clearing of the genomic DNA band	(Janda *et al*,1981; Neela, *et al*. 2012)
Gelatinase	Organisms were inoculated on 0.4% gelatin agar. The plates were incubated at 37°C for 24 h followed by which the plates were flooded with mercuric chloride solution.	Appearance of opaque zone around the inoculum	(Frazier *et al*1926; Mc and Weaver 1959)
Hemolysis	Trypticase soy agar containing 5% sheep blood was evaluated at room temperature after 24 h of growth.	Appearance of clear zone	(Travassos, *et al*. 2004)
Heparinase	Heparin was diluted in distilled water to a final concentration of 5 U/ml followed by filter sterilization (0.45 pm) before dispensing 20 µl into 96 well micro titration plate; each well contained 30 µl of the test bacteria, incubated overnight at 37°C. 20 µl of aqueous toluidine blue 0.01% was added to each well.	Blue color indicated positive result, while pink indicated negative	(Riley 1987)
Hyaluronidase	Incorporation of aqueous solutions of hyaluronic acid into Muller Hinton agar supplemented with bovine serum albumin (final concentration, 1%). After being inoculated and incubated for 48 h, each plate was flooded with 2 N acetic acid, which was removed after 10 min.	The appearance of a clear zone around the inoculum.	(Smith and Willett 1968)
Lecithinase	Ten millilitres of the 50% egg yolk was added to 90 ml of sterilized tryptic soya agar and served as the substrate (29).	A white precipitate around or beneath an inoculum spot indicated lecithinase formation.	(Nord, *et al* 1975; Edberg, *et al* 1996)
Lipase	Lipase activity was detected by the on Trypticase soy agar plates supplemented with 1% Tween 80 .	Appearance of a turbid halo around the inocula	(Rollof, *et al*. 1987)
Proteinase	Casein hydrolysis and was tested on Mueller–Hinton agar containing 3% (w/v) skimmed milk .	The presence of a transparent zone around the inoculum spot	(Burke *et al* 1991; Edberg*et*

Study 1: Extracellular enzyme profile of *S.maltophilia*



Frequency among clinical isolates (n = 108)						
	Tracheal				Wound	
Enzymes	Aspirate	Blood	CSF	Sputum	Infection	Urine
DNase	42 (100)	37 (94.8)	6 (100)	5 (100)	13 (100)	3 (100)
Gelatinase	42 (100)	39 (100)	6 (100)	5 (100)	13 (100)	3 (100)
Hemolysin	42 (100)	39 (100)	6 (100)	5 (100)	13 (100)	3 (100)
Heparinase	30 (71.42)	27 (69.2)	4 (66.6)	4 (80)	9 (69.2)	0
Hyaluronidase	42 (100)	39 (100)	6 (100)	5 (100)	13 (100)	0
Lipase	42 (100)	39 (100)	6 (100)	5 (100)	13 (100)	3 (100)
Lecithinase	34 (80.95)	17 (43.5)	6 (100)	5 (100)	13 (100)	0
Proteinase	42 (100)	39 (100)	6 (100)	5 (100)	13 (100)	3 (100)
Pyocyanin	0	0	0	0	0	0
Flourescein	0	0	0	0	0	0

Lecithinase and heparinase – significantly associated with invasive orig

Study 1: Extracellular enzyme profile of *S.maltophilia*

	Melanin		Biofilm		Motility	
	+ve	-ve	High	Low	Motile	Non-motile
Invasive (n = 45)	41 (91.1)	4 (8.8)	9 (20)	36 (80)	100	0
Non-Invasive (n =	60 (95.2)	3 (4.7)	11 (17.)	52 (82.5)	100	0

	Melanin		Biofilm		Motility	
	+ve	-ve	High	Low	Motile	Non – motile
Device Related (n= 71)	65 (91.5)	6 (8.4)	14 (19.7)	57 (80.2)	100	0
Non-Device Related (n = 37)	36 (97.2)	1 (2.7)	7 (18.9)	30 (81)	100	0

- Irrespective of Invasive/Non-invasive – All Isolates produces factors that destroy cell components.
- Infections are multifactorial events and secreted or non-secreted components contribute equally in pathogenesis.

Frequency among clinical isolates n=108		
Enzymes	Device Related (n = 71)	Non- Device Related (n = 37)
DNase	71 (100)	69 (97.1)
Gelatinase	71 (100)	37 (100)
Hemolysin	71 (100)	37 (100)
Heparinase	52 (73.2)	23 (62.1)
Hyaluronidase	71 (100)	37 (100)
Lipase	71 (100)	37 (100)
Lecithinase	49 (69)	27 (73)
Proteinase	71 (100)	37 (100)
Pyocyanin	0	0
Flourescein	0	0

- Certain enzymes like lecithinase and lipase might play important role in certain type of infections – Lining of lungs mainly composed of lecithin.
- Reservoir for pathogenic potential enzymes.

RESULTS(Cont.)

Study 1 : Extracellular enzyme profile of *S.maltophilia*

Virulence 5:2, 326–330; February 15, 2014; © 2014 Landes Bioscience

Extracellular enzyme profiling of *Stenotrophomonas maltophilia* clinical isolates

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Keywords: *Stenotrophomonas maltophilia*, virulence factors, hydrolytic enzymes, pathogenicity, nosocomial pathogen, gram-negative

Abbreviations: NETs, neutrophil extracellular traps; DNase, deoxyribonuclease; CSF, cerebrospinal fluid; UV, ultraviolet; PCR, polymerase chain reaction; LB, Luria bertanii; OD, optical density

Stenotrophomonas maltophilia, which is still defined as an organism of limited pathogenicity, has risen prominently as a nosocomial pathogen. Despite the increase in the spectrum of clinical syndromes associated with *S. maltophilia*, very little is known about the extracellular enzymes profile, pigment production and motility patterns which may have potential roles in pathogenesis. In this study, we screened and compared an array of extracellular enzymes in *S. maltophilia* collected from invasive and non-invasive clinical specimens by substrate plate assays. We also grouped the isolates as device related and non-device related and compared the enzyme profile. Our study showed all clinical isolates irrespective of source produced substantial levels of enzymes assayed, produced melanin and exhibited swimming and swarming motility pattern. These data suggests clinical isolates of *S. maltophilia* is a reservoir for pathogenic potential enzymes.

Stenotrophomonas maltophilia plays a major role as an opportunistic pathogen in immunocompromised individuals. from different anatomical sites are not clear. Therefore, the main aims of the present study were to screen for the production of extracellular enzymes (protease, lipase, lecithinase, gelatinase, deoxyribonuclease [DNase], hyaluronidase, hemolysin) by *S. maltophilia* isolates, to compare the enzyme profiles of invasive (e.g., blood and cerebrospinal fluid) and non-invasive (e.g., sputum, tracheal aspirate, wound infection, and urine) isolates and also in device and non-device related isolates.

A total of 108 *S. maltophilia* clinical isolates collected from patients admitted for various underlying diseases in tertiary care hospitals in the central region of Malaysia were investigated. Isolates were confirmed as *S. maltophilia* by lavender green colonies on blood agar, motility test, and standard biochemical assays such as DNase, catalase and oxidase activities. The isolates were genotypically confirmed by species specific polymerase chain reaction (PCR) that targeted the 23s RNA. The isolates were further reconfirmed using the VITEK® Mass Spectrometry System and an array of 64 biochemical assays. *S. maltophilia*

Study2: Prevalence of Putative Virulent Genes in *S. maltophilia* infections.

Virulence Genes Identified from closely related species

BLAST

***S. maltophilia* K279a(Clinical origin)**

Primers Designed

PCR Amplification → Electrophoresis → Analysis

Real Time- PCR

- Shares 86 to 90% similarities with *P.aeruginosa*

✓Positive control: *S. maltophilia* ATCC 13637

✓Negative control: *P.aeruginosa*: ATCC2785

PCR primers and cycling parameters for virulence genes

Genes	Initial Denaturation	Denaturation	Annealing	Extention	Final Extention	Reference
Lipase	5 min at 95°C	30 s at 95.1°C	20 s at 64.2°C	40 s at 72°C	2 min at 72°C	This study
ICOM	5 min at 95°C	20 s at 94.1°C	15 s at 59.9°C	30 s at 72°C	2 min at 72°C	This study
Lux R	5 min at 95°C	30 s at 95.2°C	20 s at 59.8°C	30 s at 72°C	2 min at 72°C	This study
Side	5 min at 95°C	30 s at 94.4°C	20 s at 59°C	40 s at 72°C	2 min at 72°C	This study
PiliZ	5 min at 95°C	34 s at 95.1°C	24 s at 64.2°C	44 s at 72°C	2 min at 72°C	This study
TatD	5 min at 95°C	30 s at 94.7°C	20 s at 51.9°C	40 s at 72°C	2 min at 72°C	This study
Tox A	5 min at 95°C	30 s at 95.1°C	20 s at 64.2°C	40 s at 72°C	2 min at 72°C	This study

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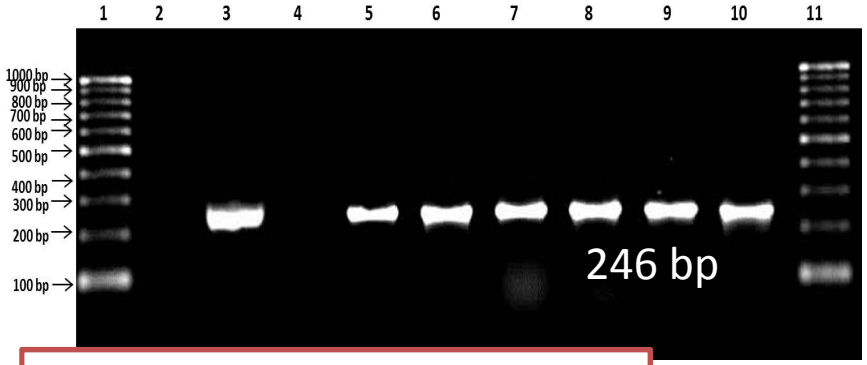
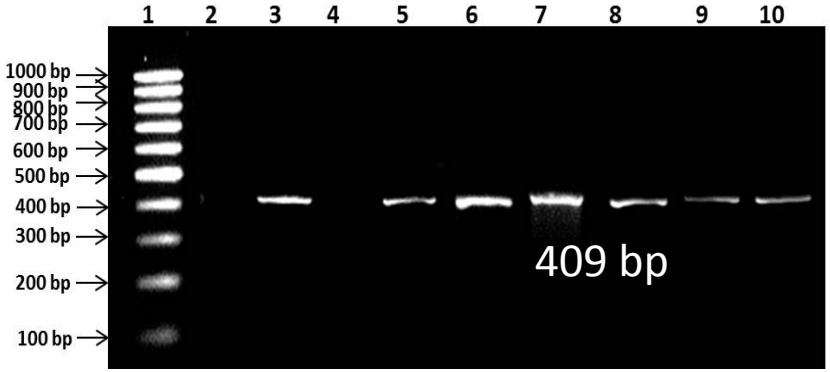
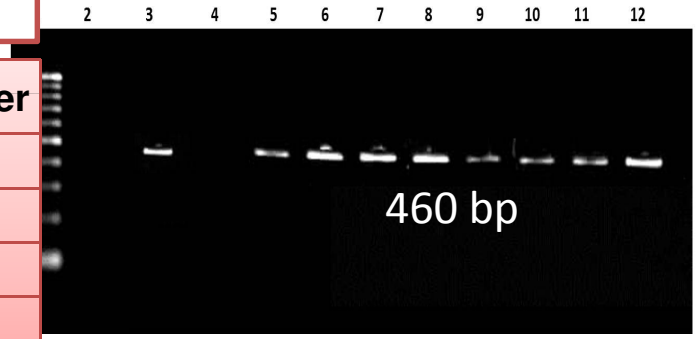




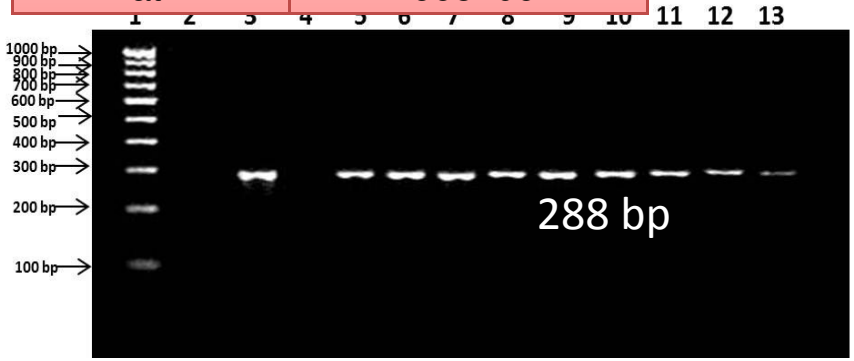
PCR confirmation of *tatD* gene.

GENES DEPOSITED IN GENBANK - NCBI

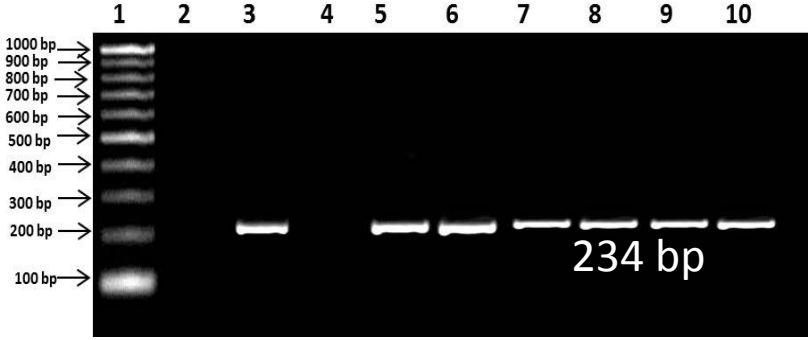
Genes	Accession Number
Lipase	KJ684062
ICOM	KJ577137
Lux R	KJ684060
Side	KC751544
TatD	KJ684061



PCR confirmation of *siderophore* gene.



PCR confirmation of *luxR* gene



PCR confirmation of *lipase* gene

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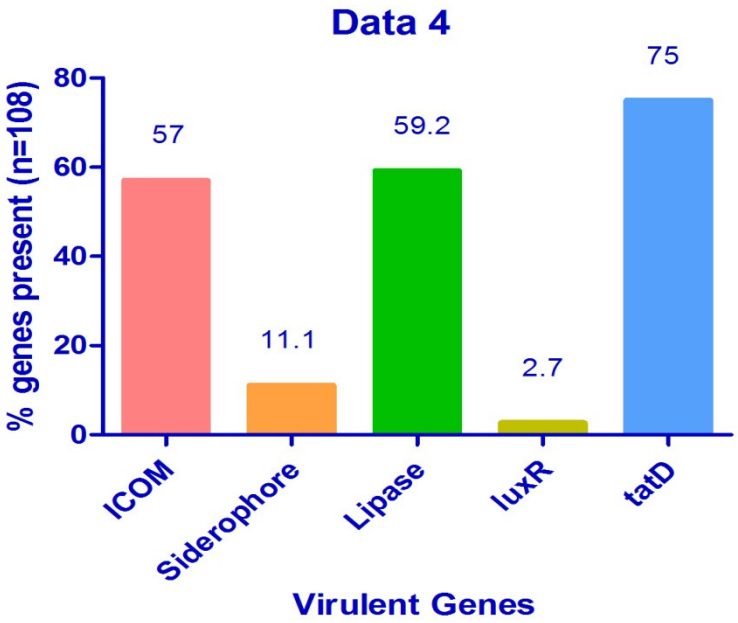
Virulent gene profile in *S.maltophilia* isolates

	ICOM	SID	LUX R	LIPASE	TOX A	PILI Z	TAT D
Blood (n = 39)	23(59)	4 (10.3)	1 (2.6)	24 (61.5)	0.0	0.0	30 (76.9)
CSF(n = 6)	6 (100)	0.0	0.0	3 (50)	0.0	0.0	3 (50)
Sputum (n = 5)	2 (40)	0.0	0.0	2 (40)	0.0	0.0	4 (80)
Tracheal Aspirate (n = 42)	23 (54.8)	7 (16.7)	2 (4.8)	3 (54.8)	0.0	0.0	28 (66.7)
Wound Swabs (n = 13)	6 (46.2)	1 (7.7)	0.0	9 (69.2)	0.0	0.0	13(100)
Urine (n = 3)	2(66.7)	0.0	0.0	3 (100)	0.0	0.0	3(100)



- Iron essential for metabolism.
- Lipase - correlated to pulmonary infection.
- *DNase* evades host immune response.

59.2% Isolates (n = 108) has Lipase.

- Hydrolyzes Lipid rich pulmonary tissues
- Triggers Inflammatory response

[Lanon *et al.* . 1992]

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METHODOLOGY(Cont.)

➢ Study3: *C.elegans* as an *In vivo* model of infection

C.elegans culture

↓

Age Synchronization

↓

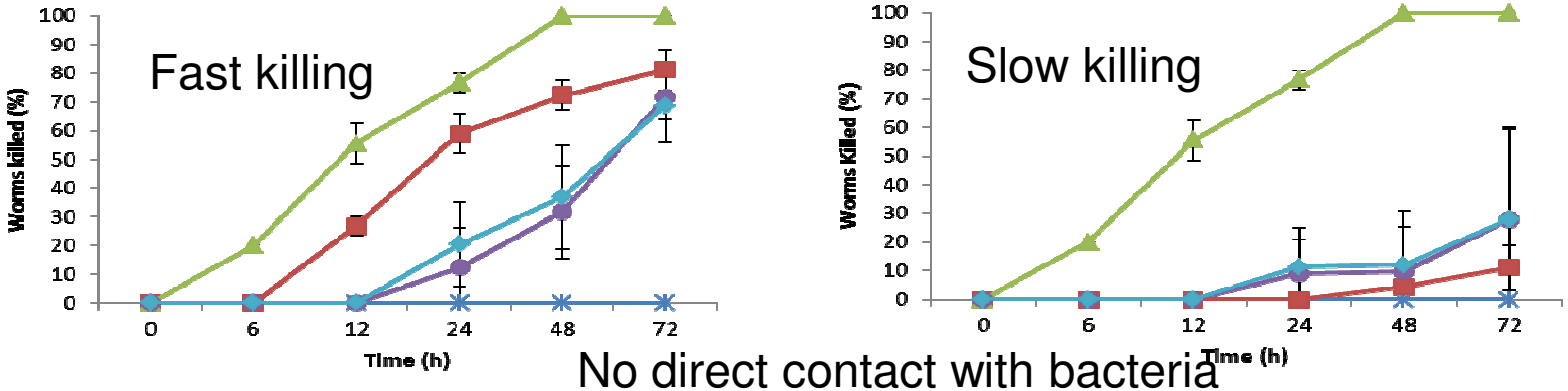
L4 stage larvae

↓

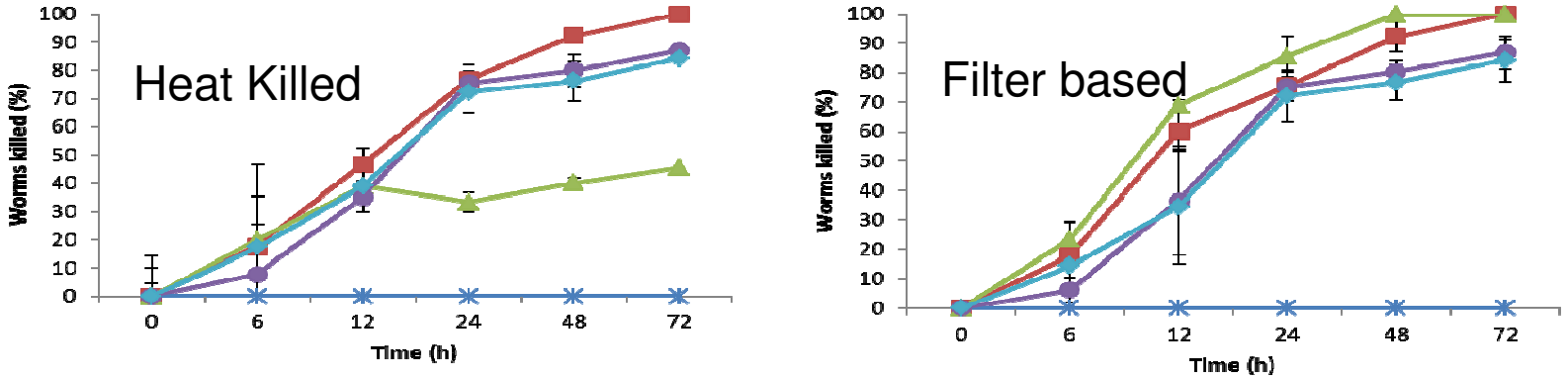
Toxicity Assay

RESULTS (Cont.)

Direct contact with bacteria

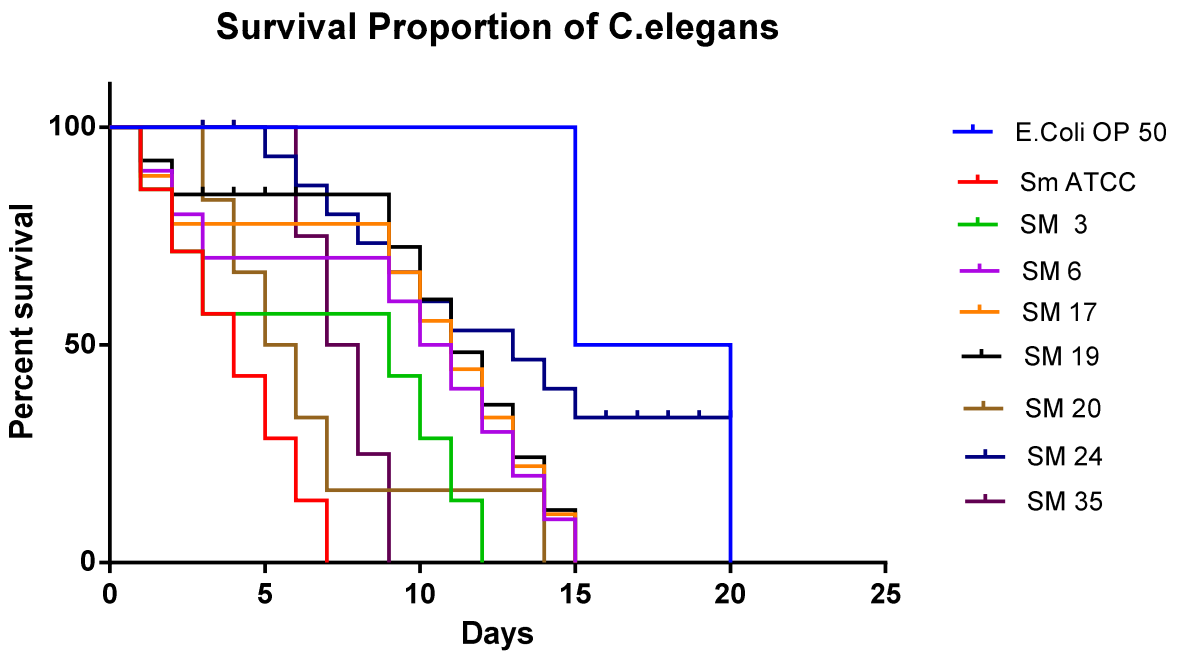


No direct contact with bacteria



Different methods employed in *C. elegans* killing. *C. elegans* killing assay using: (a) the fast killing method, (b) slow killing method, (c) heat-killed method and (d) filter-based method. Vertical bar represents SD. Experiments were conducted in triplicate. *, *E. coli* OP50 strain; ■, *S. maltophilia* ATCC 13637; ▲, *P. aeruginosa* ATCC 27853; ●, invasive strains; ♦, noninvasive strains

RESULTS (Cont.)



Survival curve analysis of *C.elegans* using graphpad prism software version 6.

- **Clinical isolates of *S.maltophilia* are detrimental**
- **Different methods of infecting the *C.elegans* with test bacteria – Different Time point.**
- **Filter based and Heat killed method – complete killing of *C.elegans* at 24hr.**
- **Clinical isolates of *S.maltophilia* effectively kills the nematodes – Filter based and Heat killed compared to fast and slow killing**

JMM Correspondence

Stenotrophomonas maltophilia: pathogenesis model using *Caenorhabditis elegans*

Stenotrophomonas maltophilia plays an important role as an opportunistic pathogen in immunocompromised individuals. Despite its clinical implications, information regarding its pathogenicity remains unclear. Various methods have been employed to demonstrate that this bacterium is pathogenic. However, the debate as to whether *S. maltophilia* is a true pathogen or a colonizer still continues, as effective killing was not seen in earlier experiments with different animal models of infection (Denton and Kerr, 1998; Adamak *et al.*, 2011; Pompilio *et al.*, 2011). A study using a murine lung infection model illustrated that different strains of mice exhibited different outcomes after *S. maltophilia* infection (Rouf *et al.*, 2011). Strains such as A/J and DBA/2 were permissive for clinical isolates of *S. maltophilia* and showed higher levels of pro-inflammatory

elegans, especially the mouth, by producing biofilm (Darby *et al.*, 2002; Joshua *et al.*, 2003). *Burkholderia* species tend to kill *C. elegans* either by infection or intoxication, or even by both methods (Darby, 2005).

Sixty-nine isolates of *S. maltophilia* obtained from various clinical sources such as tracheal aspirate, urine and pus were investigated. All isolates previously identified to the species level by phenotypic methods were reconfirmed genotypically as *S. maltophilia* by species-specific PCR using primers (SM1 5′-CAGCCTGCGAAAAGTA-3′ and 5′-SM4 TTAAGCTTGCCACGAACAG-3′) as described by Whitby *et al.* (2000). These clinical strains were further classified as invasive or non-invasive based on the anatomical site of isolation. Invasive strains included in the study were those that were isolated from sterile sites such as

performing a timed egg-laying experiment using fluorodeoxyuridine treatment of gravid adults, hatching of the eggs overnight in M9 minimal medium and plating first larval stage worms onto lawns of *E. coli* on NGM plates (Sutphin & Kaeberlein, 2009). Synchronized worms were grown to the fourth larval stage or young adult stage at 25 °C for use in the killing assays.

The *in vivo* killing efficiency of *S. maltophilia* was evaluated by four different methods: a classical fast killing assay, a fast killing assay using a nitrocellulose filter, a slow killing assay and a virulence assay using heat-killed strains. The classical fast killing assay was conducted according to the method of Mahajan-Miklos *et al.* (1999) where 50 µl of the bacterial test culture grown overnight in LB broth were spread on NGM plates containing 0.15 M

CONCLUSION

Final Conclusion

- From this study we conclude that *S.maltophilia* is a serious nosocomial pathogen due to the facts that they harbour virulent factors such as the extracellular enzymes and gene products that have deleterious effect.

- Lethal to nematodes makes this bacterium a potent nosocomial pathogen with high virulence potential.

ACKNOWLEDGEMENTS

Research Grants

Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for research facilities
Ministry of Higher education through Fundamental research Grant Scheme
Ministry od Science, Technology and Innovations through Escience

Collaborators

Professor Alex van Belkum
(Erasmus MC, The Netherlands, bioMérieux, France)
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(Creighton University, Omaha, Nebraska, USA)

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Hospital Kuala Lumpur


Thank You