

Title: Cross-reaction with *Stenotrophomonas* and *Xanthomonas* species in a 23S rDNA-directed PCR for the detection of *S. maltophilia*

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Stenotrophomonas maltophilia is an opportunistic pathogen that occupies a variety of niches both inside and outside hospitals, mostly in moist environments (1). Whitby *et al.* recently developed species-specific PCR (SS-PCR) primers directed at a region of the *S. maltophilia* 23S rRNA gene to overcome problems with definitive identification of the species in the clinical laboratory (5). Several pulmonary pathogens including *Alcaligenes xylosoxidans*, *Burkholderia* sp., *Elizabethkingia meningosepticum*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Proteus mirabilis*, *Pseudomonas* sp. and *Ralstonia* sp. were tested in this (5) and other (3) studies. In addition, environmental strains of *E. meningosepticum*, *Pseudomonas* sp., *Sphingomonas paucimobilis* and *Wautersia paucula* have been tested and no cross-reactivity was reported (2). In the present study, we examined cross-reactions with other *Stenotrophomonas* species and closely related genera occupying similar environmental niches.

Crude bacterial DNA extracts from *B. cepacia* genomovar I ATCC 25416, *P. aeruginosa* NCTC 10662, *S. acidaminiphila* ATCC 700916^T, *S. maltophilia* ATCC 13637^T, *S. nitritireducens* ATCC BAA-12^T, *S. rhizophila* ATCC BAA-473^T, *Xanthomonas axonopodis* pv. *citri* DAR 65864, and *Xanthomonas campestris* pv. *campestris* DAR 65808 (*Xanthomonas* strains were provided by the Australian Collection of Plant Pathogenic Bacteria, Orange, Australia), were prepared as described elsewhere (2). The SS-PCR protocol described by Whitby *et al.* (5) was used with modifications. The reaction mixture (50 µl) contained 1 × reaction buffer II (Applied Biosystems, USA), 3 mM MgCl₂, 200 µM of each deoxyribonucleotide triphosphate, 1.25U AmpliTaq Gold® polymerase (Applied Biosystems), 1 µM of each primer (SM1 and SM4) and 5 µl of DNA extract. Amplifications were performed

on a Gene Amp® PCR System 2700 thermocycler (Applied Biosystems) as follows: 95°C for 10 min, then 45 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 60 s, with final extension 72°C for 7 min. A product of approximately 531-bp was expected.

All *Stenotrophomonas* and *Xanthomonas* species tested gave a positive reaction in the *S. maltophilia* SS-PCR. *P. aeruginosa* NCTC 10662 and *B. cepacia* ATCC 25416 were PCR-negative; clinical isolates of *P. aeruginosa* and *B. cepacia* genomovar I were PCR-negative elsewhere (5). Results were reproducible over two or more replicates. Sequencing of products was performed (ABI PRISM 3100 Avant Genetic Analyzer, Applied Biosystems) and the *S. acidaminiphila* ATCC 700916^T, *S. nitritireducens* ATCC BAA-12^T, *S. rhizophila* ATCC BAA-473^T, *Xanthomonas axonopodis* pv. *citri* DAR 65864, and *Xanthomonas campestris* pv. *campestris* DAR 65808 sequences have the GenBank accession numbers EU878276-EU878280. All were highly homologous to the 23S rRNA partial gene sequence of *S. maltophilia* ATCC 13637, GenBank accession number AF273255 (5) (Table 1). The partial nucleotide sequence of the product from ATCC 13637 (481 bp) was 99% homologous to AF273255, with a single polymorphism (A567G).

The *S. maltophilia* 23S target sequence from GenBank sequence AF273255 (nucleotide positions 62 to 593) was used to query the GenBank database to identify other possible cross-reactivity of the PCR. *S. maltophilia* K279a genome (AM743169) and 23S rRNA genes from three other *S. maltophilia* had 97-99% identity to the target sequence (532 nucleotides) (E value, 0.0). The next highest homology (93% identity; 99% query coverage; E value, 0.0) was in 23S rDNA from

complete genome sequences of *X. axonopodis* pv. *citri* (1 strain), *X. campestris* pv. *campestris* (3), *X. campestris* pv. *vesicatoria* (1), and *X. oryzae* pv. *oryzae* (3). A single mismatch (A:C; primer:template) exists at the 3'-terminus of the forward primer (SM1) amongst *Xanthomonas* spp., while this mismatch and a second internal mismatch with the reverse primer (SM4) (T:G) exists in *Xylella fastidiosa* (23S rDNA from complete genome sequences of four strains; 89% identity; 99% query coverage; E value, 0.0) (Figure 1). Single internal mismatches and single A:C mismatches at the 3'-terminal nucleotide of a primer may not reduce amplification efficiency (4). Though 23S rRNA gene sequences from *P. aeruginosa*, *P. fluorescens* and *P. stutzeri* (PCR-negative species (5)) were of the next highest identity to the query sequence (83-85% identity; 99% query coverage; E value, 2e-163 to 6e-158), they had more significant mismatches in their primer binding regions.

S. maltophilia is the only *Stenotrophomonas* species to cause infection in humans. With the potential for use of *Stenotrophomonas* species as biological control agents (1, 6), it is important to be able to distinguish between *S. maltophilia* and other apparently less harmful *Stenotrophomonas* species sharing the same habitat and possessing similar biological control capacity. Molecular methods are increasingly used to identify or confirm the identity of bacterial isolates. Our findings emphasize the need to thoroughly evaluate SS-PCR methods using related species, particularly when such species may share an ecological niche.

References

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TABLE 1. Sequence similarity of SS-PCR products of *Stenotrophomonas* and *Xanthomonas* spp. to *S. maltophilia* ATCC 13637 23S rRNA partial gene sequence (GenBank accession number AF273255 (5)).

Strain	Sequence length ^a	Similarity to AF273255 ^b		
		Query coverage	E value	Maximum identity
<i>S. maltophilia</i> ATCC 13637	481 bp	100%	0.0	99%
<i>S. acidaminiphila</i> ATCC 700916	483 bp	100%	0.0	95%
<i>S. rhizophila</i> ATCC BAA-473	423 bp	100%	0.0	95%
<i>S. nitritireducens</i> ATCC BAA-12	486 bp	100%	0.0	93%
<i>Xanthomonas axonopodis</i> pv. <i>citri</i> DAR 65864	257 bp	100%	7e-102	92%
<i>Xanthomonas campestris</i> pv. <i>campestris</i> DAR 65808	133 bp	100%	6e-42	89%

^a Sequence length is of edited sequence confirmed by forward and reverse sequencing; primer and ambiguous terminal sequences were not included.

^b Determined by BLASTn search of GenBank database (June 2008)

A

SM1 (5'-3')	1	CAGCCTGCGAAAAGTA	16
S. maltophilia (AF273255)	62	77
S. maltophilia (AM743169)	418538	418553
S. maltophilia (AF175765)	62	77
S. maltophilia (AF175763)	62	77
S. maltophilia (AF175764)	62	77
Xanthomonas oryzae pv. oryzae (AP008229)	522971G	522985
Xanthomonas axonopodis pv. citri (AE012038)	12186G	12172
Xanthomonas oryzae pv. oryzae (AE013598)	307830G	307844
Xanthomonas oryzae pv. oryzae (CP000967)	4860844G	4860830
Xanthomonas campestris pv. vesicatoria (AM039952)	4604008G	4603994
Xanthomonas campestris pv. campestris (AM920689)	4951496G	4951482
Xanthomonas campestris pv. campestris (AE012504)	12644G	12630
Xanthomonas campestris pv. campestris (CP000050)	4635402G	4635388
Xylella fastidiosa (CP000941)	67823G	67837
Xylella fastidiosa (CP001011)	173127G	173141
Xylella fastidiosa (AE003849)	68630G	68644
Xylella fastidiosa (AE009442)	68112G	68126
Pseudomonas aeruginosa (CP000438)	735155CT	735169
Pseudomonas aeruginosa (AE004091)	724165CT	724179
Pseudomonas aeruginosa (AY116913)	2380CT	2366
Pseudomonas aeruginosa (Y00432)	63CT	77
Pseudomonas aeruginosa (CP000744)	809153T...CT	809167
Pseudomonas stutzeri (CP000304)	1799181CT	1799195
Pseudomonas fluorescens (AF134704)	2577CT	2591

B

SM4 (reverse complement)	514	CTGTTC-GTGGCAAGCTTAA	532
S. maltophilia (AF273255)	575-	593
S. maltophilia (AM743169)	419051-	419069
S. maltophilia (AF175765)	576-	594
S. maltophilia (AF175763)	572-	590
S. maltophilia (AF175764)	579-	597
Xanthomonas oryzae pv. oryzae (AP008229)	523481-	523499
Xanthomonas axonopodis pv. citri (AE012038)	11676-	11658
Xanthomonas oryzae pv. oryzae (AE013598)	308340-	308358
Xanthomonas oryzae pv. oryzae (CP000967)	4860334-	4860316
Xanthomonas campestris pv. vesicatoria (AM039952)	4603498-	4603480
Xanthomonas campestris pv. campestris (AM920689)	4950986-	4950968
Xanthomonas campestris pv. campestris (AE012504)	12134-	12116
Xanthomonas campestris pv. campestris (CP000050)	4634892-	4634874
Xylella fastidiosa (CP000941)	68333-.....G.....	68351
Xylella fastidiosa (CP001011)	173637-.....G.....	173655
Xylella fastidiosa (AE003849)	69140-.....G.....	69158
Xylella fastidiosa (AE009442)	68622-.....G.....	68640
Pseudomonas aeruginosa (CP000438)	735673	-A...A.....	735691
Pseudomonas aeruginosa (AE004091)	724683	-A...A.....	724701
Pseudomonas aeruginosa (AY116913)	1862	-A...A.....	1844
Pseudomonas aeruginosa (Y00432)	582	-A...A.....	600
Pseudomonas aeruginosa (CP000744)	809671	-A...A.....G.....	809689
Pseudomonas stutzeri (CP000304)	1799700	T.T.CA-.....G.....	1799718
Pseudomonas fluorescens (AF134704)	3096	T.T.CA-.....G.....	3114

FIGURE 1. Alignment of the SM1 (A) and SM4 (B) primer sequences with high scoring sequences from a BLASTn query (June 2008) of the AF273255 SS-PCR target sequence. Sequences known to be from the same strain were not included. Dots indicate identity with the primer sequence.