# Title

Whole-genome sequencing identifies emergence of a quinolone resistance mutation in a case of *Stenotrophomonas maltophilia* bacteremia

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## Keywords

Whole genome sequencing, *Stenotrophomonas maltophilia*, resistance, quinolone, antibiotic

## Running title

Emergence of quinolone resistance in *S. maltophilia*

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# Abstract

Whole genome sequences for *Stenotrophomonas maltophilia* serial isolates from a bacteremic patient before and after development of levofloxacin resistance were assembled *de novo* and differed by one single-nucleotide variant in *smeT*, a repressor for multidrug efflux operon *smeDEF.* Along with sequenced isolates from five contemporaneous cases, they displayed considerable diversity compared against all published complete genomes. Whole genome sequencing and complete assembly can conclusively identify resistance mechanisms emerging in *S. maltophilia* strains during clinical therapy.

*Stenotrophomonas maltophilia* is an aerobic, non-fermenting, and motile Gram-negative bacterium that is increasingly recognized as a cause of hospital-acquired infections with crude mortality rates of 14–69% in cases of bacteremia \autocite{Brooke2012}. Treatment of *S. maltophilia* infections is challenging due to the pathogen’s intrinsic resistance to many antibiotic classes via drug efflux pumps, beta-lactamase production, and decreased membrane permeability \autocite{Brooke2012}. Resistance phenotypes are known to change during the course of treatment, which complicates interpretation of automated drug susceptibility testing (DST) results \autocite{Garrison1996}. A mutant strain of *S. maltophilia* with emerging resistance to tetracycline, chloramphenicol, and quinolones was previously characterized following *in vitro* tetracycline selection \autocite{Alonso1997,Sánchez2002}. However, little is known about the genetic and molecular mechanisms underlying acquired resistance in the clinical setting—particularly for quinolones, where in contrast to other Gram-negatives, the quinolone-resistance determining region (QRDR) of topoisomerase genes is often unaltered \autocite{Valdezate2005}. In this report, we describe the first reported use of whole genome sequencing (WGS) in serial clinical isolates to definitively identify an acquired quinolone resistance mutation in *S. maltophilia*.

WGS was performed for the initial and subsequent *S. maltophilia*blood culture isolates from a patient where acquired quinolone resistance was observed (Patient 1) and five other patients (Patients 2-6) selected from a two-month period in 2013 at The Mount Sinai Hospital. Patient 1 was a 56 year-old man requiring urgent placement of an intrahepatic shunt for complications of a Whipple procedure, which was followed by several episodes of bacteremia that were treated with multiple courses of antimicrobials, including levofloxacin. Two months later, another bacteremia developed, and blood cultures intermittently grew *S. maltophilia*despite antimicrobial therapy. Automated DST by Vitek2**®**(bioMérieux, Marcy-l’Etoile, France) showed that the first *S. maltophilia*isolate acquired (ISMMS2) was susceptible to fluoroquinolones and trimethoprim/sulfamethoxazole (SXT). After treatment with ciprofloxacin the bacteremia initially cleared, but blood cultures nine days later again grew *S. maltophilia*(isolated as ISMMS2R), now resistant to fluoroquinolones while still susceptible to SXT. Ciprofloxacin was stopped and intravenous SXT was given; subsequent cultures did not grow *S. maltophilia.*

Standard culturing and susceptibility testing for levofloxacin and SXT were performed by automated microbroth dilution with Vitek2**®**. Antimicrobial sensitivities were reported and interpreted according to the 2015 CLSI guidelines for *S. maltophilia* \autocite{Clinical and Laboratory Standards Institute2015}*.* Isolates were then stocked and frozen at -80°C. Levofloxacin and SXT susceptibilities for all isolates in this study were later confirmed by Etest (bioMérieux) at 24 hours. To prepare for sequencing, isolates were grown from single colonies in tryptic soy broth, and DNA extraction was performed as previously described \autocite{Altman2014}.

Sequencing was performed on the PacBio RSII platform (Pacific Biosciences, Menlo Park, CA) and reads were assembled *de* novo using PacBio’s Hierarchical Genome Assembly Process (version 3) \autocite{Chin2013}. Pairwise comparison and variant calling were performed with MUMmer 3.23 \autocite{Delcher2003}. Mugsy 2.2 \autocite{Angiuoli2011} was used to align whole genome sequences for phylogenetic reconstruction with RAxML-8.0.2 \autocite{Stamatakis2014}, and rearrangements were visualized with Mauve 2.4.0 \autocite{Darling2004}. Additional details, including Sanger validation methods and sequence accession numbers, are in Supplementary Methods.

Two complete whole genome sequences were derived from Patient 1’s isolates before and after the change in levofloxacin MIC and compared to whole genome sequences of five control *S. maltophilia* isolates (Patients 2-6). All sequences were *de novo* assembled, i.e., without regard to reference assemblies. Table 1summarizes the relative dates of collection, antimicrobial susceptibility results, and assembly statistics.

Assembled genome sequences for Patient 1’s isolates before (ISMMS2) and after (ISMMS2R) observation of levofloxacin resistance were compared directly and were identical except for one single-nucleotide variant (SNV) and five one-base indels. Sanger sequencing confirmed the presence of the SNV, but identified the indels as homopolymer assembly errors. Coding domain sequence predictions for the surrounding locus (Figure 1A) revealed that the SNV was inside *smeT*, a *tetR*-like repressor upstream of the structural operon for the *smeDEF* genes, which encode a multidrug efflux pump. The SNV is an A>T substitution at position 497 of *smeT* causing a nonsynonymous Leu-166→Gln mutation.

The same nonsynonymous mutation has been previously observed in an *in vitro* strain of *S. maltophilia*, D457R, created by selecting single-step tetracycline-resistant mutants from the antibiotic-susceptible clinical strain D457 \autocite{Alonso1997,Sánchez2002}. The mutation is in the eighth α-helix of the *smeT* protein \autocite{Hernández2009}, which homodimerizes to repress transcription of the *smeDEF* operon \autocite{Hernández2009,Sánchez2002}. Although the mutation is not in the DNA-binding region, it has been shown to disable the repressor activity of *SmeT* \autocite{Sánchez2002}, leading to upregulation of *SmeDEF* and conferring an MDR phenotype \autocite{Alonso2001}.

Figure 1B shows an amino-acid sequence alignment comparing *SmeT* in D457 and D457R to aligned sequences from our seven isolates. Notably, while none of the remaining isolates shared the same Leu-166→Gln (c.497A>T) mutation, another isolate resistant to levofloxacin, ISMMS4, displayed a C>T mutation at position 388 of *smeT* that creates a premature stop codon that likely disrupts *smeT* function (Figure 1A and 1B).

The QRDR are loci within genes encoding topoisomerase II and IV subunits known for mutations that confer quinolone resistance in Gram-negative bacteria, although they appear to play a secondary role to efflux systems for resistance emerging during treatment of *S. maltophilia* infection \autocite{Valdezate2005}. An amino-acid sequence alignment of the *gyrA*, *gyrB*, and *parC* genes of our seven isolates and the reference clinical isolates D457 and K279a revealed no differences in the QRDR. Some variants were observed within the QRDR of *parE* (Supplementary Figure 1), all of which were consistent with past observations in clinical isolates \autocite{Valdezate2002} except for an Ile-599→Val variant observed in three of our isolates and the D457 reference sequence.

Significant genomic diversity was observed among the *S. maltophilia* isolates from all six patients. Supplementary Figure 2 shows a maximum-likelihood phylogeny with branch lengths scaled to SNV distances. Our isolates distribute widely among all four reference assemblies for complete *S. maltophilia* genomes in GenBank. The distances of tens of thousands of SNVs seen in our phylogeny suggest that the natural diversity of pathogenic *S. maltophilia* is greater than that captured by the current set of reference assemblies, even within a single hospital setting.

Recombination is not an obvious source of diversity in our *S. maltophilia* isolates. Supplementary Figure 3 depicts whole genome alignments between the four clinical isolates where assembly produced a circularized chromosome and the four GenBank references, showing small areas of non-homology separating large regions of significant homology occurring generally in the same order for each genome. ISMMS2 and ISMMS2R are structurally identical, as expected for serial isolates, while recombination events among other strains are limited to small 1-2kb segments. Epigenetics motif analysis also suggests that the isolates are not related.Supplementary Table 1 shows different motifs in isolates from separate patients, implicating differences in type II & III restriction modification systems between the isolates more likely to be caused by inter-strain/species horizontal transfer of methyltransferases than by intra-strain mutations \autocite{Srikhanta2010}. Together, this demonstrates that transmission did not occur among these six cases and that whole-genome sequencing can comprehensively capture genetic distances and structural variants among diverse clinical isolates of *S. maltophilia*.

This is the first report of WGS on serial isolates to characterize the emergence of a resistance mutation in *S. maltophilia* during antibiotic treatment of an active infection. In contrast to studies sequencing highly resistant strains of *S. maltophilia* to reveal various intrinsic and acquired antibiotic resistance genes \autocite{Crossman2008,Zhao2015}, where it remains difficult to assess their relative importance to the phenotype, performing WGS on serial isolates as resistance emerges *in vivo* allows the causative mutation(s) to be captured. In our patient, the mutation was a SNV that replicates a variant observed in an *in vitro* model strain created to study the MDR phenotype in 1997 \autocite{Alonso1997}. Using WGS and susceptibility testing, we can confirm that this SNV was the only variant to emerge and that it was sufficient to confer quinolone resistance in a clinical case. This underscores the need for clinicians to consider repeating DST during monotherapy if clinical signs suggest therapy failure.

*smeT* appears to play a central role in adaptive resistance to quinolones and other antibiotics effluxed by *smeDEF*, like tetracycline, chloramphenicol, erythromycin, and aminoglycosides. Since any mutation that inactivates this protein would be able to derepress *smeDEF* and confer resistance, *smeT* is under intense selective pressure in the presence of these drugs. In this study, we observed not only a deleterious SNV in the strain that displayed resistance (ISMMS2R), but a premature stop codon in *smeT* in a strain that was already resistant at first isolation (ISMMS4). Certain nucleotide positions appear to be under greater selective pressure than others, as evidenced by our observation of the same mutation that occurred in D457R, and a relative paucity of nonsynonymous coding mutations in *smeT* observed among clinical *smeT* isolates \autocite{Sanchez2004}. Since sustained overexpression of *smeDEF* is physiologically unfavorable \autocite{Alonso2004}, it is possible that pathogenic strains of *S. maltophilia* rely on natural diversity of mutations in the *smeT* locus to activate or deactivate *smeDEF* expression, allowing for rapid adaptation to antibiotic stress, though further study is needed.

Since resistance from a single SNV emerged during a short course of ciprofloxacin, clinicians should be cautioned about using quinolone monotherapy for *S. maltophilia* bacteremia, as highlighted in recent retrospective studies \autocite{Cho2014,Wang2014}. The wide variety of MDR phenotypes and unreliability of DST results has created uncertainty about appropriate treatment for *S. maltophilia*, but SXT remains the most common choice for monotherapy \autocite{Brooke2012,Cho2014,Wang2014}. SXT resistance in *S. maltophilia* is not known to be caused by efflux systems but has been linked to Class 1 integrons and IS*CR* elements \autocite{Brooke2012}. This suggests that spontaneous resistance is less likely to emerge with SXT monotherapy, although a clinical trial comparing the two antibiotics is warranted \autocite{Cho2014,Wang2014}.

In conclusion, characterizing the full extent of genetic alterations that *S. maltophilia* utilizes to develop antibiotic resistance *in vivo* and improving genomic surveillance of clinical strains will help refine antibiotic selection criteria available to clinicians. This study furthermore highlights the utility of WGS for profiling the precise mutations underlying emerging antibiotic resistance in clinical cases of bacteremia.

# Acknowledgement

This work was supported by the Icahn Institute for Genomics and Multiscale Biology at Mount Sinai, and also in part by the NIAID-supported NRSA Institutional Research Training Grant (5 T32 AI 7647-13) for Global Health Research (DRA), and in part by the resources and expertise of the Department of Scientific Computing at the Icahn School of Medicine at Mount Sinai. We thank Timothy O’Donnell, Tavi Nathanson, Jose Clemente, Flora Samaroo, Angelo Rendo, and members of the clinical microbiology laboratory at Mount Sinai for their contributions.

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# Tables

**Table 1.** Sequenced clinical isolates and their antimicrobial susceptibilities.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Patient | Time of collection[[4]](#footnote-4) (days) | Isolate name | Levo susceptibility (MIC, mg/L) | | SXT susceptibility (MIC, mg/L) | | Assembly quality | Depth of coverage |
| Vitek2 | Etest | Vitek2 | Etest |
| 1 | 0 | ISMMS2 | S (0.5)[[5]](#footnote-5) | S (1) | S (<20) | S (0.19) | 1 circular 4.51Mbp chromosome | 160x |
| 1 | +10 | ISMMS2R | R (>32)b | R (16) | S (1) | S (0.38) | 1 circular 4.51Mbp chromosome | 403x |
| 2 | -26 | ISMMS3 | S (0.25) | S (0.38) | U  (80, <20)[[6]](#footnote-6) | S (0.75) | 1 circular 4.80Mbp chromosome | 153x |
| 3 | +14 | ISMMS4 | R (>8) | R (>12) | U  (0.5, 80)c | S (0.75) | 3 contigs (4.73Mbp, 6.5kbp, 11.2kbp) | 303x |
| 4 | -32 | ISMMS5 | S (1) | S (1) | S (<20) | S (0.25) | 18 contigs | 270x |
| 5 | 0 | ISMMS6 | S (<0.12) | S (0.125) | S (<20) | S (1.5) | 10 contigs | 262x |
| 6 | +2 | ISMMS7 | S (1) | S (0.75) | S (<20) | S (1.5) | 1 circular 4.69Mbp chromosome, 1 additional 17.7kbp contig | 318x |

Abbreviations: Levo, levofloxacin; SXT, trimethoprim/sulfamethoxazole; S, susceptible; R, resistant; U, undetermined; Mbp, million base pairs; kbp, thousand base pairs.

# Figure Legends

**Figure 1.** Single-nucleotide variants (SNVs) observed in quinolone-resistant *S. maltophilia* clinical isolates. ***A***,assembled circular chromosome for ISMMS2, including predicted coding domain sequence (CDS) and noncoding RNA (ncRNA) features drawn with ChromoZoom \autocite{Pak2013}. Horizontal position corresponds to base pair location. The *smeDEF* operon is shown in the detail callout, which highlights both the *smeT* c.497T>A SNV that emerged in ISMMS2R and the aligned location of the *smeT* c.388C>T SNV (encoding a premature stop codon) in ISMMS4. ISMMS2 and ISMMS2R are serial isolates from a single patient before and after development of quinolone resistance, while ISMMS4 was quinolone-resistant at initial isolation from a different patient. ***B***, multiple sequence alignment of part of the predicted *smeT* product in each of the clinical isolates, the D457 reference assembly, and its quinolone resistant counterpart D457R. Predicted α-helices \autocite{Hernández2009} are labeled as grey bars below the sequence. Positions identical in all sequences are shaded with a dark gray background, equivalent substitutions are typeset in red, and non-equivalent substitutions are typeset in boldface black. The L166Q and Q130\* (\*, stop codon) polymorphisms are highlighted.

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4. Time of collection was defined in days relative to the date of collecting the initial *S. maltophilia* isolate in the case patient. [↑](#footnote-ref-4)
5. This is the change in levofloxacin susceptibility investigated in this study. [↑](#footnote-ref-5)
6. Inconsistent results were obtained in replicate. [↑](#footnote-ref-6)