Research

The complete genome, comparative and functional analysis of Stenotrophomonas maltophilia reveals an organism heavily shielded by drug resistance determinants
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Abstract

Background: Stenotrophomonas maltophilia is a nosocomial opportunistic pathogen of the Xanthomonadaceae. The organism has been isolated from both clinical and soil environments in addition to the sputum of cystic fibrosis patients and the immunocompromised. Whilst relatively distant phylogenetically, the closest sequenced relatives of S. maltophilia are the plant pathogenic xanthomonads.

Results: The genome of the bacteremia-associated isolate S. maltophilia K279a is 4,851,126 bp and of high G+C content. The sequence reveals an organism with a remarkable capacity for drug and heavy metal resistance. In addition to a number of genes conferring resistance to antimicrobial drugs of different classes via alternative mechanisms, nine resistance-nodulation-division (RND)-type putative antimicrobial efflux systems are present. Functional genomic analysis confirms a role in drug resistance for several of the novel RND efflux pumps. S. maltophilia possesses potentially mobile regions of DNA and encodes a number of pili and fimbriae likely to be involved in adhesion and biofilm formation that may also contribute to increased antimicrobial drug resistance.

Conclusion: The panoply of antimicrobial drug resistance genes and mobile genetic elements found suggests that the organism can act as a reservoir of antimicrobial drug resistance determinants in a clinical environment, which is an issue of considerable concern.

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Background
The rise of antimicrobial drug resistance in bacteria is one of the biggest threats to healthcare provision in the developed world. Few new antimicrobials are undergoing clinical trials, and almost none are effective against Gram-negative multi-drug resistant (MDR) pathogens [1]. A return to the pre-antibiotic era is a possibility, and for some infections is the current reality [2].

Antimicrobial resistance in historically common pathogens is usually either acquired on a mobile genetic element or results from a mutation [3]. However, some opportunistic pathogens are intrinsically resistant to the actions of a number of antimicrobial classes. These tend to be of environmental origin, and their intrinsic drug resistance determinants either provide resistance to antibiotics produced by competitors, or represent broad-spectrum methods for removing toxic compounds or waste products that, by chance, protect against antimicrobials [3,4]. It is known that established opportunistic infections are very difficult to treat due to the MDR nature of the causative bacteria [5].

The most common intrinsically MDR opportunistic pathogens are the non-fermenting Gram-negative bacilli typified by Pseudomonas aeruginosa. In this case, intrinsic resistance is due to a battery of efflux pumps, specific antibiotic hydrolyzing enzymes, and intrinsically low outer membrane permeability. When intrinsically MDR bacteria then acquire resistance to those few drugs that can kill them, the result is an isolate resistant to all clinically available antimicrobials. This pan-resistant phenotype is observed in P. aeruginosa isolates with increasing frequency [6].

S. maltophilia is the third most common nosocomial non-fermenting Gram-negative bacilli [7]. A recent study of intensive care patients in the USA found that 4.3% of almost 75,000 Gram-negative infections studied were caused by S. maltophilia [8]. Isolates are intrinsically resistant to β-lactams, aminoglycosides, macrolides, and many older quinolones [7].

S. maltophilia is found in soil and water, and routinely resides in showerheads and other moist places where it grows as biofilm. It is a truly opportunistic pathogen, and patient to patient spread has not been reported, though small outbreaks have been seen due to contaminated water sources [9]. Consistent with this, we find that isolates are generally genotypically and phenotypically diverse [10-12]. However, there is phylogenetic clustering, with about half of clinical isolates being very similar to each other, even across a wide geographical range. Members of this group, termed phylogenetic group A, may be better at causing infections than other S. maltophilia isolates [13]. The two most common diseases caused by S. maltophilia are bacteremia and pneumonia with infection being via an indwelling catheter or ventilator, respectively [9]. Respiratory tract colonization is seen in about a third of all cystic fibrosis (CF) patients; nevertheless, there is controversy as to whether this leads to a poorer clinical outcome or morbidity [14,15].

Bioinformatic and functional genomic analyses on the complete genome sequence emphasize factors with proven or potential contribution to antibiotic resistance, persistence and virulence. The findings reveal the remarkable capacity of S. maltophilia for multidrug resistance and environmental adaptability that underpins its importance as an emerging opportunistic nosocomial pathogen.

Results and discussion
Total genome overview
The sequenced isolate is from a typical presentation: an elderly male patient undergoing chemotherapy at the Bristol Oncology Unit, Bristol, UK in 1998 developed a bloodstream infection that did not respond to therapy with piperacillin/tazobactam, ceftazidime or imipenem. S. maltophilia K279a was cultured from a blood sample taken shortly before death [16]. K279a falls into phylogenetic group A, and has typical antimicrobial resistance properties [13,17,18]. Accordingly, it was thought suitable as a representative genome sequence strain.

The genome consists of a single circular chromosome; no plasmids were detected (Figure 1). The total size is 4,851,126 bp with a G+C content of 66.7% G+C. Four copies of the rRNA operon and 74 tRNAs are present. These data have been submitted to EMBL under accession number AM743169.

Drug resistance
In Gram-negative nosocomial pathogens, MDR is usually mediated by the over-production of resistance-nodulation-division (RND) type efflux pumps. These pumps tend to have broad substrate profiles, including organic solvents, disinfectants and antimicrobial drugs from a number of different classes. Cytoplasmic efflux is driven by dissipation of the proton motive force across the inner membrane. Two additional components are needed to remove substrates from the cell, forming a tripartite efflux pump complex that spans the envelope. A particular periplasm-spanning membrane-fusion protein (MFP) is usually specific to each RND efflux protein, and it is common to find the pair encoded as part of an operon. A third component, the outer membrane protein (OMP), can be encoded in the same operon, but there tend to be fewer different OMPs than RND/MFP pairs in a cell, meaning that the OMPs are often promiscuous [19].

The K279a sequence carries nine RND-type efflux pump genes that fall into the drug resistance type based on sequence homology. Homologues of two known S. maltophilia tripartite efflux pump operons are present, smeABC (Smlt4474-6) and smeDEF (Smlt4070-2), representing MFP, RND and OMP genes, respectively, in each case. SmeABC was first characterized in the clinical S. maltophilia isolate ULA511.
[20], which is phylogenetically closely related to K279a [21]. Disruption of smeAB in ULA-511, or in a hyper-resistant mutant background, had no effect on drug resistance. However, disruption of smeC reduced the minimum inhibitory concentration (MIC) of a variety of antimicrobials against ULA-511, so SmeC may act as an OMP in at least one functional tripartite antimicrobial efflux pump [20].

SmeDEF is over-produced in a hyper-resistant mutant of the clinical isolate S. maltophilia D457 [22], which is phylogenetically quite distinct to K279a [21]. SmeDEF over-expression causes hyper-resistance to fluoroquinolones, chloramphenicol and tetracycline in K279a [17]. Hyper-expression occurs either through loss-of function mutations in the locally encoded TetR-type transcriptional repressor, smeT [17,23].

**Figure 1**
Circular diagram of the main features of K279a. The circles show (outermost to innermost): 1. DNA coordinates (black); 2. color coded annotation (the CDSs are color coded according to function: blue = pathogenicity/adaptation; dark grey = essential metabolism; red = DNA replication/transcription/restriction-modification; green = transmembrane/outer membrane; cyan and magenta = degradation of large and small molecules, respectively; yellow = intermediary metabolism; light green = hypothetical; light blue = regulators; orange = conserved hypothetical; brown = pseudogenes; pink = transposons and phage); 3. laterally transferred regions (determined by Alien Hunter with a cut-off score of 15); 4. transposons and phage (pink); 5. pili and fimbriae (blue); 6. RND efflux transporters (green); 7. GC skew; 8. GC deviation.
or through undefined mutations, which may affect another regulator of the concentration of the activator [17,24]. Characteristics of the nine S. maltophilia RND efflux pumps are described in more detail in Table 1.

To determine involvement of the seven novel RND efflux pumps in intrinsic antimicrobial drug resistance in K279a, their genes were disrupted by suicide gene replacement to cause a significant intragenic deletion and frameshift mutation. MICs of a variety of antimicrobials were determined against the mutants in comparison to wild-type K279a. From this experiment, we conclude that SmeZ, SmeJ and SmeK are involved in intrinsic antimicrobial drug resistance in K279a (Table 2). A smeJK double mutant behaved identically to the individual mutants, leading to the conclusion that, as with their homologues mtdBC in Escherichia coli [25], their products cannot work separately. Disruption of smeZ markedly affects only aminoglycoside MICs; disruption of smeJ and/or smeK has a more general but subtle effect on resistance, lowering MICs of some aminoglycosides, fluoroquinolones and tetracyclines, but none dramatically.

Other known and putative antibiotic resistance genes in the genome specify resistance via a number of mechanisms to \( \beta \)-lactams, chloramphenicol, aminoglycosides, fluoroquinolones and macrolides (Table 3). Many of the resistance genes are located on small islands with no obvious mobile DNA features (determined by Alien Hunter [26]), and may not all be expressed. Experimentally determined antibiotic modifying enzymes produced by K279a are \( \beta \)-lactamases L1 and L2 specifying resistance to all clinically available \( \beta \)-lactams except the monobactams [27], and the aminoglycoside modifying enzymes APH 3’II and AAC 6’I that together confer resistance to all clinically available aminoglycosides except gentamicin [18]. It has been reported previously that the S. maltophilia L1 and L2 \( \beta \)-lactamases might be encoded on a large ‘plasmid-like element’, but this was not confirmed using pulse-field gel electrophoresis [27]. Given that there is no plasmid in S. maltophilia isolate K279a, and that L1 and L2 are not encoded on a region of the chromosome that resembles an integrated plasmid, it is likely that the result reported previously reflected chromosomal contamination of a plasmid preparation, giving a false PCR positive for the L1 and L2 genes.

### Table 1

Characteristics of Sme efflux transporters in S. maltophilia K279a

| Systematic ID | Name       | Known or putative regulation mechanism | Closest match to a known antimicrobial efflux protein |
|---------------|------------|---------------------------------------|-----------------------------------------------------|
| Smr4474-4476  | SmeABC     | Two component regulator (SmeSR, Smr4477-8) | S. maltophilia SmeABC [20]                          |
| Smr4070-4072  | SmeDEF     | Tet-R type (SmeT, Smr4073)             | S. maltophilia SmeDEF [17,22]                       |
| Smr1829-1833  | SmeVWX     | LysR type (Smr1827)                   | 51%, 56% and 48% amino acid identity, respectively, to P. aeruginosa MexEF-OprN [64] |
| Smr2201-2202  | SmeYZ      | Two component regulator (Smr2199-30)   | 44% and 59% amino acid identity, respectively, to AdeAB of A. baumannii [65] |
| Smr3170-3171  | SmeGH      | TetR type (Smr3169)                   | 39% and 49% amino acid identity, respectively, to AcrAB of M. morganii [66] |
| Smr3788-3787  | SmeMN      | ?                                     | <30% identity to other known antimicrobial efflux proteins |
| Smr3925-3924  | SmeOP      | TetR type (Smr3926)                   | <30% identity to other known antimicrobial efflux proteins |
| Smr4279/4281  | SmeIJK     | ?                                     | 41%, 50% and 44% amino acid identity, respectively, to MtdABC of E. coli [25] |

### Table 2

MICs of a variety of antimicrobials against S. maltophilia K279a and derivatives lacking specific functional RND efflux pump genes

|        | Gent | Kan | Ami | Tob | Ery | Chor | Mero | Imi | Azt | Ctz | Pip | Tet | Min | Trim | Sul | Cipro | Levo | Nor |
|--------|------|-----|-----|-----|-----|------|------|-----|-----|-----|-----|-----|-----|------|-----|-------|-----|-----|
| K279a  | 16   | 256 | 64  | 32  | >1,024 | 6   | 32  | 256 | 256 | 8   | 64  | 16  | 0.25 | 32  | 64   | 2   | 4    | 32  |
| smeJ   | 8    | 256 | 32  | 32  | >1,024 | 6   | 32  | 256 | 512 | 8   | 64  | 8   | 0.125 | 32  | 64   | 1   | 4    | 32  |
| smeK   | 8    | 256 | 32  | 32  | >1,024 | 6   | 32  | 256 | 512 | 8   | 64  | 8   | 0.125 | 32  | 64   | 1   | 4    | 32  |
| smeJK  | 8    | 256 | 32  | 32  | >1,024 | 6   | 32  | 256 | 512 | 8   | 64  | 8   | 0.125 | 32  | 64   | 1   | 4    | 32  |
| smeZ   | 1    | 128 | 32  | 32  | >1,024 | 6   | 32  | 256 | 512 | 8   | 64  | 16  | 0.25 | 32  | 64   | 2   | 4    | 32  |

Gent, gentamicin; Kan, kanamycin; Ami, amikacin; Tob, tobramycin; Ery, erythromycin; Chor, chloramphenicol; Mero, meropenem; Imi, imipenem; Azt, aztreonam; Ctz, ceftazidime; Pip, piperacillin; Tet, tetracycline; Min, minocycline; Trim, trimethoprim; Sul, sulphamethoxazole; Cipro, ciprofloxacin; Levo, levofloxacin; Nor, norfloxacin.

Entries in bold indicate changes of the MIC in the mutants compared to wild type K279a. The units used are in mg/L.
Genes encoding six other putative RND family tripartite efflux pumps are found in the K279a genome sequence. However, these are more closely related to cation/metal efflux pumps than to antimicrobial RND efflux pumps, and are designated SmmABC to TUV. K279a additionally encodes several alternative heavy metal resistance mechanisms that are associated with a complex mobile region of DNA. These include arsenic, mercury, and copper resistance. Alternative copper resistance genes are specified elsewhere in the genome. Heavy metal resistance (to cadmium via an efflux protein) has been described in S. maltophilia D457R [28].

DNA acquired by lateral gene transfer was identified using Alien Hunter [26]. Putative transposons, both conjugative and complex as well as insertion sequence (IS) elements were found in K279a. Throughout the genome there are seven intact copies of a single unique IS element related to ISXac3 of Xanthomonas campestris pv campestris (X. campestris) 8004, and two pseudogenic copies. Additional IS elements present in the sequence include ISHneg3/ISst11A-like and ISPsyg-like (Table 4). Intriguingly, a single putative streptomycin 3” phosphotransferase gene (Smlt2336) has inserted between genes clpS and clpA relative to the X. campestris genome. To one side of Smlt2336 is a set of 36 and 18 bp direct repeats, perhaps suggestive of a footprint of a mobile element that may have inserted then excised.

There is no evidence in K279a for a class one integron specifying sulfonamide resistance as has recently been seen in a number of S. maltophilia isolates [29], and K279a is sensitive to trimethoprim-sulphamethoxazole.

### Table 3
Putative and known antimicrobial drug and heavy metal resistance genes in the S. maltophilia K279a genome sequence

| Substrate      | Gene            | Putative gene product                         |
|----------------|-----------------|-----------------------------------------------|
| Aminoglycoside | Smlt0191        | Putative aminoglycoside phosphotransferase    |
| Aminoglycoside | Smlt1669        | Putative aminoglycoside 2’ N-acetyltransferase|
| Aminoglycoside | Smlt2120        | Known aminoglycoside 3’ phosphotransferase    |
| Streptomycin   | Smlt2336        | Putative streptomycin 3” phosphotransferase/  |
|                |                 | kinase                                        |
| Aminoglycoside | Smlt3615        | Known aminoglycoside 6’N acetyltransferase    |
| Spectinomycin  | Smlt2125/spcN   | Putative spectinomycin phosphotransferase     |
| Chloramphenicol| Smlt0620/cat    | Putative chloramphenicol acetyltransferase    |
| Fluoroquinolone| Smlt1071/qnrB   | Putative quinolone resistance protein         |
| Macrolides     | Smlt0032        | Putative MFS-type tripartite efflux transporter|
| Macrolides     | Smlt1337-9      | Putative ABC-type tripartite efflux transporter|
| Macrolides     | Smlt2642-3      | Putative ABC efflux transporter and MFP       |
| Multidrug      | Smlt528-30/SmeR3 | Putative MFS-type tripartite efflux transporter|
| Multidrug      | Smlt1830-31:33/SmeVWX | Putative RND-type tripartite efflux transporter|
| Multidrug      | Smlt2201-2/SmeYZ | Putative RND-type efflux protein and MFP      |
| Multidrug      | Smlt2796-8      | Multidrug/fusaric acid resistance protein     |
| Multidrug      | Smlt3170-1/SmeGH | Putative RND-type efflux protein and MFP      |
| Multidrug      | Smlt3787-78/SmeMN| Putative RND-type efflux protein and MFP      |
| Multidrug      | Smlt3924-25/SmeQP | Putative RND-type efflux protein and MFP      |
| Multidrug      | Smlt4072-74/SmeDEF | Known RND-type tripartite efflux protein      |
| Multidrug      | Smlt4279-81/SmeJKL | Putative RND-type tripartite efflux proteins  |
|                |                 | and MFP                                       |
| Multidrug      | Smlt4474-76/SmeABC | Known RND-type tripartite efflux protein      |
| Beta-lactams   | bla11           | Known Beta-lactamase - L1                     |
| Beta-lactams   | bla12           | Known Beta-lactamase - L2                     |
| Kasugamycin    | ksgA            | Putative kasugamycin resistance protein       |
| Organic solvents| otaA           | Organic solvent tolerance protein             |
| Peroxide       | ohrA            | Organic hydroperoxide                         |
| Mercury        | merRTPA         | Mercury resistance                            |
| Copper         | copLABMGCDF Smlt2445/Smlt2443 copL2A2B2 copCD | Copper resistance                            |
| Arsenic        | arsRCHR2C2B Smlt2420 | Arsenic resistance                            |
S. maltophilia harbor giant phage [30]; although potential prophages were identified in K279a, there is no evidence for giant lysogenic phage.

**Secretion systems and extracellular enzymes**

Type I, II (sec), IV and V (autotransporter) as well as the twin arginine secretion systems are present in the K279a genome sequence. Surprisingly, there are no type III secretion genes in K279a. Type III secretion components are related to the flagella apparatus [31]. The flagella apparatus of S. maltophilia is highly conserved with the X. campestris system and there is no evidence to suggest that these components could function in type III secretion. Secreted extracellular enzyme genes were found in the genome. K279a encodes non-hemolytic phospholipase C (plcN1, Smlt1755) as well as enzymes from the phospholipase D family. Phospholipase cleaves phospholipids to fatty acids and is implicated in virulence due to its ability to degrade cell membranes. There is evidence that phospholipases contribute towards virulence in Burkholderia pseudomallei [32]. Other extracellular enzymes, including DNase, gelatinase, hemolysin, lipases, proteinase K and proteases, have been characterized and implicated in disease in S. maltophilia [33]. The major extracellular protease of K279a, StmPr1 (Smlt0861), has also been implicated as a virulence determinant [34].

**Pili, fimbriae and adhesins**

S. maltophilia produces various pili/fimbriae that are implicated in adhesion and biofilm formation [35]. This type of aggregative behavior is likely to be associated with colonization of biotic and abiotic surfaces, evasion of the host immune response as well as increased drug resistance.

The Smf-1 fimbrial operon includes Smlt0706-Smlt0709. These 17 kDa subunit fimbriae mediate adherence, participate at early stages of biofilm formation [36] and can agglutinate red blood cells. Smf-1, seen as peritrichous semiflexible fimbriae of 5-7 nm under electron microscopy, are produced at 37°C but not 18°C. Two distinct loci, Smlt1508-12 and Smlt0732-6, comprise further sets of putative pili/fimbrial genes that include fimbrial subunit and chaperone/usher proteins. A TadE-like pili/fimbrial gene cluster is located at Smlt2867-Smlt2875. In Actinobacillus, bundled Flp pili are required for tight adherence and strongly attached biofilm on solid surfaces in vitro, which is likely to be required in oral cavity colonization and initiation of periodontal disease [37].

Type IV pili are implicated in adherence and autoaggregation in enteropathogenic E. coli. In some species they have been associated with twitching motility and biofilm formation (for example, the obligate plant pathogen Xylella fastidiosa and P. aeruginosa). Subunits and associated apparatus specifying

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**Table 4**

| Mobile region | Putative length, approx. (bp) | G+C content (%) | Putatively bounded by (repeat length, bp) | Major characteristics |
|---------------|-------------------------------|-----------------|------------------------------------------|----------------------|
| Potential conjugative transposon | 43,769 | 62.7 | 19 | Hypotheticals, lipoproteins and an efflux protein cargo |
| Potential complex transposon insertion | 97,538 | 61.9 | 18 | Efflux transporters, mercury, arsenic and copper resistance, co-integrate resolution and integrases. May be a multiple insertion* |
| Potential complex transposon insertion | 52,344 | 60.5 | 20 | Tra genes and adhesins, DNA repair, conserved and unique hypotheticals. May be a multiple insertion. Carries IS elements and Tn5044 similarity |
| ISXac3-like | 1,157 | 65.1 | ND | Seven intact copies and two pseudogenic copies |
| ISHne3/I/S1 I/A-like | 915 | 61.7 | ND | Eleven intact copies |
| ISpsi9 | 1,352 | 58.8 | ND | Four intact copies |
| Phage cluster 1 | 118,000 | 63.7 | ND | Putative pseudogenic phage. Putative IS insertion and tRNA located centrally |
| Phage cluster 2 | 37,992 | 63.2 | ND | Putative intact phage |

Mobile regions were determined using an approach that combined using the Alien Hunter program, repeat analysis and by-eye comparisons between K279a and the X. campestris genome sequence by ACT analysis. *CDS (Smlt2465) in this feature shares 72.5% identity with a previously characterized transposase, Tn5044, from a Xanthomonas spp. isolated from a heavy metal mine in Russia [67]. Stenotrophomonas was at that time classified as Xanthomonas. ND, not detected.
the type IV pilus are scattered throughout the genome of K279a. K279a also carries a gene cluster that shares significant similarity with a locus specifying the giant cable pilus of *Burkholderia cepacia*. This pilus has been implicated in the pathogenicity of *B. cepacia* in CF patients [38]. However, not all pathogenic CF isolates of *B. cepacia* carry *cbl* genes; this can also be the case in other *Burkholderia* spp. [39]. Alternative potential adhesins are encoded in the genome, including an afimbrial adhesin and Hep-Hag family adhesins.

In this bacteremia-associated isolate, K279a, there are three members of the YadA family of BuHA proteins that contain numerous Hep-Hag repeat domains [40]. Two hemagglutinin/hemolysin family proteins are present as pseudogenes. Hemolysin activator Smlt1389, and outer membrane surface filamentous hemagglutinin (FHA) Smlt1390 and Smlt4452 are present. Filamentous hemagglutinin is an important virulence factor in * Bordetella pertussis*, being involved in related adhesion and spread of bacteria through the respiratory tract [41].

**Intercellular and intracellular signaling**

Quorum sensing (cell-cell signaling) is important in infection models of *P. aeruginosa*, and quorum-sensing signals that coordinate biofilm formation have been identified in CF sputum along with biofilm-like structures [42]. *S. maltophilia* also carries out cell-cell signaling; however, the *S. maltophilia* system does not employ the usual LuxIR regulators [43,44]. Instead, *S. maltophilia* uses the Xanthomonas and *Xylella* signaling system mediated by a diffusible signal molecule, DSF [45,46]. DSF activity has been detected in a number of strains of *S. maltophilia*, including K279a, and controls resistance to several antibiotics, aggregative and biofilm behavior and virulence in a nematode model [47]. The K279a proteome contains no n-acyl homoserine lactone (N-AHL) synthases of either the LuxI or LuxM type and no LuxS protein (implicated in autoinducer 2 synthesis in a wide range of bacteria). K279a does encode a single LuxR type regulator with an N-AHL autoinducer-binding domain. Such orphan LuxR-like proteins have been described in *Xanthomonas oryzae* pv *oryzae* (*X. oryzae*) [48] and *X. campestris* [49], which do not synthesize N-AHLS. These proteins may interact with a plant host component rather than bind N-AHLS.

DSF perception in *X. campestris* is linked to altered levels of the second messenger cyclic di-GMP through the action of the HD-GYP phosphodiesterase domain regulator RpfG [50]. Cyclic-di-GMP regulates a range of functions, including developmental transitions, adhesion, biofilm formation and virulence in diverse bacteria [51]. Cyclic-di-GMP levels are influenced by synthesis and degradation acted on by the protein domains GGDEF, EAL and HD-GYP. K279a encodes 33 proteins with a potential role in cyclic di-GMP turnover: 3 proteins with an EAL domain; 18 with a single GGDEF domain; 10 with GGDEF and EAL domains; and two HD-GYP domain proteins, including RpfG. Most of these proteins contain additional signal input domains, suggesting that their activities (and therefore cyclic di-GMP levels) are responsive to diverse environmental cues.

**Polysaccharides**

Polysaccharides are integral components of the extracellular matrix of bacterial biofilms and may play a role in resistance of bacteria to antibiotics. In xanthomonads, the *gun* gene cluster specifies production of the exopolysaccharide xanthan that is important in biofilm formation as well as being a commercially important product. *X. fastidiosa* produces fastidin gum, a truncated xanthan that is encoded by a reduced *gun* gene cluster [52]. There are no *gun* gene cluster orthologues in K279a; hence, this strain does not produce either xanthan or a modified version. Additionally, K279a does not carry genes for cellulose production, nor the exopolysaccharide cepacian, produced by some strains of *B. cepacia*.

Gene products implicated in the formation of intermediates of lipopolysaccharides and exopolysaccharides have been identified in K279a. XanAB are involved in UDP-glucose and GDP-mannose biosynthesis whilst RmlAC are involved in the synthesis and interconversion of TDP-sugars. XanB shares significant homology with phosphomannose isomerase, a key enzyme in the biosynthesis of *P. aeruginosa* alginate. Alginate is a key polysaccharide and is upregulated in CF sputum isolates from patients that have been infected with *P. aeruginosa* over a considerable length of time. Mutations in *xanB* and *rpmAC* affect biofilm formation and twitching motility in *S. maltophilia* WR-C [53]. The xanA gene, also known as *spgM*, is a phosphoglucuronase that shares similarity with *P. aeruginosa* algC [54]. K279a also specifies an orthologue of alginate lyase (Smlt1473), which is intriguing, since in CF lungs, the organisms are likely to be in contact with alginate-producing *P. aeruginosa*.

**Comparing the genomes of *S. maltophilia* and X. campestris - two sides of the same coin?**

The K279a genome sequence was compared to that of *X. campestris* and *X. oryzae* using the Artemis Comparison Tool (ACT) (Figure 2). The extent of conserved regions between K279a and 8004 are difficult to visualize by ACT, mainly due to multiple chromosomal rearrangements. The first side of the ‘coin’ is illustrated by the use of K279a as a reference genome with a comparison of orthologous genes shared between K279a and sequenced xanthomonads on a circular genome representation. This comparison allows islands unique to K279a to be more clearly seen, the most obvious being the acquisition of a phage sited at 1,922,800 bp (Figure 3). Predicted functions of coding sequence (CDS) unique to K279a and those shared between 8004 and K279a are shown in Additional data file 1. Genes present in K279a that were not found in *X. campestris* may be applicable to human disease and are briefly described below.
The major regions of difference are phage and mobile elements; these encode both hypothetical and conserved hypotheticals as well as phage structural components (Table 3). In addition, several efflux transporter proteins in K279a are not present in 8004. Fimbrial/pili gene clusters are either divergent or not present in 8004. Other K279a acquisitions include...
hemagglutinins and hemolysins, two proteins with F5/8 type C coagulase domains, along with pseudogenes with hemagglutinin domains and similarities. Myosin cross-reactive antigen has also been acquired relative to *X. campestris*. Heavy metal resistance on a complex mobile element was acquired compared to 8004, as were some antibiotic resistance genes, especially those for aminoglycoside resistance. Although *S. maltophilia* is an obligate aerobe, the membrane-bound nitrate reductase that supports growth in the absence of oxygen with nitrate as a terminal electron acceptor is present in some strains [55]. The potential for microoxic growth is suggested in K279a, with the putative acquisition of formate dehydrogenase (*fdh*), the selenocysteine tRNA synthesis genes required for the Sel codon in *fdnG*, and the membrane-bound nitrate reductase (*nar*). Nar employs a molybdenum cofactor, and K279a *nar* genes cluster with Mo cofactor bio-

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**Figure 3**
Circular diagram showing xanthomonad orthologues. Circles show (outermost to innermost): 1, DNA coordinates of the reference genome K279a; 2, color-coded annotation file, all reading frames in the same circle; 3-7, orthologous genes determined by reciprocal best match analysis (3, *X. campestris* pv *campestris* 8004 (NC_007086); 4, *X. campestris* pv *campestris* 3391 (AE008922); 5, *X. campestris* vesicatoria (NC_007508); 6, *X. axonopodis* citri (NC_003919); 7, *X. oryzae* pv *oryzae* KACC10331 (NC_006834)); 8, GC skew; 9, GC deviation.
synthesis and transport genes, and a member of the FNR/CRP family of transcriptional regulators (fnr2, Smlt2767). An FNR homologue present in the K279a gene cluster suggests that the associated genes are only produced under limiting oxygenation since E. coli FNR regulates the aerobic-anaerobic switch [56]. Microarray analysis of P. aeruginosa under conditions encountered in CF lung (modeled by growth in CF lung sputum) indicates that nar gene expression is elevated [57]. Another FNR family member (Smlt2159) is located elsewhere. The potential for growth under microoxic conditions may enhance the pathogenicity of this organism, for example, by increasing its ability to grow in biofilm. K279a has gained some heat shock proteins that may be needed during pathogenic growth. Both genera share a high number of TonB dependent receptor proteins, a peculiarity of xanthomonads and epsilon proteobacteria [58]. Using X. campestris 8004 as the reference genome in comparison to K279a, there are no large islands of acquisitions or losses. The flip side of the ‘coin’ is that genes present in K279a are of relevance in plant disease (Figure 4). We can see the lack of the type III secretion system and gun gene cluster relative to X. campestris. Other plant pathogenic virulence determinants that are not present in K279a include the extracellular enzymes endoglucanase, polygalacturonate lyase, pectate lyase and cellulase. The avr genes involved in gene-for-gene resistance, such as avrBS1 [59], are also not present in K279a. Further studies of the genomic comparisons between X. campestris and S. maltophilia may reveal additional genes of medical interest or of interest in plant pathogenesis.

Conclusion

The genome sequence of the bacteremia-associated S. maltophilia isolate K279a carries a startling array of antimicrobial resistance gene determinants. Knockout mutagenesis confirms the involvement of a number of novel RND efflux genes in resistance to a variety of different classes of antimicrobials.

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 [29,60]. Other drugs with reasonable activity against S. maltophilia are minocycline and newer fluoroquinolones [60]. 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 [17].

The panoply of antimicrobial drug resistance genes and mobile genetic elements is an issue of clinical concern. S. maltophilia can also provide antibiotic resistance protection for sensitive P. aeruginosa and Serratia marcescens strains growing nearby [61]. Even more importantly, the organism potentially acts as a reservoir of antibiotic resistance determinants in medically relevant environments.

K279a possesses an unusual cell density-signaling pathway like that of its plant pathogenic xanthomonad relatives. K279a does produce extracellular enzymes such as protease StmPr1 and phospholipases; however, previous studies on clinical isolates have reported the production of other extracellular enzymes by S. maltophilia, suggesting that such virulence factors may be strain-specific. Comparison of K279a with X. campestris illustrates the movements of mobile genetic elements, the acquisition of potentially human pathogenic factors such as hemagglutinin, hemolysins and the loss of plant pathogenic factors such as the extracellular enzyme polygalacturonate lyase.

In conclusion, the S. maltophilia genome sequence reveals the capacity of this organism for environmental adaptations that presumably contribute to its persistence in vivo. As expected of a true opportunist pathogen, the S. maltophilia genome does not suggest a highly virulent organism. However, the large number of pili/fimbrial genes does indicate a strong ability to attach to catheters and ventilators, from which infections of the blood or lungs arise. With its MDR phenotype and ability to attach, it is clear why this organism is persistent and difficult to eradicate. We are starting to build up a picture of an organism that is a true opportunist, which, while lacking many conventional key virulence determinants, has nevertheless emerged as a considerable threat.

Materials and methods

Sequencing strategy and annotation

S. maltophilia K279a was grown on Nutrient broth (Oxoid Cambridge, Cambridgeshire, UK) and genomic DNA was isolated using cetyltrimethylammonium bromide.

DNA was sonicated, size selected, and libraries were constructed in pUC19, pMAQ1b and pBACe3.6. The genome assembly was based on 3,381, 41,541 and 21,977 paired end-reads, respectively, from pUC19 libraries (of insert sizes 1.4-2.0 kb, 2.0-2.8 kb and 3.0-3.3 kb) and from 6,890, 314 and 69 paired end-reads, respectively, from pMAQ1b libraries (of insert sizes 5.5-6.0 kb, 9-10 kb and 10-12 kb), to give a 10.76-fold sequence coverage of the genome. We generated 1,250 and 106 reads, respectively, to produce a scaffold from 15-18 and 20-25 kb libraries in pBACe3.6. The genome was sequenced, finished and annotated as previously described [62]. To ensure that all bases were covered by reads on both strands or with different sequencing chemistries, and to fill gaps, 789 extra reads were generated. Repeats were bridged using read-pairs or end-sequenced PCR products. The total shotgun size was 53,580,262 Mb with a total genome coverage of 11.05-fold. Orthologous genes were determined by reciprocal best match analysis.
Disruption of putative efflux pump genes and MIC determination

Genes were disrupted using a modified method of that previously described [17]. Genes were amplified by PCR in two non-overlapping fragments, with HindIII being introduced such that the two fragments could be ligated together, resulting in a mutant gene having a large deletion and a frameshift mutation. The primers used are listed in Additional data file 2. Mutated genes were used to replace wild-type sequences on the chromosome of K279a using the gene replacement approach described previously. Agar dilution MICs of antimicrobials against K279a and its derivatives were determined according to British Society for Antimicrobial Chemotherapy (BSAC)-approved methods [63].
Abbreviations
ACT, Artemis Comparison Tool; BSAC, British Society for Antimicrobial Chemotherapy; CDS, coding sequence; CF, cystic fibrosis; IS, insertion sequence; MDR, multi-drug resistant; MFP, membrane fusion protein; MIC, minimum inhibitory concentration; N-AHL, n-acyl homoserine lactone; OMP, outer membrane protein; RND, resistance-nodulation-division.

Authors’ contributions
LCC, MBA, JMD and JP wrote the paper. GSV, AO, NP, AK, TC and EA provided DNA or analysis tools. VCG, DS, CA, and MAQ carried out experiments. LCC, VCG, JMD, GSV, MS, DS, AL, LM, KS, RS, SR, MAJ, DH, CC, SDB, JP, NRT and MBA analyzed data.

Additional data files
The following additional data are available with the online version of this paper. Additional data file 1 shows the shared genes between K279a and X. campestris, and the genes unique to K279a determined by reciprocal best match analysis. Additional data file 2 is a table listing the primer sequences used in the generation of gene knock-outs.

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References
1. Fernandes P: Antibacterial discovery and development - the failure of success? Nat Biotechnol 2006, 24:1497-1503.
2. Paterson DL, Lipman J: Returning to the pre-antibiotic era in the critically ill: the XDR problem. Crit Care Med 2007, 35:1789-1791.
3. Martinez JL, Baquero F, Andersson DI: Predicting antibiotic resistance. Nat Rev Microbiol 2007, 5:958-965.
4. Torres JA, Villegas MV, Quinn JP: Current concepts in antibiotic-resistant gram-negative bacteria. Expert Rev Anti Infect Ther 2007, 5:833-843.
5. Falagas ME: Kopterides P: Risk factors for the isolation of multi-drug-resistant Acinetobacter baumannii and Pseudomonas aeruginosa: a systematic review of the literature. J Hosp Infect 2006, 64:7-15.
6. Mesaros N, Nordmann P, Plisiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, Van Laethem Y, Jacobs F, Lebecque P, Malfroot A, Coigny M, Acheman M, Berg G: A molecular biological protocol to distinguish potentially human pathogenic Stenotrophomonas maltophilia from plant-associated Stenotrophomonas rhizophila. Environ Microbiol 2005, 7:1853-1858.
7. Berg G, Roskot N, Smalla K: Genotypic and phenotypic relationships between clinical and environmental isolates of Stenotrophomonas maltophilia. J Clin Microbiol 2001, 39:139-145.
8. Berg G, Roskot N, Smalla K: Genotypic and phenotypic relationships between clinical and environmental isolates of Stenotrophomonas maltophilia. J Clin Microbiol 1999, 37:3594-3600.
9. Gould VC, Okazaki A, Avison MB: Beta-lactam resistance and beta-lactamase expression in clinical Stenotrophomonas maltophilia isolates having defined phylogenetic relationships. J Antimicrob Chemother 2002, 49:239-243.
10. Ribbeck-Busch K, Roder A, Hasse D, de Boer W, Martinez JL, Hegemann M, Berg G: A molecular biological protocol to distinguish potentially human pathogenic Stenotrophomonas maltophilia from plant-associated Stenotrophomonas rhizophila. Environ Microbiol 2005, 7:1853-1858.
11. Berg G, Roskot N, Smalla K: Genotypic and phenotypic relationships between clinical and environmental isolates of Stenotrophomonas maltophilia. J Clin Microbiol 2001, 39:139-145.
12. Berg G, Roskot N, Smalla K: Genotypic and phenotypic relationships between clinical and environmental isolates of Stenotrophomonas maltophilia. J Clin Microbiol 1999, 37:3594-3600.
13. Gould VC, Okazaki A, Avison MB: Beta-lactam resistance and beta-lactamase expression in clinical Stenotrophomonas maltophilia isolates having defined phylogenetic relationships. J Antimicrob Chemother 2002, 49:239-243.
14. Avison MB, von Heldenreich CJ, Higgins CS, Bennett PM, Walsh TR: A TEM-2beta-lactamase encoded on an active Tn 1-like transposon in the genome of a clinical isolate of Stenotrophomonas maltophilia. J Antimicrob Chemother 2000, 46:879-884.
15. Gould VC, Avison MB: SmeDEF-mediated antimicrobial drug resistance in Stenotrophomonas maltophilia clinical isolates having defined phylogenetic relationships. J Antimicrob Chemother 2006, 57:1070-1076.
16. Okazaki A, Avison MB: Aph(3’)-Iic, an aminoglycoside resistance determinant from Stenotrophomonas maltophilia. Antimicrob Agents Chemother 2007, 51:1339-1340.
17. Poole K: Efflux pumps as antimicrobial resistance mechanisms. Ann Med 2007, 39:162-176.
18. Li XZ, Zhang L, Poole K: SmeC, an outer membrane multidrug efflux protein of Stenotrophomonas maltophilia. Antimicrob Agents Chemother 2002, 46:332-343.
19. Gould VC, Okazaki A, Howe RA, Avison MB: Analysis of sequence variation among smeDEF multi drug efflux pump genes and flanking DNA from defined 16S rRNA subgroups of clinical Stenotrophomonas maltophilia isolates. J Antimicrob Chemother 2004, 54:348-353.
20. Alonso A, Martinez JL: Cloning and characterization of SmeDEF, a novel multidrug efflux pump from Stenotrophomonas maltophilia. Antimicrob Agents Chemother 2000, 44:3079-3086.
21. Sanchez P, Alonso A, Martinez JL: Cloning and characterization of SmeT, a repressor of the smeDEF operon in Stenotrophomonas maltophilia strains expressing different amounts of the multidrug efflux pump SmeDEF. Antimicrob Agents Chemother 2004, 48:2274-2276.
22. Baranova N, Nikaido H: The baeSR two-component regulatory system activates transcription of the vegaMNOB (mtABoCD) transporter gene cluster in Escherichia coli and increases its resistance to novobiocin and deoxycholate. J Bacteriol 2002, 184:4168-4176.
23. Vernikos GS, Parkhill J: Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the Salmonella pathogenicity islands. Bioinformatics 2006, 22:2196-2203.
24. Avison MB, Higgins CS, von Heldenreich CJ, Bennett PM, Walsh TR: Plasmid location and molecular heterogeneity of the L1 and L2 beta-lactamase genes of Stenotrophomonas maltophilia. Antimicrob Agents Chemother 2001, 45:413-419.
25. Alonso A, Sanchez P, Martinez JL: Stenotrophomonas maltophilia D457R contains a cluster of genes from gram-positive bacteria involved in antibiotic and heavy metal resistance. Antimicrob Agents Chemother 2000, 44:1778-1782.
26. Toleman MA, Bennett PM, Bennett DM, Jones RN, Walsh TR: Global emergence of trimethoprim/sulfamethoxazole resistance in Stenotrophomonas maltophilia mediated by acquisition of sul2 genes. Emerg Infect Dis 2007, 13:559-565.
27. Chang HC, Chen CR, Lin JW, Shen GH, Chang KM, Tseng YH, Weng SF: Isolation and characterization of novel giant Stenotropho-
mons maltophilia phage phiSMAS. Appl Environ Microbiol 2005, 71:1387-1393.

31. Willingham V, Lehmann V, Krauss K, Lehner B, Richter S, Ruckdeschel K, Heesemann J, Trutlczk K: Yersinia enterocolitica type III secretion depends on the proton motive force but not on the flagellar motor components MotA and MotB. Infect Immun 2004, 72:4004-4009.

32. Korbitz S, Tomaras AP, Dammin S, Clumdeed J, Srinon V, Lengvehasatit I, Vasil ML, Suparak S: Characterization of two distinct phospholipase C enzymes from Burkholderia pseudomallei. Microbiology 2007, 153:1907-1915.

33. Travassos LH, Pinheiro MN, Coelho FS, Sampaio JL, Merquior VL, M. L. Queses EA: Hypotonic propitiation: drug susceptibility and genetic relatedness of Stenotrophomonas maltophilia clinical strains from seven hospitals in Rio de Janeiro, Brazil. J Appl Microbiol 2004, 96:143-150.

34. Windhorst S, Frank E, Georgievas DN, Genov N, Buck F, Borowski P, Windhorst S, Frank E, Georgievas DN, Genov N, Buck F, Borowski P: The major extracellular protease of the nosocomial pathogen Stenotrophomonas maltophilia: characterization of the protein and molecular cloning of the gene. J Biol Chem 2002, 277:11042-11049.

35. de Oliveira-Garcia D, Dall’Agnol M, Rosales M, Azzuz AC, Martinez MB, Giron JA: Characterization of flagella produced by clinical strains of Stenotrophomonas maltophilia. Emerg Infect Dis 2002, 8:918-923.

36. de Oliveira-Garcia D, Dall’Agnol M, Rosales M, Azzuz AC, Alcantara N, Martinez MB, Giron JA: Fimbriae and adherence of Stenotrophomonas maltophilia to epithelial cells and to abiotic surfaces. Cell Microbiol 2003, 5:625-636.

37. Tomich M, Planet PJ, Figurski DH: The tad locus: postcards from the widespread colonization island. Nat Rev Microbiol 2007, 5:363-375.

38. Urban TA, Goldberg JB, Forstner JF, Sajan US: Cable pilus and the 22-kilodalton adhesin are required for Burkholderia cenocefaciation to and transmigration across the squamous epithelium. Infect Immun 2003, 71:5342-5347.

39. Carvalho AP, Ventura GM, Pereira CB, Leko RS, Folescu TW, Higa L, Teixeira LM, P. BM, Micocci MC, Merquior VL, Albano RM, Marques E: Characterization of Burkholderia cepacia, B. multivorans, B. ambifaria and B. vietnamiensis isolates from cystic fibrosis patients have different profiles of exoenzyme production. APMS 2007, 105:11-31.

40. Tytwisutris R, Holden MT, Tumapa S, Rengpipat S, Clarke SR, Foster SJ, Nierman WC, Day NP, Peacock SJ: Burkholderia Hep_Hag autotransporter (BuHaH) proteins elicit a strong antibody response against elevator-grown clinical isolates but not during chronic infection. BMC Microbiol 2007, 7:19.

41. Colombi D, Oliveira ML, Campos IB, Monedero V, Perez-Martinez G, Ho PL: Haemagglutination induced by Bordetella pertussis filamentous haemagglutinin adhesin (FHA) is inhibited by antibodies produced against FHA (340-873) fragment expressed in Escherichia coli. Curr Microbiol 2006, 53:462-466.

42. Pearson JP, Feldman M, Iglweski BH, Prince A: Pseudomonas aeruginosa cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infect Immun 2000, 68:4331-4334.

43. Eberl L: Quorum sensing in the genus Burkholderia. Int J Med Microbiol 2006, 296:103-110.

44. Bjarnsholt T, Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He YW, Zhang LH, Heeb S, Camicia M, Williams P, Dow JM: Cell-cell signaling in Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic-di-GMP turnover. Proc Natl Acad Sci USA 2006, 103:6712-6717.

45. Jenal U, Malone J: Mechanisms of cyclic-di-GMP signaling in bacteria. Annu Rev Genet 2006, 40:385-407.

46. da Silva FR, Vettore AL, Kemper EL, Leite A, Arruda P, Fastidi gum: The Xylella fastidiosa exopolysaccharide possibly involved in bacterial pathogenicity. FEMS Microbiol Lett 2001, 203:165-171.

47. Huang TP, Somers EB, Wang AC: Differential biofilm formation and motility associated with lipopolysaccharide/exopolysaccharide-charide-coupled biosynthetic genes in Stenotrophomonas maltophilia. J Bacteriol 2006, 188:3116-3120.

48. Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He YW, Zhang LH, Heeb S, Camicia M, Williams P, Dow JM: Cell-cell signaling in Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic-di-GMP turnover. Proc Natl Acad Sci USA 2006, 103:6712-6717.

49. Jenal U, Malone J: Mechanisms of cyclic-di-GMP signaling in bacteria. Annu Rev Genet 2006, 40:385-407.

50. Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He YW, Zhang LH, Heeb S, Camicia M, Williams P, Dow JM: Cell-cell signaling in Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic-di-GMP turnover. Proc Natl Acad Sci USA 2006, 103:6712-6717.