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Stenotrophomonas maltophilia isolated from patients affected by cystic fibrosis: genotyping analysis and molecular characterization of virulence determinants

Stenotrophomonas maltophilia isolato da pazienti affetti da fibrosi cistica: analisi genotipica e caratterizzazione molecolare di determinanti di virulenza

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ABSTRACT

Stenotrophomonas maltophilia is a an environmental organism, that in recent years has been isolated with increasing frequency as an opportunistic pathogen in nosocomial infections as well as from the airways of cystic fibrosis (CF) patients. Despite its growing clinical importance, the mechanisms adopted by this organism to establish persistent infections have not been clarified yet. The availability of information on genome sequence of two *S. maltophilia* strains (K279a and R551-3), allow development of new post-genomic approaches direct to understanding of key factors involved in the colonization of respiratory tract of CF patients and of cellular and molecular mechanisms of its pathogenicity. Identification of *S. maltophilia* genes expressed inside host, is essential for development of new antimicrobial compounds. Moreover, molecular and genetic characterization of *S. maltophilia* clinical strains is essential to understand pathogenicity mechanism of these organisms and to build detailed epidemiological maps to draw their spread in CF patients.

The present study is aimed at determining the genomic and epidemiological relatedness of 52 *S. maltophilia* strains. Strains included 43 clinical collected, between January 2003 and December 2005, from 43 independent patients (41 were from CF patients, and two from blood cultures of two non-CF patients) attending the Paediatric Hospital "Bambino Gesù" of Rome. Other strains were: K279a and LMG958, two *S. maltophilia* reference clinical strains, and seven strains of environmental origin (two isolated within the Hospital "Bambino Gesù"). In these strains the presence and expression of the virulence-associated genes were evaluated and their contribution to virulence was assessed using larvae of the wax moth *Galleria mellonella* as an infection model. To confirm a role of the putative virulence factors identified we constructed K279a (the clinical reference strain whose genome sequence is available) mutants and performed complementation assay with the wild type functional gene. Moreover, to study genomic evolution of *S. maltophilia* toward a pathogenic lifestyle, we compare the genome of an environmental strain with that of the clinical strain K279a.

Genotyping analysis. Data that we obtained indicates PFGE fingerprinting as the most discriminatory technique to characterize genomospecies present in *S. maltophilia*. All strains produced well-defined PFGE profiles: forty-seven different PFGE pulsotypes were identified, indicating a remarkable genomic diversity among the strains analysed. Three clusters (pulsotype 1, 17 and 33) showed a banding pattern identical to at least 1 other isolate likely indicating either cross-transmission among

patients or infection from a common source. However, the results obtained with the RFLP of *gyrB* gene and with the variable regions of the 16S rRNA gene clearly indicated that certain phylogenetic groups are likely better able to cause infection than others.

Identification and characterization of virulence genes involved in *S. maltophilia* pathogenicity. All 52 *S. maltophilia* strains were characterized for the presence and expression of type-1 fimbria, esterase, proteases (StmPr1 and StmPr2). Regarding the type-1 fimbria, only the clinical strains showed the presence of the gene coding for this surface structure, indicating that it might be an important virulence factor of this opportunistic pathogen. The expression of esterase activity by most CF-derived *S. maltophilia* strains, reinforces the hypothesis that esterase, such as fimbria, may play some role in the virulence. In order to verify the role of these potential virulence factors in *S. maltophilia* pathogenesis, representative *S. maltophilia* strains unable to express proteases or esterase were assayed in *G. mellonella* infection. The results indicate that proteases may be relevant virulence factors of *S. maltophilia.* Moreover, the type-1 fimbria could play an important role since the environmental strains, lacking of smf-1, show the higher lethal dose (LD_{50}).

To analyze the relative role of the two proteases (StmPr1 and StmPr2), the OBGTC23 strain, naturally impaired in protease expression, was complemented with the StmPr1 or StmPr2 functional genes and tested in *G. mellonella* assay. Data obtained showed that StmPr1 protease play a major role in *S. maltophilia* pathogenesis.

Analysis of the role of *S. maltophilia* virulence factors by mutants construction. In the complex, the results that we obtained using different clinical OBGTC strains, impaired in the expression of one or more hypothetical virulence factors, indicates proteases as important virulence determinants for *S. maltophilia*. Nevertheless, these natural mutant strains could lack of many others virulence determinants that may be involved in pathogenesis process. Then, to study the effect of the single factors identified and to confirm their role, deletions encompassing different putative virulence genes are introduced by allelic exchange in reference strain K279a, whose genome sequence is available. The proteases mutants showed a strong reduction of the protease activity and were less virulent, compared to wild-type, in *G. mellonella* larvae, confirming that proteases, particularly StmPr1, are important virulence factors for *S. maltophilia*. The proteases expression is controlled by the diffusible signal factor (DSF) a quorum sensing signal molecule that controls the expression of several virulence factors. Moreover, the DSF mutant reveals a greater reduction of virulence in *G. mellonella* infection model, compared to that of proteases

mutants, suggesting a DSF involvement in the control of proteases but also of other virulence factors. Finally, despite fimbriae are important to establish chronic infection in airways CF patients, this surface structures may be not important in *G. mellonella* infection model.

Genomic evolution of *S. maltophilia* toward a pathogenic lifestyle. To identify the specific genetic determinants acquired from *S. maltophilia* during its evolution towards a more pathogenic lifestyle, we performed a Suppression Subtractive Hybridization: this technique allowed us to identify the genetic sequences present in the clinical strain K279a and absent in the environmental strain LMG959. The majority of the subtracted sequence may have been acquired from other organisms by horizontal gene transfers, since their different G+C content and since the presence of several IS elements. Among the sequence, particularly interesting are genes coding for haemagglutinin, Clp protease, TonB dependent receptor protein, and a putative ankyrin repeat-containing protein, which are known to be important virulence factors in many gram-negative bacteria. Moreover, many of the subtracted sequence represent genes involved in metabolism, DNA restriction/modification system, transmembrane proteins, hypothetical proteins and proteins with unknown function. These differences between *S. maltophilia* K279a and *S. maltophilia* LMG959 could be related to niche adaptation or host preference and this accessory genome may represents an advantage for pathogen evolution driven by the need for continuous adaptation to the host in order to evade or suppress coevolving host defense mechanisms, making it an emergent opportunistic pathogen in nosocomial infections.

RIASSUNTO

Stenotrophomonas maltophilia è un microrganismo ambientale, che negli ultimi anni è stato isolato con una frequenza crescente come patogeno opportunista in infezioni nosocomiali così come dalle vie respiratorie di pazienti affetti da fibrosi cistica (FC). Nonostante la sua crescente importanza clinica, i meccanismi adottati da questo organismo per stabilire infezioni croniche non sono ancora stati chiariti. La disponibilità delle informazioni sulla sequenza genomica di due ceppi di *S. maltophilia* (K279a e R551-3), permette lo sviluppo di nuovi approcci post genomici diretti alla comprensione dei fattori chiave necessari per colonizzazione del tratto respiratorio dei pazienti FC, nonché l'analisi dei meccanismi molecolari e cellulari che contribuiscono alla patogenicità di tale microrganismo. L'identificazione dei geni di *S. maltophilia* espressi all'interno dell'ospite, è essenziale per lo sviluppo di nuovi composti antimicrobici. Infine, la caratterizzazione genetica e molecolare dei ceppi clinici di *S. maltophilia* è essenziale per comprendere i meccanismi della patogenicità di questi organismi e per costruire mappe epidemiologiche dettagliate per monitorare la loro diffusione tra i pazienti FC.

Lo scopo di questo progetto è determinare le relazioni genetiche ed epidemiologiche di 52 ceppi di *S. maltophilia*. Tali ceppi comprendono 43 ceppi clinici collezionati, tra gennaio 2003 e dicembre 2005, da 43 pazienti indipendenti (41 da pazienti FC e 2 da emocolture di pazienti non FC) ricoverati all'Ospedale Pediatrico "Bambino Gesù" di Roma. Sono stati inoltre analizzati due ceppi clinici di *S. maltophilia* di riferimento (K279a e LMG958), e sette ceppi di origine ambientale (di cui 2 isolati all'interno dell'Ospedale "Bambino Gesù"). In questi ceppi è stata determinata la presenza e l'espressione di geni associati alla virulenza, ed il loro ruolo nella virulenza è stato determinato utilizzando larve di *Galleria mellonella* come modello di infezione *in vivo*. Per confermare il ruolo dei potenziali fattori di virulenza identificati, abbiamo costruito mutanti del ceppo K279a (il ceppo di riferimento clinico il cui genoma è stato sequenziato) e abbiamo effettuato test di complementazione con i geni funzionali selvatici. Inoltre, allo scopo di studiare l'evoluzione genomica di *S. maltophilia* verso uno stile di vita più patogeno, abbiamo comparato il genoma di un ceppo ambientale con quello del ceppo clinico K279a.

Analisi genotipica. I risultati ottenuti indicano che l'elettroforesi in campo pulsato (PFGE) è la tecnica a maggior potere discriminatorio per caratterizzare i diversi ceppi di *S. maltophilia*. Tutti i ceppi hanno prodotto un profilo di PFGE ben definito: abbiamo infatti identificato 47 pulsotipi di PFGE differenti, indicando una grande diversità genomica tra i ceppi

analizzati. Inoltre, abbiamo identificato tre cluster (pulsotipi 1, 17 e 33) che hanno un profilo identico ad almeno un altro ceppo, indicando la possibilità di contagio tra pazienti oppure infezione dalla stessa sorgente. Il risultato che abbiamo invece ottenuto dall'analisi RFLP del gene *gyrB* e dall'analisi delle regioni variabili del gene 16S rRNA indicano chiaramente che alcuni gruppi filogenetici sono più adatti a causare infezioni che altri.

Identificazione e caratterizzazione di geni di virulenza coinvolti nella patogenicità di *S. maltophilia*. Tutti i 52 ceppi di *S. maltophilia* sono stati caratterizzati per la presenza ed espressione della fimbria di tipo 1, esterasi e proteasi (StmPr1 e StmPr2). Per quanto riguarda la fimbria tipo-1, solo i ceppi di origine clinica hanno mostrato la presenza del gene che codifica per questa struttura di superficie, che potrebbe costituire un importante fattore di virulenza per questo patogeno opportunista. L'espressione di esterasi da parte della maggior parte dei ceppi FC, rinforza l'ipotesi che l'esterasi, come la fimbria, potrebbe svolgere un ruolo nella virulenza di *S. maltophilia*. Allo scopo di valutare il ruolo di questi potenziali fattori di virulenza nella patogenicità di *S. maltophilia*, alcuni ceppi rappresentativi di *S. maltophilia*, incapaci di esprimere proteasi o esterasi sono stati saggiati in vivo in larve di *Galleria mellonella*. I risultati ottenuti indicano che le proteasi potrebbe essere fattori di virulenza rilevanti per *S. maltophilia*. Inoltre, la fimbria tipo-1 potrebbe svolgere un ruolo importante in quanto i ceppi ambientali, che non hanno tale fimbria, hanno mostrato la più alta dose letale (DL_{50}).

Per analizzare il ruolo relativo delle due proteasi (StmPr1 e StmPr2), il ceppo OBGTC23, naturalmente incapace di esprimere attività proteasica, è stato complementato con il gene funzionale *StmPr1* o *StmPr2* di K279a e testato in *G. mellonella*. I risultati ottenuti confermano che la proteasi StmPr1 svolge un ruolo primario nella patogenesi di *S. maltophilia*.

Analisi del ruolo dei fattori di virulenza di *S. maltophilia* tramite costruzione di mutanti. Nel complesso, i risultati ottenuti analizzando i differenti ceppi clinici OBGTC, incapaci di esprimere uno o più degli ipotetici fattori di virulenza, indicano che le proteasi rappresentano determinanti di virulenza importanti per *S. maltophilia*. Tuttavia, questi ceppi mutanti naturali, potrebbero non avere molti altri fattori di virulenza che sono coinvolti nella patogenicità. Quindi, allo scopo di studiare l'effetto dei singoli fattori identificati e per confermare il loro ruolo, sono stati costruiti dei mutanti di delezione nei diversi geni di virulenza, del ceppo di riferimento K279a, il cui genoma è stato sequenziato. I mutanti nelle proteasi hanno mostrato una forte riduzione nella loro attività proteasica ed erano anche meno virulenti rispetto al selvatico, in *G. mellonella*, confermando che le proteasi, ed in particolare la StmPr1, sono fattori di

virulenza importanti nella patogenicità di *S. maltophilia*. L'espressione di queste proteasi è regolata dal DSF ("diffusible signal factor") una molecola segnale del "quorum sensing" che controlla l'espressione di vari fattori di virulenza. Inoltre, il mutante DSF ha mostrato una maggiore DL_{50} rispetto a quella osservata per i mutanti nelle proteasi, suggerendo un coinvolgimento del DSF nel controllo delle protease ma anche di altri fattori di virulenza. Infine, nonostante le fimbrie siano importanti per stabilire infezioni croniche nelle vie respiratorie dei pazienti FC, queste strutture di superficie potrebbero non essere fondamentali nel modello di infezione *G. mellonella*.

Evoluzione genomica di *S. maltophilia* verso uno stile di vita patogeno. Allo scopo di individuare specifiche sequenze genetiche acquisite da *S. maltophilia* durante la sua evoluzione verso uno stile di vita più patogeno, abbiamo applicato la tecnica della "Suppression Subtractive Hybridization". Questa tecnica ci ha permesso di identificare le sequenze genetiche, presenti nel ceppo clinico K279a e assenti nel ceppo ambientale LMG959. La maggior parte di esse sono stati acquisite da altri organismi tramite trasferimento genico orizzontale, in quanto abbiamo osservato una differenza nel loro contenuto medio in G+C rispetto al genoma di K279a e la presenza di alcuni elementi IS. Tra le sequenze sottratte, particolarmente interessanti sono quelle che rappresentano geni che codificano per una emoagglutinina, per una proteasi Clp, per un recettore TonB e per una proteina contenente la ripetizione ankirina, i quali sono noti come importanti fattori di virulenza in molti altri batteri gram-negativi. Inoltre, molte delle sequenze rappresentano geni coinvolti nel metabolismo, nei sistemi di modificazione/restrizione del DNA, proteine transmebrana, proteine ipotetiche e proteine con funzione sconosciuta. Tutte queste differenze tra i ceppi di *S. maltophilia* K279a e LMG959 potrebbero essere correlate all'adattamento a diverse nicchie o ospiti, e questo genoma accessorio potrebbe rappresentare un vantaggio per l'evoluzione patogena di *S. maltophilia*, che lo ha trasformato in un patogeno opportunista.

INTRODUCTION

Cystic Fibrosis (CF) is one of the most common inherited genetic diseases in Caucasian population and affects approximately 30,000 individuals in the United States and 60,000 individuals worldwide with an estimated incidence in the U.S. white population ranging from 1 in 1,900 to 1 in 3,700. CF results in the production of a thick viscous layer of mucus that covers the lungs of patients with this disease. This promotes respiratory tract infections by a variety of microorganisms, including *Pseudomonas aeruginosa*, *Staphylococcus aureus, Haemophilus influenzae*, *Burkholderia cepacia* complex (Figure 1). Inflammation and chronic bacterial infections are responsible for most of the morbidity and mortality of CF patients. Aggressive use of antibiotics has made a major contribution to improve prognosis, however it has been also associated with a change in the pattern of infecting organisms over decades. When CF was first described, *Staphylococcus aureus* was the major pathogen and subsequently *Pseudomonas aeruginosa* became increasingly common (Gibson et al., 2003).

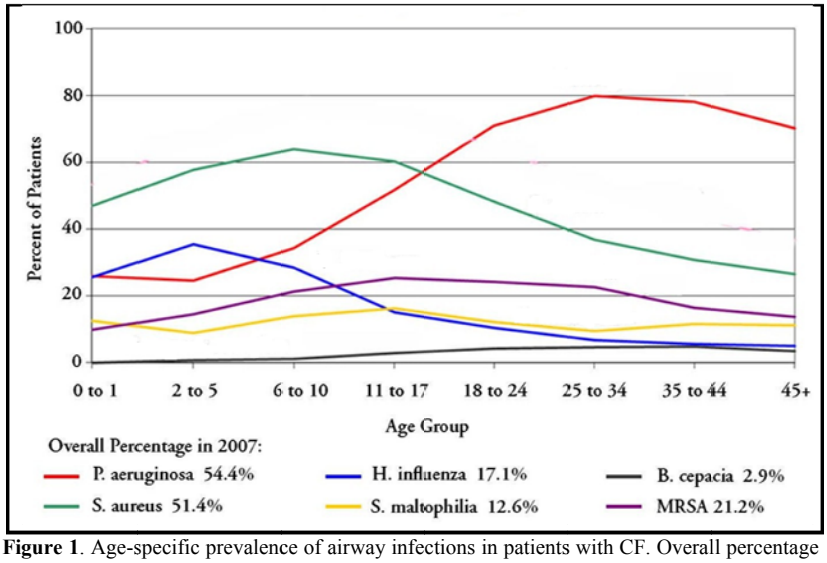


Figure 1. Age-specific prevalence of airway infections in patients with CF. Overall percentage of patients (all ages) who had at least one respiratory tract culture that was positive for the following organisms: *Pseudon* ; *Staphylococcus aureus* (*green line*), 51.4%; *Haemc* 7.1%; *Stenotrophomonas maltophilia* (*yellow line*), 12.6%; MRSA (*violet*), 21.2%; *Burkholderia cepacia* (*black line*), 2.9% (U.S. CF patients registry 2007).

The use of specific anti-pseudomonal therapies has caused a selective pressure on the CF bacterial polymicrobial community resulting in the emergence of new pathogens, including *S. maltophilia* (Denton and Kerr, 1998). Persistent colonization by *P. aeruginosa* and damage of the epithelial mucosa has been suggested to enhance the chance of *S. maltophilia* to colonize the respiratory epithelium of CF patients where a progressive deterioration of pulmonary functions has been observed, particularly in patients colonized for longer periods (Goss et al., 2004).

From the first report, the isolation of *S. maltophilia* has continued to raise. Currently it is isolated from the lungs of approximately 10% of the CF patients in USA and up to 25% in Europe (Looney et al., 2009; Steinkamp et al., 2005). Considering its innate resistance to antibiotics, this microorganism has become a cause of great concern within the international CF community (Avison et al., 2001; Canton et al., 2003; Crossman et al., 2008), but despite its high incidence, its virulence factors have been poorly characterized.

The versatility and adaptation of bacteria from the genus *Stenotrophomonas*

The genus *Stenotrophomonas* (*Stenos*, Greek: narrow; *trophos*, Greek: one who feeds; *monas*, Greek: a unit, monad; i.e., a unit feeding on few substrates) is phylogenetically placed in the Gammaproteobacteria and its name was intended to highlight the limited nutritional range of the bacterium. However, several studies subsequently demonstrated that the genus is capable of great metabolic versatility and intraspecific heterogeneity (Berg et al., 1999; Hauben et al., 1999).

The genus was proposed in 1993 by Palleroni and Bradbury after many years of debate regarding the appropriate taxonomic position of this organism. The *S. maltophilia* type strain (ATCC 13637) was isolated in 1958 by Hugh from an oropharyngeal swab from a patient with an oral carcinoma and named *Pseudomonas maltophilia* (Hugh et al., 1963). Subsequently, Hugh and Ryschenkow reclassified "*Bacterium bookeri*", which had been isolated from pleural fluid in 1943, as *P. maltophilia*. Later, in 1981 *P. maltophilia* was reclassified in the genus *Xanthomonas* as *X. maltophilia*. Continuing dissatisfaction with the classification of this organism finally gave rise to the proposal in 1993 to create the new genus *Stenotrophomonas* with *S. maltophilia* as the sole member. *Stenotrophomonas* currently comprises 12 species, *S. maltophilia, S. nitritireducens*, *S. rhizophila*, *S. acidaminiphila*, *S. koreensis*, *S. chelatiphaga*, *S. terrae, S. humi, S. pavanii, S. ginsengisoli, S. panacihumi* and *S. daejeonensis* (Svensson-Stadler et al., 2011). Phenotypic and genotypic studies as well as analysis of the ecological and metabolic diversity of these bacteria have revealed further differentiation at the species level (Ryan et al., 2009).

Although *Stenotrophomonas* spp. occur ubiquitously in the environment, soil and plants are their main environmental reservoirs (Denton and Kerr, 1998). Members of the genus *Stenotrophomonas* have an important ecological role in the nitrogen and sulphur cycles and several *Stenotrophomonas* species, especially *S. maltophilia* and *S. rhizophila,* can engage in beneficial interactions with plants. Moreover, *Stenotrophomonas* spp. have many traits that could be used in different biotechnological processes. Some *Stenotrophomonas* spp. can produce antimicrobial compounds that protect plants, as well as generate factors that can promote plant growth. Also, many *Stenotrophomonas* spp. have a high level of intrinsic resistance to heavy metals and antibiotics and have been shown to degrade a wide range of compounds, including pollutants, and could

potentially be used in bioremediation and phytoremediation. *Stenotrophomonas maltophilia* is also known to cause human disease as a result of its ability to colonize immunocompromised patients, and has been shown to be virulent in a nematode model (Figure 2).

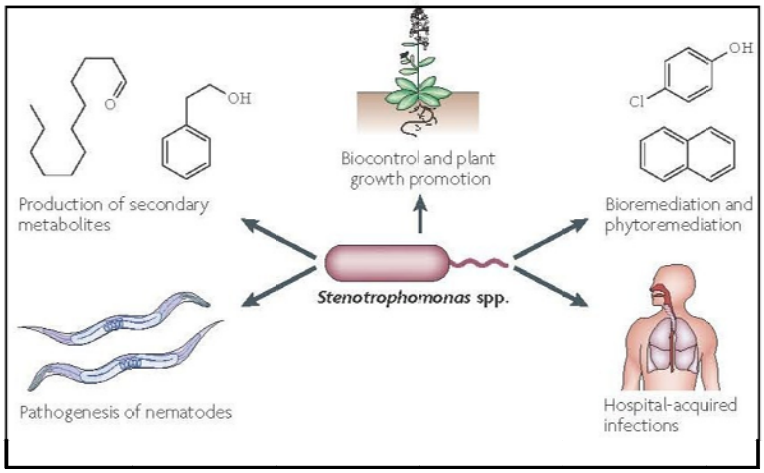


Figure 2. Behavior diversity of *Stenotrophomonas* spp. (Ryan et al., 2009).

The recently published sequence of the *S. maltophilia* genome is a landmark in our understanding of this organism and should greatly improve our ability to understand drug resistance and pathogenicity, and develop new strategies directed at preventing and treating infection. The information currently available on the genomics of *S. maltophilia* are mainly from the genome sequencing of two strains: *S. maltophilia* R551-3 and *S. maltophilia* K279a. The R551-3 strain was isolated as a symbiont from *Populus trichocarpa*. The endophytic microorganisms have a fundamental role in plant growth as provide for training of growth factors, nutrients and eliminate toxic compounds to the plant. The genome of *S. maltophilia* R551-3 (Lucas et al., 2008) is composed of 4,573,969 base pairs with a high content of G + C, equal to 66.3%. The strain of *S. maltophilia* K279a was isolated, from a patient with cancer. The genome of this strain is longer than that of strain R551-3 (4,851,126 base pairs) and has a G + C content of 66.7%. Comparative genome wide analysis revealed that strains R551-3 and K279a have a conserved backbone, but contain different sets of genomic island (Rocco et al., 2009). Both strains contain genes encoding for many

extracellular enzymes (nucleases, elastases, lipases and proteases etc.) that may be involved in the spreading and colonization process (Crossman et al., 2008). Genetic analysis suggests that *S. maltophilia* has adapted to human colonisation, having lost certain plant pathogenic traits and gained potential human virulence factors (Crossman et al., 2008). *S. maltophilia* has been recognized as an important opportunistic pathogen affecting primarily the hospitalized and debilitated host. *S. maltophilia* is frequently isolated from the lungs of cystic fibrosis patients, and its multidrug-resistance phenotype makes *S. maltophilia* infection often difficult to be eradicated (Ryan et al., 2009). In essence, *S. maltophilia* is an emerging human pathogen that increasingly challenges clinicians, microbiologists, and infection-control specialists with difficult situations (Looney et al 2009).

1.1 Associations of *Stenotrophomonas* with plants

Stenotrophomonas species, especially *S. maltophilia* and *S. rhizophila*, are often found in association with plants. These bacteria can be isolated from the rhizosphere or from internal plant tissues, particularly from the vascular tissues of the root and stem. Endophytic strains of *S. maltophilia* have been isolated from the roots of many plant species (Berg et al., 1996).

Most *Stenotrophomonas* spp. are highly adaptable to hostile and nutrient-limited environments. Several factors have a known or suggested influence on the ability of *Stenotrophomonas* spp. to colonize and survive on plant surfaces. The establishment of interactions between plants and microorganisms in the rhizosphere is preceded by the movement of free-living microorganisms towards the plant roots and can involve chemotaxis towards attractants that are present in plant root exudates. In addition to flagellar motility, other contributing factors to the colonization of plant tissues include a high bacterial growth rate, vitamin B1 synthesis, the exudation of NADH dehydrogenase and bacterial lipopolysaccharides (LPSs), particularly the O antigen (Denton and Kerr, 1998). Furthermore, many *Stenotrophomonas* spp. can produce extracellular enzymes (proteases, lipases, nucleases, chitinases and elastases) that have been shown to be important in plant colonization by other rhizosphere microorganisms.

S. maltophilia produces various pili or fimbriae that have been implicated both in adhesion to surfaces and in the formation of complex biofilms (de Oliveira-Garcia et al., 2003). Adhesion and biofilm formation might contribute to the ability of *S. maltophilia* to compete with other microorganisms on the surface of plant roots, and are certainly important for the colonization of medical devices that leads to infection in humans. Type-

1 fimbriae (SMF-1) has been characterized from *S. maltophilia* type strain ATCC 13637. SMF-1 fimbriae are composed of a 17 kDa fimbrin subunit that shares significant amino-terminal amino acid sequence similarity to the CupA fimbriae of *Pseudomonas aeruginosa* and to several fimbriae from pathogenic *Escherichia coli*. All of the clinical *S. maltophilia* isolates that were tested produced the 17 kDa fimbrin. The genomes of *S. maltophilia* strains K279a and R551-3 contain also genes that encode type I pili and type IV pili: the first have been implicated in adhesion and in the early stages of biofilm formation, while the second have been implicated in adherence, auto-aggregation, twitching motility and biofilm formation. These gene clusters are distributed throughout each genome of the two strains in a similar manner, which may indicate that there are some similarities in the plant and animal colonization strategies (Ryan et al., 2009).

Stenotrophomonas epiphytic bacteria (that grows on or attaches to the surface of a living plant) can also alter the properties of the leaf surface to which they attach. Has been demonstrated that a strain of *S. maltophilia* can increase the water permeability of *Hedera* and *Prunus* cuticles, which in turn should increase the availability of water and dissolved compounds in the phyllosphere, thereby enhancing the environmental growth conditions for the bacteria (Schreiber et al., 2005). The molecular mechanism (or mechanisms) responsible for the observed effects is as yet unknown, although it has been proposed that extracellular enzymes that degrade the cutin polymer and/or plasticizers, such as biosurfactants, could be responsible (Ryan et al., 2009).

The synthesis of compatible solutes by bacteria contributes to their survival under the changing osmolarities that occur in the rhizosphere. *S. maltophilia* accumulates trehalose as the only compatible solute, whereas *S. rhizophila* produces glucosylglycerol in addition to trehalose. These sugars often accumulate intracellularly and protect against various stresses. *S. maltophilia* K279a encodes proteins, which are involved in the biosynthesis of trehalose through the degradation of glycogen. Genes encoding the enzymes for trehalose biosynthesis that involves the conversion of maltose, are carried on another region of the *S. maltophilia* K279a genome, which is absent from *S. maltophilia* R551-3. Both *S. maltophilia* K279a and *S. maltophilia* R551-3 can also produce trehalose from glucose through the trehalose-6 phosphate synthase- trehalose-6-phosphate phosphatase pathway (encoded by *otsA* and *otsB*). However, unlike the clinical isolate *S. maltophilia* K279a, the environmental isolate *S. maltophilia* R551-3 does not encode the pathway to use trehalose and therefore cannot use this sugar as a sole carbon source (Taghavi et al., 2009).

S. maltophilia might also have the capacity to protect itself from predation by soil protozoa, which could confer a selective advantage over other bacteria. The genome of the environmental isolate *S. maltophilia* R551-3 contains a cluster of genes, which is absent from the *S. maltophilia* K279a isolate. The *rebA-C* genes encode refractile inclusion bodies, known as R bodies, which are toxic to sensitive species of *Paramecium*, a genus of unicellular ciliate protozoa that live in freshwater environments. These proteins could conceivably have a role in the defence of bacteria against predation by protozoa in the rhizosphere or bulk soil (Ryan et al., 2009).

1.2 Biotechnological uses of *Stenotrophomonas* spp.

Stenotrophomonas spp. are promising candidates for biotechnological applications in agriculture; treatment with *Stenotrophomonas* spp. can result in enhanced plant growth and can influence plant development on marginal soil. For example, plant growth promotion of up to 180% was observed for many vegetables in the highly salinated soils of Uzbekistan (Berg et al., 2010). *Stenotrophomonas* spp. also have promising applications in bioremediation and phytoremediation, as these bacteria can metabolize a large range of organic compounds that are present in the rhizosphere, including phenolic compounds found in plant root exudates. These metabolic properties could provide plants with protection against the phytotoxic effects of these compounds. *Stenotrophomonas* spp. can enhance plant productivity by several mechanisms, including the production of the plant growth hormone, nitrogen fixation and the oxidation of elemental sulphur, which in turn provides sulphate for the plants (Ryan et al., 2009).

Many *S. maltophilia* strains have intrinsic resistance to various heavy metals and are than able to tolerate various toxic metals, such as cadmium, lead, cobalt, zinc, mercury and silver. The tolerance of *S. maltophilia* strains to heavy metals could be useful in the bioremediation of soils that are polluted with heavy metals and xenobiotics (Page et al., 2008). A different complement of genes that specify metal tolerance has been identified in the *S. maltophilia* R551-3 and K279a genomes. Genes coding for copper and mercury resistance, located on a genomic island in *S. maltophilia* K279a, were absent from the *S. maltophilia* R551-3 genome. Conversely, loci coding for arsenic resistance and two tellurium resistance proteins were identified in the *S. maltophilia* R551-3 genome but were absent from the *S. maltophilia* K279a genome (Ryan et al., 2009).

The potential of *Stenotrophomonas* spp. for the biocontrol of fungal and bacterial pathogens has been demonstrated in several systems that include both monocotyledonous and dicotyledonous crops as hosts.

Stenotrophomonas spp. can prevent the growth or activity of plant pathogens by production of antifungal compounds (maltophilin and xanthobaccin) and volatile organic compounds with antifungal activity. *S. maltophilia* strains have an extraordinarily high hydrolytic potential; they produce diverse proteases, chitinases, glucanases, DNases, RNases, lipases and laccases. Both chitinolytic and proteolytic activities contribute to the biocontrol activity of *S. maltophilia*. Chitinases might protect plants against fungal pathogens through fungal cell wall lysis but might also have a role in triggering plant defence mechanisms. Another factor that is important for the control of fungal infection is competition for iron. *Stenotrophomonas* cells can efficiently capture siderophores that are produced by other microorganisms, such as ferrichrome, which is produced by fungi of several genera. Both of the sequenced *S. maltophilia* genomes encode many TonB-dependent receptors (TBDRs), outer membrane proteins that are primarily known for the active transport of iron-siderophore complexes in Gram-negative bacteria. There are major differences, however, in the complement of genes encoding TBDRs; the *S. maltophilia* R551-3 genome carries 82 such genes compared with 65 in the *S. maltophilia* K279a genome. Many of the genes that are present in *S. maltophilia* R551-3 but absent from *S. maltophilia* K279a are linked to genes encoding proteins that are involved in iron transport and siderophore uptake. Such an over-representation of TBDRs is found in only a limited number of organisms, but is common in *Xanthomonas* spp. and in aquatic bacteria that scavenge complex carbohydrates. Although both *S. maltophilia* R551-3 and K279a produce the siderophore enterobactin, this additional capacity for iron uptake suggests that iron competition with other organisms for endophytic (or rhizospheric) growth is important.

S. maltophilia strains also produce bioactive compounds, including antibiotics and enzymes. Several proteases produced by *Stenotrophomonas* spp. are so much more effective than those currently in use in industry that it is thought these proteases could 'revolutionize' washing agents. Although a wide range of biologically active compounds has been isolated from *Stenotrophomonas* spp., these organisms still remain an untapped source of novel natural products (Ryan et al., 2009).

1.3 Antimicrobial resistance

Many strains of *S. maltophilia* are also well known for their multiple antibiotic resistance phenotypes, which is consistent with the elevated antibiotic and bactericidal selection pressure that is found in their biotopes. Multiple antibiotic resistance could help *S. maltophilia* to compete in the

rhizosphere (Crossman et al., 2008), which supports intense microbiological activity and competition in comparison to the nutrient-limited bulk soil. Comparative genomic analysis and experimental data of the clinical K279a and endophytic R551-3 *S. maltophilia* showed that many of the genes that encode antimicrobial drug resistance are conserved. The presence of these genes indicates that the endophytic and clinical strains have a similar level of antibiotic resistance, with possibly an even broader resistance spectrum for the endophytic strain *S. maltophilia* R551-3. In both strains, most antibiotic resistance genes are not associated with mobile genetic elements, which makes it unlikely that *S. maltophilia* K279a acquired its antibiotic resistance genes in the clinical environment (Ryan et al., 2009).

S. maltophilia exhibits high-level intrinsic resistance to a variety of structurally unrelated antibiotics, disinfectants, and heavy metals. Sequencing of the *S. maltophilia* K279a genome showed numerous resistance genes, such as genes encoding for multidrug-efflux pumps, β-lactamases, and aminoglycoside-modifying enzymes. Multidrug-efflux pumps and low permeability of the outer membrane are major determinants of the intrinsic antibiotic resistance of this opportunistic pathogen. *S maltophilia* can also acquire resistance through the uptake of resistance genes located on integrons, transposons, and plasmids (Looney et al., 2009).

Resistance to β-lactams arises from the expression of two inducible β-lactamases: L1 and L2. L1 is a class B Zn^{2+}-dependent metalloenzyme that hydrolyses all classes of β-lactams except the monobactams (Okazaki and Avison, 2008). L2 is a class A serine active site β-lactamase that is inhibited by clavulanic acid. The production of both β-lactamases, when cells are exposed to β-lactams, is controlled by the same regulator (AmpR); however, L1 production requires more AmpR than does L2 production.

Resistance to quinolones is mediated primarily by over expression of efflux pumps (in particular SmeDEF) and possibly low permeability of the outer membrane (Looney et al., 2009).

Resistance of *S. maltophilia* to aminoglycosides can be due to aminoglycoside-modifying enzymes, efflux pumps, and temperature-dependent resistance due to outermembrane protein changes. The temperature-dependent variation in susceptibility to aminoglycosides and polymyxin B has been linked to outer-membrane lipopolysaccharide characteristics. *S. maltophilia* can alter the size of the O-polysaccharide and the phosphate content of lipopolysaccharide at different temperatures, and shows greater resistance to aminoglycosides and polymyxin B at 30°C than at 37°C (Elbain et al., 2003).

S. maltophilia also possesses numerous heavy-metal resistance mechanisms, and can tolerate silver-lined catheters.

Selection of an appropriate antimicrobial regimen for the treatment of *S. maltophilia* infection is a challenge given the high-level intrinsic resistance and increasing resistance prevalence of this opportunistic pathogen and the uncertainties related to in-vitro susceptibility testing.

1.4 Adaptation and metabolic versatility

Stenotrophomonas spp. can efficiently colonize such different biotopes as plants, humans and marine environments. Comparative studies of the recently determined genome sequence of the endophytic *S. maltophilia* strain R551-3 with that of the clinical isolate *S. maltophilia* K279a have provided insight into functions that could be associated with adaptation to these different niches. Approximately 85% of the 4,175 *S. maltophilia* R551-3 genes are homologous to genes from *S. maltophilia* K279a, and have the same organization in both strains. This indicates that the ancestral *S. maltophilia* core genome had to be well equipped to allow survival, colonization and competition for resources in such a wide range of biotopes. Divergence in the gene complement of the two organisms might reflect adaptation to specific niches (Ryan et al., 2009).

The *S. maltophilia* R551-3 genome contains 27 regions that are absent from *S. maltophilia* K279a; the size of these regions ranges from 1.7 kb to 61.5 kb. Six of these regions represent putative genomic islands, as characterized by different codon usage from the rest of the genome, location next to a tRNA gene and/or the presence of a flanking integrase gene. By contrast, the genome of *S. maltophilia* K279a contains 19 regions that are absent from *S. maltophilia* R551-3, five of which are putative genomic islands (Crossman et al., 2008). These islands are often inserted at the same positions in both the *S. maltophilia* R551-3 and K279a genomes, which points to the existence of insertion hotspots in the core genome of this species (Figure 3). In addition to genomic islands, the *S. maltophilia* R551-3 genome carries 14 complete insertion sequence (IS) elements: five copies of the IS*481* family, three copies of the IS*3* family (identical to IS*Psy9* from K279a) and six intact copies and two truncated copies of the IS*110* family. In addition, two phages were found, one of which also occurs in *S. maltophilia* K279a (Ryan et al., 2009).

The genomic comparison between *S. maltophilia* R551-3 and K279a, showed a different distribution of many genes potentially involved in colonization and adaptation to different host. *S. maltophilia* R551-3 encodes three filamentous haemagglutinins. One of these has a low level of amino acid sequence similarity (only 53% identity) with its *S. maltophilia* K279a

homologue compared with the average similarity of proteins that are shared by the two strains. An adhesin that is specific to *S. maltophilia* K279a is located on a putative complex transposon. Filamentous haemagglutinin proteins are adhesins that are secreted by the type V protein secretion pathway and are involved in cell-cell aggregation. These filamentous haemagglutinins have also been shown to mediate contact between the phytopathogen *Xylella fastidiosa* and plant cells and are important virulence factors that are involved in the adhesion of *Bordetella pertussis* to mammalian host cells.

The differences between *S. maltophilia* K279a and *S. maltophilia* R551-3 could be related to niche adaptation or host preference (Ryan et al., 2009).

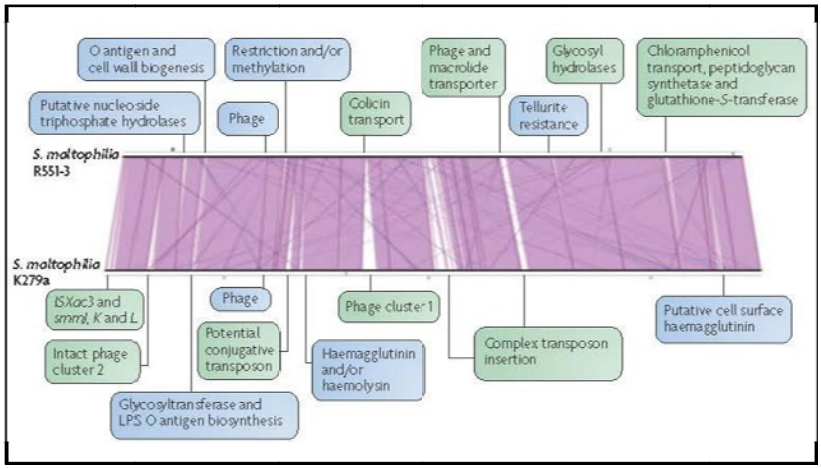


Figure 3. Comparison of gene content and organization in the genomes of *Stenotrophomonas maltophilia* R551-3 and K279a. Syntenic regions between *S. maltophilia* R551-3 and K279a are displayed in purple, and the location and putative functions of selected genes within regions that are specific to each strain are indicated. The green boxes represent putative genomic islands whereas the blue boxes represent virulence genes. LPS, lipopolysaccharide (Ryan et al., 2009).

Many studies have examined the lipopolysaccharide (LPS) in *S. maltophilia* in an effort to assess its contribution to antimicrobial resistance

in this organism and as a basis for serotyping. Mutations in *manA*, *rmlA* and *rmlC*, which encode enzymes involved in the biosynthesis of LPS, affect LPS structure in *S. maltophilia*. Adjacent to these genes in the sequenced genomes is a locus that is also probably involved in LPS biosynthesis and that shows considerable variability between the two *S. maltophilia* strains. Differences in the complement and nature of the genes in this locus are indicative of alterations in the structure of LPS, particularly the O antigen moiety. Serotyping of heat-stable O antigens from *S. maltophilia* has revealed a considerable level of variation between isolates, with 31 defined serotypes. Variation in LPS biosynthetic gene clusters between strains is common in bacterial pathogens of animals, for which it might have a role in evading the host immune system. The role of differences in O antigen or LPS structure between related plant pathogenic or plant associated bacteria is less certain, although involvement in host-range selection and specificity has been proposed. Serotype analysis of a range of *S. maltophilia* strains, however, shows no clear delineation between clinical and environmental isolates. Furthermore, other roles for variations in O antigen structure, such as promoting insensitivity to phage infection, should not be overlooked (Ryan et al., 2009).

The R551-3 genome encode genes that are known to be involved in phytopathogenesis *Xanthomonas* spp., such as cellulase (glycosyl hydrolase family 5) type I, type II (*Sec*), type IV, type V and the twin arginine transporter (TAT) secretion systems. Neverthless, the *S. maltophilia* K279a and R551-3 genomes do not seem to encode the virulence factor type III and type VI secretion system, as well as certain plant cell wall degrading enzymes, such as pectinases. In addition, neither genome carries the zonula occludens toxin (*zot*) gene that was identified in several clinical isolates of *S. maltophilia*. This gene is similar to the *zot* gene in *Vibrio cholerae*, which encodes the major virulence factor enterotoxin (Hagemann et al., 2006). Functional genomic analysis will allow an investigation of the factors that are important for the association of *Stenotrophomonas* spp. with plants (Ryan et al., 2009).

1.5 *S. maltophilia*: an opportunistic pathogen

S. maltophilia is an important opportunistic pathogen, affecting the hospitalised and debilitated host, that in recent years has been isolated with increasing frequency from the airways of cystic fibrosis (CF) patients.

S. maltophilia is the only species of *Stenotrophomonas* that is known to cause human disease, but there is considerable phylogenetic and phenotypic variability among *S. maltophilia* isolates, including those from patients in a single hospital. This is probably the result of the many environmental niches of this bacterium; most infections are likely to reflect contact with separate environmental sources (Ryan et al., 2009). Indeed, there are few instances of outbreaks of *S. maltophilia*, and those that occur are caused by a single contaminated source, such as a water source. Despite this, there is evidence that certain phylogenetic groups are better able to cause infection than others (Looney et al., 2009). There are three major risk factors for infection: severe debilitation and/or neutropenia; the presence of indwelling devices such as ventilator tubes and/or intravenous catheters for prolonged periods; and multiple and/or prolonged courses of broad-spectrum antimicrobial drugs. The bacteraemia isolate *S. maltophilia* K279a carries several genes that encode factors that could allow this strain to adhere to surfaces and form biofilms, which are both key factors in the colonization of indwelling devices. Studies have shown that *S. maltophilia* isolates have cytotoxic effects *in vitro* against Hep-2, Vero and Hela cell lines after 24 hours (Figuereido et al., 2006), and *S. maltophilia* K279a killed almost all of the N2 *Caenorhabditis elegans* in the assay within 24 hours (Fouhy et al., 2007). However, as one might expect of a true opportunist, *S. maltophilia* has no type III secretion system (Crossman et al., 2008). A recent study by Waters and colleagues (Waters et al., 2007) tried to address the lack of documentation of the potential of *S. maltophilia* for virulence by investigating the immunostimulatory properties of 24 *S. maltophilia* clinical respiratory and non-respiratory isolates (from blood, skin and soft tissue). In this study, which involved a neonatal mouse model of pneumonia and macrophage cell lines, they determined the rates of pneumonia, bacteraemia and mortality, as well as the inflammatory response that is elicited by *S. maltophilia* infection. They demonstrated that the respiratory and non-respiratory *S. maltophilia* isolates were highly immunostimulatory but weakly invasive, which indicates that these isolates could contribute to airway inflammation.

Whether *S. maltophilia* clinical isolates are colonizers or true pathogens is still controversial in some cases. This is particularly the case for pneumonia, because it is rare to culture pure *S. maltophilia* from the lungs,

and severely debilitated patients are often colonized asymptomatically (Ryan et al., 2009).

The attributable mortality for *S. maltophilia* pneumonia (as far as this can be accurately quantified in patients with multiple and complex pathologies) is estimated at 20-30%. As many as 25% of adult patients with cystic fibrosis carry *S. maltophilia* in their lungs at any one time, although these numbers are highly variable (Steinkamp et al, 2005). Although the rates are variable, approximately 1% of all nosocomial bacteraemias are caused by *S. maltophilia* and the attributed mortality has been estimated at approximately 25% (Ryan et al., 2009).

The primary reason for the increase in *S. maltophilia* infections is the intrinsic resistance of this species to many front-line antimicrobials, such as β-lactams, including carbapenems, aminoglycosides (except gentamicin), macrolides, tetracycline, chloramphenicol and older quinolones (Zhang et al., 2001). Furthermore, *S. maltophilia* isolates can rapidly develop resistance to newer fluoroquinolones, gentamicin and minocycline through mutation; the underlying mechanisms are not certain, but are likely to be the result of the overproduction of intrinsic efflux pumps. Typically, empiric therapy for *S. maltophilia* is trimethoprim-sulphamethoxazole (TMP-SMX), to which > 95% of isolates are sensitive. However, resistance is increasing as a result of the spread of acquired mobile resistance determinants and, in many patients, TMP-SMX therapy is contra-indicated. For how long these drugs would remain clinically efficacious if they were used as front-line therapy is uncertain, however, as resistant mutants arise spontaneously at high rates *in vitro*.

The *de novo* mobilization of antibiotic resistance genes from environmental bacteria or opportunistic pathogens has contributed greatly to the increase in antibiotic resistance in more common and important pathogens. One potential consequence of the increased colonization of patients by *S. maltophilia* is the mobilization of its L1 metallo-β-lactamase gene onto a plasmid that can then confer broad-spectrum β-lactam resistance, including to the last-line carbapenems, in *P. aeruginosa* and even the enterobacteriaceae. Another possibility is the mobilization of a potential quinolone resistance determinant, *qnr*, that was identified in the *S. maltophilia* K279a and R551-3 genome sequences.

Several factors may contribute to the pathogenicity of *S. maltophilia*, as the ability to elaborate a wide range of extracellular enzymes, such as lipases, fibrolysin, and proteases, potentially involved in the colonization process (Crossman et al., 2008), adhere to and form biofilm on epithelial cells (Pompilio et al., 2010), replicate and persist in the lung (Rouf et al., 2011). Deterioration of lung function associated to *S. maltophilia* infections

is widely documented by infection assays carried out in mice (Di Bonaventura et al., 2010).

Adhesion to airway epithelial cells has been shown to require type-1 fimbriae and flagella components (de Oliveira-Garcia et al., 2002, 2003). Moreover, *S. maltophilia* elaborates a wide range of extracellular enzymes, including proteases and esterase, the roles of which in the colonization of pulmonary tissues of CF patients remain largely unexplored (Crossman et al., 2008). The major extracellular protease of *S. maltophilia* (StmPr1), has been purified and shown to be able to degrade in vitro several human proteins, to damage cultured human fibroblasts and to inactivate components of the host immune response. The gene, termed *StmPr1,* codes for a 63-kDa precursor that is processed to the mature protein of 47 kDa. The enzyme is an alkaline serine protease that, by sequence homology and enzymic properties, can be further classified as a new member of the family of subtilases. It differs from the classic subtilisins in molecular size, in substrate specificity, and probably in the architecture of the active site (Windhorst et al., 2002). Esterases in general represent a group of hydrolytic enzymes of broad natural variety concerning substrate and reaction type. However, the physiological functions of many esterases are not clear. Some of these enzymes are known to be involved in metabolic pathways connected to virulence (Talker-Huiber et al., 2003).

There is an increasing appreciation of the polymicrobial nature of human infections and of the potentially important role for interspecies interactions in bacterial virulence and response to therapy. *S. maltophilia* can be found together with the opportunistic pathogen *P. aeruginosa* in diverse niches, including the lungs of patients with cystic fibrosis, where *S. maltophilia* can protect antibiotic-sensitive strains of *P. aeruginosa* by degrading antibiotics. *S. maltophilia* possesses a cell-cell signalling system that is mediated by a diffusible signal factor (DSF) that was first identified in the related plant pathogen *X. campestris*. In *S. maltophilia* the DSF can be one of eight structurally related fatty acids that include *cis*-11-methyl-2-dodecenoic acid, the DSF signal from *X. campestris*. Genome analyses revealed that *S. maltophilia* does not synthesize *N*-acyl homoserine lactones (*N*-AHls) or autoinducer 2 (AI2), which are signal molecules that are commonly found in other Gram-negative organisms, and *N*-AHls have not been detected in *S. maltophilia* cultures. In the clinical isolate *S. maltophilia* K279a, DSF signalling controls several functions, including the production of extracellular enzymes, aggregative behaviour and virulence in a nematode model. Interspecies signalling can also influence *P. aeruginosa*: although *P. aeruginosa* does not synthesize DSF, it can respond to the signal that is produced by *S. maltophilia* by altering biofilm architecture and increasing

tolerance to the cationic antimicrobial peptides polymyxin B and colistin. These observations indicate that modulation of bacterial behaviour through DSF-mediated interspecies signaling with *S. maltophilia* is a phenomenon that could also occur in a non-pathogenic context in rhizospheric or endophytic communities (Ryan and Dow, 2008; Ryan et al., 2008).

AIM OF WORK

Stenotrophomonas maltophilia is a an environmental organism, that in recent years has been isolated with increasing frequency as an opportunistic pathogen in nosocomial infections as well as from the airways of cystic fibrosis (CF) patients. Despite its growing clinical importance, the mechanisms adopted by this organism to establish persistent infections have not been clarified yet. The availability of information on genome sequence of two *S. maltophilia* strains (K279a and R551-3), allow development of new post-genomic approaches direct to understanding of key factors involved in the colonization of respiratory tract of CF patients and of cellular and molecular mechanisms of its pathogenicity. Identification of *S. maltophilia* genes expressed inside host, and so involved in colonization, is essential for development of new antimicrobial compounds. Moreover, molecular and genetic characterization of *S. maltophilia* clinical strains is essential to understand pathogenicity mechanism of these organisms and to build detailed epidemiological maps to draw their spread in CF patients.

This project will follow three main lines of research.

Genotyping analysis and genetic relatedness of *S. maltophilia* strains. Due to the enormous genomic variability showed by clinical strains, it is very important the identification of clones able to cause disease in CF patients. The genetic relatedness of 52 *S. maltophilia* strains, collected from various environmental and clinical sources, including cystic fibrosis patients, will be evaluated by different molecular techniques: macrorestriction analysis of chromosomal DNA by Pulsed Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphisms (RFLP) of the *gyrB* gene and characterization of the variable regions within the 16S rDNA.

Identification and characterization of virulence genes involved in *S. maltophilia* pathogenicity. To get further insights on the virulence of *S. maltophilia* we will characterize the strains for the presence (by PCR amplification) and expression (by biological assay) of some virulence-associated genes, known in *S. maltophilia* or in other pathogens associated with CF: type-1 fimbria, proteases, esterase and other extracellular enzymes. Representative *S. maltophilia* strains impaired in the expression of the virulence factors investigated, will be assayed by *in vivo* tests by using *Galleria mellonella* larvae as an infection model.

In order to determine the relative importance of each potential virulence factor identified and to clarify the role in *S. maltophilia* pathogenesis, we will construct K279a (the clinical reference strains) mutants in the putative virulence genes.

Genomic evolution of *S. maltophilia* toward a pathogenic lifestyle. It is widely accepted that the evolution of bacterial pathogens from harmless ancestors mainly depends on the acquisition of virulence gene clusters by horizontal gene transfer. In order to identify the specific genomic fragments acquired during the evolution toward the pathogenicity, we will compare the genome of the environmental strain LMG959 to that of the clinical strain K279a, by Suppression Subtractive Hybridization. This technique will allow us to isolate specific DNA sequences that could differentiate K279a from the avirulent LMG959.

RESULTS

1) GENOTYPING ANALYSIS AND GENETIC RELATEDNESS OF *S. maltophilia* STRAINS

Molecular and genetic characterization of *S. maltophilia* clinical strains is essential to understand the pathogenicity mechanism of these organisms and to build detailed epidemiological maps to draw their spread in CF patients.

A total of 52 *S. maltophilia* strains were analysed in this study (Table 1). Strains included 43 clinical isolates collected, between January 2003 and December 2005, from 43 independent patients (41 were from CF patients, and two from blood cultures of two non-CF patients) attending the Paediatric Hospital "Bambino Gesù" of Rome, Italy. Other strains were: K279a (Avison et al., 2001) and LMG958/ATCC13637 (Hugh and Leifson, 1963), two *S. maltophilia* reference clinical strains, and seven strains of environmental origin (two isolated within the Hospital "Bambino Gesù" and five reference strains from the LMG Collection, Belgium). Isolates were identified as *S. maltophilia* by the API-20NE (bioMerieux, La Balme Les Grottes, France) system and 5' end sequencing of the 16S rRNA gene.

To investigate on the genetic relatedness among the 52 *S. maltophilia* strains analyzed in this study (Table 1), we used PFGE, RFLP of the *gyrB* gene profiles and sequencing of the hypervariable regions of the 16S rRNA gene.

1.1 *S. maltophilia* macrorestriction profile analysis

Of the many molecular methods currently available, macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) has been shown to be particularly useful for the clustering and differentiation of many bacterial pathogens. Although the sensitivity and discriminatory power of macrorestriction profile analysis depend on the organism being subtyped and the restriction enzyme used, its high epidemiologic relevance has made this technique the "gold standard" for subtyping bacterial pathogens (Hunter et al., 2005).

Total DNA preparations of all strains tested were digested with the endonuclease *Xba*I and subjected to PFGE analysis. Fifteen to 20 bands were used for the scoring and computer analysis of each *S. maltophilia* strain. All strains produced well-defined PFGE-profiles and a similarity dendrogram was generated (Figure 4). Forty-seven different PFGE pulsotypes were identified, indicating a remarkable genomic diversity

among the strains analyzed. Similarity ranged from 78 to 100% and Simpson's index of diversity (DI) was 0.99. Three clusters (pulsotypes 1, 17 and 33) showed a banding pattern identical to at least 1 other isolate (Figure 4), likely indicating either cross-transmission among patients or infection from a common source. One of the two pulsotype 33 strains was isolated from the sputum of a CF patient (OBGTC19) and the other from a blood culture of an non-CF patient (Stm3), both patients attending the Hospital "Bambino Gesù". Strains belonging to pulsotypes 1 (OBGTC9 and OBGTC10) and 17 (OBGTC13, OBGTC23, OBGTC30 and OBGTC42) were isolated from independent CF patients (Table 1 and Figure 4). Interestingly, the two pulsotype 1 strains were isolated, from two patients attending the same Hospital unit chronically infected by the same clone of *S. maltophilia* (100% similarity coefficient).

Table 1. Relevant characteristics of the 52 *S. maltophilia* strains.

Strain[a]	PFGE type[b]	*gyrB* RFLP[c]	16S rRNA group[d]	PCR analysis of genes encoding[e]					Activity[f]	
				smf-1	*StmPr1*		*StmPr2*	Smlt3773		
				674 bp	1621 bp	868 bp	1764 bp	1342 bp	Protease	Esterase
OBGTC1	44	1	1	+	+	-	+	+	+	+
OBGTC2	36	1	1	+	+	-	+	+	+	+
OBGTC3	30	1	1	+	+	-	+	+	+	+
OBGTC4	12	1	1	+	+	-	+	+	+	+
OBGTC5	27	1	1	+	+	-	+	+	+	+
OBGCT8	26	1	1	+	+	-	+	+	+	+
OBGTC9, 10	**1**	**1**	**1**	**+**	**+**	**-**	**+**	**+**	**+**	**-**
OBGTC12	13	1	3	+	-	-	+	+	+	+
OBGTC14	42	1	3	+	+	-	+	+	+	+
OBGTC15	4	1	3	+	-	-	+	+	+	+
OBGTC16	14	1	1	+	+	-	+	+	+	+
OBGTC17	32	1	2	+	-	-	+	+	+	-
OBGTC18	25	1	1	+	+	-	+	+	+	+
OBGTC20	21	1	1	+	+	-	+	+	+	+
OBGTC21	22	1	1	+	+	-	+	+	+	+
OBGTC22	37	1	1	+	+	-	+	+	+	+
OBGTC25	39	1	1	+	+	-	+	+	+	+
OBGTC28	9	1	3	+	+	-	+	+	+	+
OBGTC29	43	1	1	+	+	-	+	+	+	+
OBGTC31	40	1	M	+	+	-	+	+	+	+
OBGTC32	2	1	2	+	-	-	+	+	+	+
OBGTC33	20	1	1	+	+	-	+	+	+	+
OBGTC34	34	1	1	+	+	-	+	+	+	+
OBGTC36	10	1	1	+	+	-	+	+	+	+
OBGTC38	35	1	2	+	+	-	+	+	+	-
OBGTC39	16	1	2	+	+	-	+	+	+	+
OBGTC44	19	1	1	+	+	-	+	+	+	+
OBGTC45	23	1	1	+	+	-	+	+	+	+
OBGTC13, 23,30,42	**17**	**2**	**3**	**+**	**+**	**+**	**+**	**+**	**-**	**-**

OBGTC7	29	3	3	+	-	-	+	+	+	+
OBGTC24	45	3	1	+	+	-	+	+	+	+
OBGTC26	7	3	1	+	+	-	+	+	+	+
OBGTC37	24	3	2	+	+	-	+	+	+	+
OBGTC27	38	4	3	+	-	+	+	+	+	+
OBGTC6	31	5	6	+	+	+	+	+	+	+
OBGTC11	11	6	5	+	-	+	+	+	+	+
OBGTC19, Stm3	**33**	**3**	**1**	**+**	**+**	**-**	**+**	**+**	**+**	**+**
Stm2	41	1	1	+	-	+	+	+	+	+
K279a	5	1	1	+	+	-	+	+	+	+
LMG 958	18	1	1	+	+	-	+	+	+	+
LMG 959	46	4	M	-	-	+	+	+	+	+
LMG 10871	15	9	M	-	-	-	+	-	+	-
LMG 10879	3	4	M	-	-	+	+	+	+	+
LMG 11104	47	8	M	-	-	-	+	-	+	-
LMG 11108	6	7	M	-	-	+	+	-	+	-
OBGN1	8	1	M	+	-	-	+	+	+	+
OBGN2	28	4	2	+	+	-	+	+	+	+

[a]Bacterial collections: OBG, "Bambino Gesù" Hospital, Rome, Italy; LMG LaboratoriumvoorMicrobiologie Gent Culture Collection, Universiteit Gent, Belgium. The 41 OBGTC strains were isolated from independent CF patients attending the Hospital "Bambino Gesù"; Stm strains were isolated from blood-culture of non-CF patients; OBGN strains were isolated from the hospital environment; K279a and LMG958 are reference clinical strains; strains LMG959, LMG10871, LMG10879, LMG11104 and LMG11108 are reference strains of environmental origin.

[b]Based on the PFGE profiles shown in Fig. 4. Numbers indicate the 47 different PFGE pulsotypes. Only strains presenting a 100% profile similarity have been assigned the same number of pulsotype.

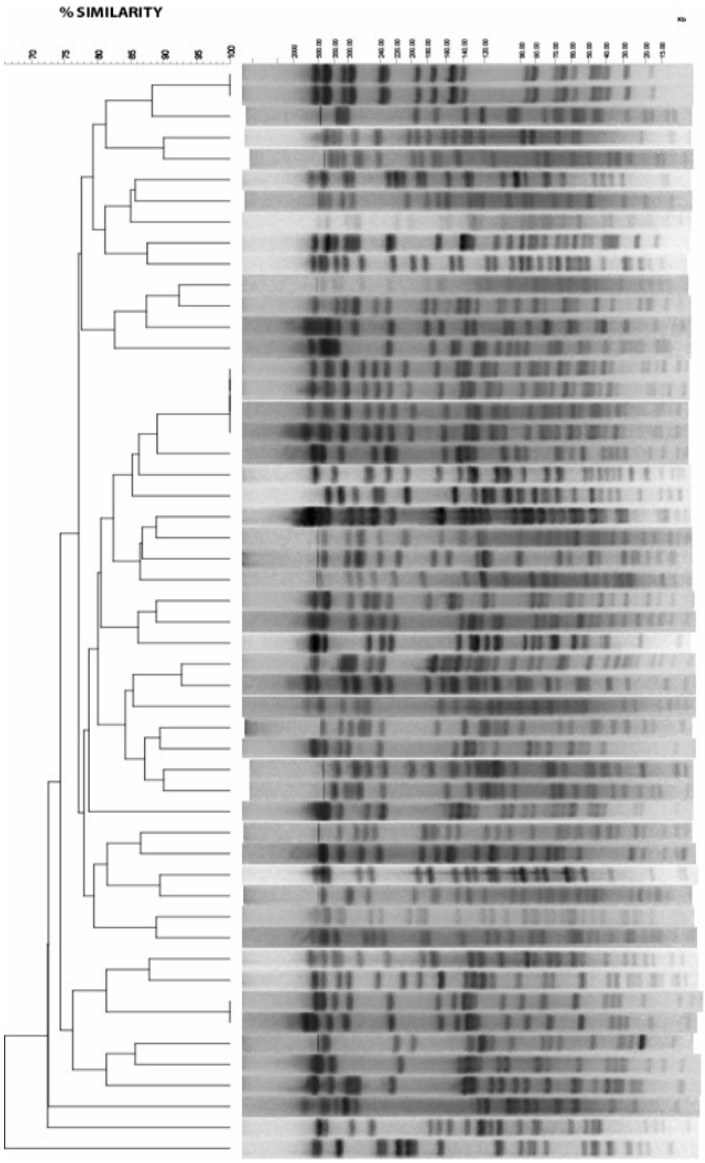
[c] Based on the RFLP profiles shown in Fig. 5. Numbers indicate the 9 different RFLP profiles. Only strains presenting a 100% profile similarity have been assigned with the same number;.

[d]Groups 1–3, 5 and 6 correspond to the 16S rRNA allelic groups previously described by Gould et al.; M, mixed sequences which presented highly divergent signature sequences, not groupable within the allelic groups described by Gould et al. (2006).

[e] +, amplification of the expected DNA fragment; −, no amplification.

[f] +, presence; −, absence of protease or esterase activity, assayed as described in Materials and methods.

Figure 4. Phylogenetic analysis of PFGE *Xba*I profiles of the 52 *S. maltophilia* strains analysed in this study



1.2 RFLP analysis of the *gyrB* gene

The genomic diversity of the 52 *S. maltophilia* strains was analyzed by RFLP analysis of the *gyrB* gene using the *Hae*III restriction enzyme (Coenye et al., 2004). The *gyrB,* is an house-keeping gene encoding the β-subunit of the DNA gyrase (DNA topoisomerase II) responsible for catalysing negative supercoiling of DNA. This gene, which is essential for DNA replication, is present in all bacteria in a single-copy and has been used to differentiate species and estimate the phylogenetic relationships within several genera (Svensson-Stadler et al., 2011).

The *Hae*III-generated *gyrB* RFLP profile consisted of seven to twelve bands in the approximate size range of 40 to 400 bp (Figure 5). Computational analysis distinguished the 52 *S. maltophilia* isolates into nine profiles, arbitrarily designated 1 to 9 (Table 1 and Figure 5). Only strains representative of each RFLP-profile are shown in Figure 5. The majority of CF isolates (38 out of 41) grouped in RFLP profiles 1 to 3. Among non-CF *S. maltophilia* isolates reference strains K279a and LMG958 showed RFLP profile 1, while the two strains isolated from the blood of non-CF hospitalized patients displayed profile 1 and 3. As far the two environmental strains isolated at the Hospital "Bambino Gesù", one showed profile 1 (as the majority of CF isolates) and the other profile 4. Interestingly, only the pulsotypes 17 strains grouped in profile 2.

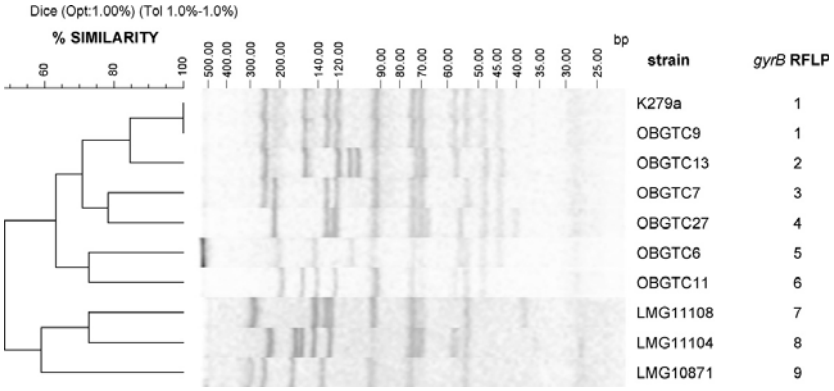


Figure 5. Genotype analysis of *gyrB* RFLP profiles of representative *S. maltophilia* strains included in the study. A percentage genetic similarity scale is shown above the dendrogram. Isolate number and *gyrB* RFLP profiles (numbered 1-9) are shown on the right-hand side of each pattern. Only strains representative of each profile are included in the figure.

1.3 Allelic variations among 16S rRNA genes

To further assess the phylogenetic divergence among strains, a fragment encompassing the hypervariable region of the 16S rRNA gene was PCR-amplified from all strains and sequenced. Sequence analysis confirmed that all strains examined were *S. maltophilia*, with a sequence similarity ranging from 98.0 to 100% (data not shown). Comparative analysis of the allelic variations indicated that all clinical isolates (with the exception of strain OBGTC31) contained signature sequences corresponding to the allelic groups previously described by Gould et al. (2006). As shown in Table 2, the majority of CF isolates (23 out of 41), strains Stm2 and Stm3 (isolated from blood cultures of non-CF patients) and the two reference clinical strains (K279a and LMG958) belonged to 16S rRNA sequence group 1. Five CF-derived strains and the environmental hospital strain OBGN2 belonged to sequence group 2; eleven CF-derived isolates and the OBGN1 environmental isolate to sequence group 3; and the CF-isolates OBTC11 and OBGTC6 to sequence group 5 and 6, respectively. No strain belonging to sequence group 4 was found, while strains indicated as mixed in Table 2 presented highly divergent sequences which were not typable according to the scheme proposed by Gould et al. (2006). As far the three clusters with an identical PFGE pattern, strains with pulsotypes 1 and 33 belonged to sequence group 1, while strains with pulsotype 17 belonged to sequence group 3. According to this scheme, the five reference environmental strains were untypable (Tables 1 and 2).

Table 2. Variable DNA sequences in the variable regions of the 16S rRNA gene of the 52 *S. maltophilia* strains.

Group[a]	Isolates in groups	41-50[b]	55-60[b]	104-109[b]
1	OBGTC1, 2, 3, 4, 5, 8, 9, 10, 16, 18, 19, 20, 21, 22, 24, 25, 26, 29, 33, 34, 36, 44, 45, STM2, STM3, K279a, LMG958	CAGGAGAG	TCTCT	TTTTT
2	OBGTC17, 32, 37, 38, 39, OBGN2	CAGGAGAG	TCTCT	TCTGT
3	OBGTC7, 12, 13, 14, 15, 23, 27, 28, 30, 42, OBGN1	CAGAGGAG	TCCTT	TCTGT
4	No isolates	CAGTAAGAG	TCTTAC	TTTTT
5	OBGTC11	CAAAGGAG	TCCTT	TCTGT
6	OBGTC6	CAGAGGAG	TCCTT	TTTTT
Mixed	OBGTC31, LMG959, 10871, 10879, 11104, 11108	NT	NT	NT

[a]Allelic groups 1-6 were essentially described by Gould et al. (2006).

[b]Nucleotide position within the 500 bp amplified DNA fragment of the 16S rRNA variable regions.

[c]Strains presenting highly divergent sequences of the variable regions. NT, not typable according the scheme described by Gould et al. (2006).

2) IDENTIFICATION AND CHARACTERIZATION OF VIRULENCE GENES INVOLVED IN *S. maltophilia* PATHOGENICITY

Identification of *S. maltophilia* genes involved in colonization, is essential for development of new antimicrobial compound. All the 52 *S. maltophilia* stains were analysed for the presence and/or the expression of the following putative virulence factors.

2.1 Type-1 fimbriae (SMF-1)

S. maltophilia produces SMF-1 fimbriae that have been implicated both in adhesion and biofilm formation (de Oliveira-Garcia et al., 2003). On the basis of the *smf-1* sequence of *S. maltophilia* strain K279a, a complete *smf-1* gene (674 bp fragment) was amplified by PCR from the 43 clinically-derived strains, and from OBGN1 and OBGN2, the two hospital-isolated environmental strains (Table 1). No amplicon was obtained from the five *S. maltophilia* reference strains of environmental origin. As a control, three amplicons obtained from three CF-derived strains were sequenced and their sequences matched (>99%) with that of the *smf-1* gene of *S. maltophilia* strain K279a. The finding that the *smf-1*-encoded fimbriae are present only in clinically-isolated strains, reinforces the hypothesis that these structures might play some role in the colonization of CF patients by *S. maltophilia* (de Oliveira-Garcia et al., 2003). This is in agreement with previous study indicating that in contrast to most *S. maltophilia* clinical isolates, the environmental strain LMG959 (used as prototype), does not produce biofilm (Di Bonaventura et al., 2007).

2.2 Proteases

An important virulence factor for many gram-negative bacteria are extracellular proteases, that are involved in bacterial survival and growth and for invasion and tissue destruction.

In silico analysis showed that an alkaline serine protease-encoding gene is present in *S. maltophilia* reference strain K279a; this gene show some degree of variability (79% DNA similarity) as compared to that of the *StmPr1* gene, encoding the major extracellular protease (an alkaline serine protease) described by Windhorst et al. 2002 (accession No. AJ291488) (Windhorst et al., 2002). Moreover, *S. maltophilia* was found to encode a minor extracellular protease-encoding gene, *StmPr2* (accession No.

AY253983). To screen for the presence of the two allelic variants of the *StmPr1* gene as well as of the *StmPr2* gene, the 52 *S. maltophilia* strains were subjected to PCR analysis by using three primer pairs: i) the first specifically amplify a 1621 bp fragment encompassing the *StmPr1* allelic variant of strain K279a; ii) the second amplify a 868 bp internal fragment of the *StmPr1* gene described by Windhorst et al. 2002**;** and iii) the third amplify a 1764 bp fragment specific for the *StmPr2* gene of strain K279a (Material and Method). As reported in Table 1, a 1621 bp amplicon was obtained from 38 *S. maltophilia* strains, while a 868 bp amplicon resulted from 11 strains (Table 1). Only five strains (OBGTC6, and the four strains with pulsotype 17) positively amplified both the 1621 bp and the 868 bp fragments, while eight strains (five CF and three environmental isolates) were PCR-negative for both fragments (Table 1). All strains positively amplified the 1764 bp fragment corresponding to the *StmPr2* gene. These results indicate that the most clinical isolates carries the *StmPr1* allelic variant present in the K279a genome, and that only six strains carry the *StmPr1* gene described by Windhorst et al. 2006. The sequences of the 1621 bp fragments of the five strains which also amplified the 868 bp fragment showed that the 868 bp fragment is contained within the 1621 bp sequence (accession Nos. FN598883 to FN598887).

Although a *StmPr2* gene is present in all strains analysed, only 48 produced proteolytic activity (Table 1). Interestingly, the four CF-derivative strains of pulsotype 17, that scored negative for protease activity (casein hydrolysis, see Material and Method), correctly amplified the 1621 bp (*StmPr1*) and the 1764 bp (*StmPr2*) fragments (Table 1). The nucleotide sequences of the 1621bp and of the 1764 bp amplicons of these strains (accession Nos. FN598884 to FN598887 and FN598891 to FN598894, respectively) evidenced the presence of frame-shift mutations within the 1621bp and the 1764 bp ORFs (Figure 6 and 7). This well accounts for the lack of protease activity of these strains. The sequence of the 1621 bp fragment of strain OBGTC6 (the other strain positive for the 1621 bp and 868 bp fragments) (accession No. FN598883) also contained mutations which impaired the expression of the major protease, while the sequence of the 1764 bp fragment (accession No. FN598895) indicated a functional *StmPr2* gene, thus accounting for the protease activity of this strain (Table 1). As controls we sequenced two independent 1621 bp amplicons (from OBGTC9 and OBGTC20), one 868 bp amplicon (from OBGTC27, a strain PCR-positive only for the 868 bp fragment) and three 1764 bp amplicons (from strains OBGTC9, OBGTC20 and OBGTC32) which all expressed protease activity (accession Nos. FN598888 to FN598890 and from FN598896 to FN598898). The sequences (Figure 6 and 7) did not show any

frame-shift mutations. Taken together, these results indicate that pulsotype 17 strains have to be considered as naturally-occurring mutants unable to produce both the major and the minor proteases. The protease-positive strains with pulsotypes 1 and 33, carry the major protease allelic variant of *StmPr1*, present on the genome of *S. maltophilia* strains K279a, and the *StmPr2* gene (Table 1).

Figure 6

```
AM743169        MIKKQNLRINVLAAAVLSLTGVGLAQAADLKANAPLSGPKTQQVDGIIVKYRAGSAAAAD 60
OBGTC20,9       MIKKQNLRINVLAAAVLSLTGVGLAQAADLKANAPLSGPKTQQVDGIIVKYRAGSAAAAD 60
AJ291488        MIKKQNLRINVLAAAVLSMTAVGAVHAAGLPTREPVRQASAAQPGTDRIIVKYRAGSAAA 60
OBGTC23         MIKKQNLRINVLAAAVLSMTAVGAVHAAGLPTREPVRQASTAQPGAERIIVKYRAGAAAA 60
OBGTC6          MIRAEPSHQCACRRGAVDDGRRCRPRRRPADP*                              32
OBGTC30         MIKRQEPSHQCACRRRAVDDGCRCRPRCWTADP*                             33
OBGTC13,42      MIRSRTFASMCLPPPCCR*                                            18

AM743169        ANAKLAVVNSAIARAVPAGTNAAARSAALRPQVARKLGIGADLIRLQGGIARAELDKVLG 120
OBGTC20,9       ANAKLAVVNSAIARAVPAGTNAAARSAALRPQVARKLGIGADLIRLQGGIARAELDKVLG 120
AJ291488        GDRSAKLSTVQSALTRASLAGGTARASTLGPQVVRRLGVGADVIRLQGRLAPAELQRVLK 120
OBGTC23         TDRSAKLSTVQSALTRASLSGGTSRASTLGPQVVRKLSTGADLIRVQGRLAPAELQRVLK 120

AM743169        ELKADPTVEYAVADAIMYPIDAASSPRADAVAKSDASPSFVPNDPYYQSHNWHFHNPVGG 180
OBGTC20,9       ELKADPTVEYAVADAIMYPIDAASSPRADAVAKSDASPSFVPNDPYYQSHNWHFHNPVGG 180
AJ291488        ELKADPAVQYAEADVKLRRSELRAGDVQPALAPNDPYYQQYQWHLHNATGGINAPSAWDV 180
OBGTC23         ELKADPSVQYAEADVKLRRTELRAGDVQPALAPNDPYYQQYQWHLHNATGGINAPSAWDV 180

AM743169        VNAPAAWDVSQGEGVVVAVLDTGILPEHPDFAAGTLLEGYDFISQASRSRRAADGRVPGA 240
OBGTC20,9       VNAPAAWDVSQGEGVVVAVLDTGILPEHPDFAAGTLLEGYDFISQASRSRRAADGRVPGA 240
AJ291488        SQGEGVVVAVLDTGILPQHPDLVGNLLEGYDFISDAETSRRATNDRVPGAQDYGDWVEND 240
OBGTC23         SQGEGVVVAVLDTGILSQHPDLVGNLLEGYDFISDAETSRRATNDRVPGAQDYGDWVEND 240

AM743169        LDYGDWMPTANACYDGSPVRDSSWHGTHVTGTIAEATNNGLHTAGLAYKAKVLPVRVLGY 300
OBGTC20,9       LDYGDWMPTANACYDGSPVRDSSWHGTHVTGTIAEATNNGLHTAGLAYKAKVLPVRVLGY 300
AJ291488        NECYTGSVAEDSSWHGTHVAGTVAEQTNNGVGMAGVAHKAKVLPVRVLGKCGGYLSDIAD 300
OBGTC23         NECYTGSVAEDSSWHGTHVARYRGRADQQRRRHGRCRAQGQGAAGPRARQVRWLPFRYRR 300

AM743169        CGGTLSDITDAITWASGGTVAGIPANQNPAEIINMSLGGSGSCDPAYQAAITGATNRGTL 360
OBGTC20,9       CGGTLSDITDAITWASGGSVAGIPANQNPAEIINMSLGGSGSCDPAYQAAITGATNRGTL 360
AJ291488        AITWASGGTVAGVPANANPAEVINMSLGGSGSCDGTYQDAINGAISRGTTVVVAAGNETD 360
OBGTC23         RHHLGVWRHGGRRTRQYQPGRGHQHEPRRQRQLRRDLPGCDQRRDLAGHHRGRGRRQRDR 360

AM743169        VVVAAGNDSMNVANARPANCDGVVSVGATGITGAMAYYSNFGTRIDLSGPGGGVTDGNPN 420
OBGTC20,9       VVVAAGNDSMNVANARPANCDGVVSVGATGITGAMAYYSNFGTRIDLSGPGGGVTDGNPN 420
AJ291488        NASKYRPASCDGVVTVGATRITGGITYYSNYGSRVDLSGPGGGGSVDGNPGGYVWQSGSD 420
OBGTC23         QRLQVPPSQLRRRGDRRRHPHHRRDHLLLELRHPCGPVRSGRWRQCGRQSGRLRLAVRLR 420

AM743169        GYVWQAVSSSKTSPPAAGSTEGYTLGGKAGTSMAAPHVAAVAALVQSALIAANRDPLAPA 480
OBGTC20,9       GYVWQAVSSSKTSPPAAGSTEGYTLGGKAGTSMAAPHVAAVAALVQSALIAANRDPLAPA 480
AJ291488        AATTPESGSYSYMGMGGTSMASPHVAAVAALVQSALIAKGKDPLAPAAMRTLLKETARPF 480
OBGTC23         CGHHAGVGQLQLHGHGRHTDGLAARGCCCCTGAERADRQGQGSAGPGRDAHPAEGDRASV 480

AM743169        GMRTLLKETARPFPVSIPSATPIGTGIVDAKAALDKALEEPCTENCGPVAKPLTNKVAIG 540
OBGTC20,9       GMRTLLKETARPFPVSIPSATPIGTGIVDAKAALDKALEEPCTEN--------------- 525
AJ291488        PVSIPTATPIGTGIVDAKAALAKALEEPCTENCGPVATPLTNKTAVGGLNGTAGSSRLYS 540
OBGTC23         PGRHSGSHPDRYRYSRCQGRAGQGTGRAVHREL--------------------------- 513
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Figure 6. Multiple alignment of StmPr1 protease from *S. maltophilia* strains. Accession numbers of the proteins used for the alignment are: AM743169 (*S. maltophilia* K279a locus Smlt0686; Crossmann et al., 2008) or AJ291488 (Windhorst et al., 2002). Residues underlined indicate regions of identity that result from alignment with the sequences of subtilisin BPN and proteinase K. The amino acids of the catalytic triad Asp, His, and Ser are highlighted in gray. Protein regions that significantly differ from reference sequences AM743169 or AJ291488 are in grey; the premature termination of truncated proteins is marked with an asterisk.

Figure 7

```
AY253983         MSQVTQPRVRRVWVVLGASVLSSLLLATPALAGDVHSAGLQSAPTHQRFIVKYRDGSAPV 60
AM743169         MSQVTQPRVRRVWVVLGASVLSSLLLATPALAGDVQLSGLQSAPTHQRFIVKYRDGSAAV 60
OBGTC9,20,6,32   MSQVTQPRVRRVWVVLGASVLSSLLLATPALAGDVQLSGLQSAPTHQRFIVKYRDGSAAV 60
OBGTC13,30,42    MSRYATACASSVGGPWCVRSVIAAAGHACAGR*                              32
OBGTC23          MSR*                                                            3

AY253983         ANTTALASSLKSAAAGLASSQGRALGLQEVRKLAVGPTLVRTDRPLDQAESELLMRKLAA 120
AM743169         ANTTALASSLKTAAAGLASSQGRALGLQQVRKLAVGPTLVKTDRPLDQAESELLMRKLAA 120
OBGTC9,20,6,32   ANTTALASSLKTAAAGLASSQGRALGLQQVRKLAVGPTLVKTDRPLDQAESELLMRKLAA 120

AY253983         DPNVEYVEVDQIMRATLTPNDTRLSEQWGFGTSNASINVRPAWDKATGTGVVVAVIDTGI 180
AM743169         DPNVEYVEVDQIMRATLTPNDTRFSEQWGFGTSNAGINIRPAWDKATGTGVVVAVIDTGI 180
OBGTC9,20,6,32   DPNVEYVEVDQIMRATLTPNDTRFSEQWGFGTSNAGINIRPAWDKATGTGVVVAVIDTGI 180

AY253983         TNHPDLNANILPGYDFISDAAMARDGGGRDNNPNDEGDWYGANECGSGIPASNSSWHGTH 240
AM743169         TNHADLNANILPGYDFISDAAMARDGGGRDNNPNDEGDWYGANECGSGIPASNSSWHGTH 240
OBGTC9,20,6,32   TNHADLNANILPGYDFISDAAMARDGGGRDNNPNDEGDWYGANECGSGIPASNSSWHGTH 240

AY253983         VAGTVAAVTNNSTGVAGTAFNAKVVPVRVLGKCGGYTSDIADAIVWASGGTVSGVPANAN 300
AM743169         VAGTVAAVTNNSTGVAGTAFNAKVVPVRVLGKCGGYTSDIADAIVWASGGTVSGVPANAN 300
OBGTC9,20,6,32   VAGTVAAVTNNSTGVAGTAFNAKVVPVRVLGKCGGYTSDIADAIVWASGGTVSGVPANAN 300

AY253983         PAEVINMSLGGGGSCSTTYQNAINGAVSRGTTVVVAAGNSNTNVSSSVPANCPNVIAVAA 360
AM743169         PAEVINLSLGGGGSCSTTYQNAINGAVGRGTTVVVAAGNSNTNVSSSVPANCPNVIAVAA 360
OBGTC9,20,6,32   PAEVINLSLGGGGSCSTTYQNAINGAVGRGTTVVVAAGNSNTNVSSSVPANCPNVIAVAA 360

AY253983         TTSAGARASFSNYGTGIDISAPGQSILSTLNTGTTTPGSASYASYNGTSMAAPHVAGVVA 420
AM743169         TTSAGARASFSNYGTGIDISAPGQSILSTLNTGTTTPGSATYASYNGTSMAAPHVAGVVA 420
OBGTC9,20,6,32   TTSAGARASFSNYGTGIDISAPGQSILSTLNTGTTTPGSATYASYNGTSMAAPHVAGVVA 420

AY253983         LMQSVAPSPLSPAQVESIIKSTARPLPGACSGGCGAGIVDANAAVAAAINGGGNPNPGGN 480
AM743169         LMQSVAPSPLSPAQVESIIKSTARPLPGACSGGCGAGIIDANAAVAAAINGGGNPNPGGN 480
OBGTC9           LMQSVAPSPLSPAQVESIIKSTARPLPGACSGGCGAGIIDANAAVAAAINGGGNPNPGGN 480
AY253983         VLQNNVPVTGLGAATGAELNYTVAVPAGSSQLRVTISGGSGDADLYVRQGSAPTDTSYTC 540
AM743169         VLQNNVPVTGLGAATGAELNYTVAVPAGSTQLRVAISGGSGDADLYVRQGSAPTDTTYTC 540
OBGTC9,20,6,32   VLQNNVPVTGLGAATGAELNYTVAVPAGSTQLRVAISGGSGDADLYVRQGSAPTDTTYTC 540

AY253983         RPYLSGNSETCTINSPAAGTWYVRVKAYSTFSGLTLNAQY 580
AM743169         RPYLSGNSETCTINSPAAGTWYVRVKAYSTFSGLTLNAQY 580
OBGTC9,20,6,32   RPYLSGNSETCTINSPAAGTWYVRVKAYSTFSGLT----- 575
```

Figure 7. Multiple alignment of StmPr2 protease from *S. maltophilia* strains. Accession numbers of the proteins used for the alignment are: AY253983 and AM743169 (*S. maltophilia* K279a locus Smlt0861; Crossmann et al., 2008).. Residues underlines indicate regions of identity that result from alignment with the sequences of subtilisin BPN and proteinase K. The amino acids of the putative catalytic triad Asp, His, and Ser are highlighted in gray. Protein regions that significantly differ from reference sequence AY253983 and AM743169 are in grey; the premature termination of truncated proteins is marked with an asterisk.

2.3 Esterase

Esterases are a group of hydrolytic enzymes that are known, in some cases, to be involved in metabolic pathways connected to virulence (Talker-Huiber et al., 2003).

S. maltophilia strain K279a appears to carry an ORF, the Smlt3773 locus, coding for a putative outer membrane esterase which shares a high degree of homology (63% identity and 76% similarity) with *estE* of *Xanthomonas vesicatoria* (Fig. 8). Grounding on the DNA sequence of the Smlt3773 locus, we designed a primer pair (Material and Method) to evaluate the presence of the esterase encoding gene in our *S. maltophilia* strains. An expected 1342 bp DNA fragment was amplified from 49 strains (Table 1). On the other hand, eleven strains failed to express esterase activity (Figure 8): the three environmental strains (LMG10871, LMG11104 and LMG11108), which failed to amplify the 1342 bp amplicon, and eight CF-derived strains, which amplified the expected fragment (Table 1). Interestingly, among the eight CF-derived strains lacking esterase activity we found the two pulsotype 1 strains and the four pulsotype 17 strains (Table 1).

To explain the lack of esterase activity of strains that were PCR-positive for the presence of the Smlt3773 locus, the 1342 bp fragments amplified from the eight esterase-negative strains were sequenced (accession Nos. FN598899 to FN598906). As expected, BLAST analysis revealed the presence of frame-shift mutations which introduced premature stop codons accounting for the lack of esterase activity, while the analysis of two independent 1342 bp amplicons obtained from the esterase-producing strains OBGTC20 and OBGTC32, chosen as positive controls, confirm the presence of wild type esterase (Fig. 8). These results indicate that the esterase encoding locus is highly conserved and also that spontaneous non-functional variants occur at a significant frequency among CF-derived clinical *S. maltophilia* isolates.

Figure 8

```
AM743169,TC20,32 MLLSKRPIRTLMAAAIALAALPAMAGESPYSKAVFFGDSLTDAGYFRPLLPADVRPVTGQ 60
OBGTC9,10        MLLSKRPIRTLMAAAIALAALPAMAGESPYSKAVFFGDSLTDAGYFRPLLPPADVRPVTGQ 60
OBGTC23          MLLSKRPIRTLMAAAIALAALPAMAGESPYSKAVFFGDSLTDAGYYRPLLPAEVRPVTGQ 60
OBGTC36          MLLSKRPIRTLMAAAIALAALPAMAGESPYSKAVFFGDSLTDAGYFRPLLDPGVQPVTGQ 60
OBGTC17          MLLSKRPIRTLMAAAIALAALPAMAGESPYSKAVFFGDSLTDAGYFRPLLPEATQGLSGQ 60
OBGTC42          MLLSKRPIRTLMAAAIALAALPAMAGESPYSKAVFFGDA*                      39
OBGTC13,30       MLLSKRPIRTLMAAAIALAALPAMAGESPYSKAVFFERQPDRQRLLPPAAAGRSASGHRP 53

AM743169,TC20,32 FTTNPGWVWSQQVANYYGLNGAANGNGQNGDNYAVGGARVGVDVPS-AMG--TIPSLKSQ 117
OBGTC9,10        FTTNPGWVWSQQVANYYGLNGAANGNGQNGDNYAVGGARVGVDVPS-AMG--TIPSLKSQ 117
OBGTC23          STTNPGWEWSQFVADYYGTNASPNGNGQTGDNYAVGGARVGVDLTQPAFGNVPVPSLKTQ 120
OBGTC36          FTTNPGWVWSQQVANYYGLNGGANGNGQKGDNYAVGGARVSVDEAG-GLG--AIPSLKSQ 117
OBGTC17          FTTNPGWVWSQQVANYYGLNKDPNGNGQNGDNYAVGGARVSVEGSG-ALG--AIPSLKSQ 117
OBGTC13,30       VHHQSGLGVVAVRRRLLRHQRQPQRQRPDR*                               90

AM743169,TC20,32 AARYLAANGGKADGNVLYTVWGGANDLFAAAAAPA------QAQAIIGAAVTDQIALVGA 171
OBGTC9,10        AARYLAANGGKADGNVLYTVWGGANDLFAAAAAPA------QAQAIIGAAVTDQIALVGA 171
OBGTC23          FANYLAANGGKADPNALYTVWGGANDLFS-ITAPA------QAPAVIGAAVTIQIGIVAS 173
OBGTC36          AARYLAANGGKADGNALYTVWGGANDLFAATRAAAGGASQAQVQGIIGAAVTDQIALVGA 177
OBGTC17          AARYLAANGGKADRNALYTVWGGANDLFAAAGAAGAGAPPAQVQGIIGAAVTDQIALVGA 177

AM743169,TC20,32 LKQAGAQYVLVPNLPNVGLTPAFRGPNAA---TATALSAGYNKALYGGLKQAGIEFIPLD 228
OBGTC9,10        LKQAGAQYVLVPNLPNVGLTPAFRGPNAA---TATALSAGYNKALYGGLKQAGIEFIPLD 228
OBGTC23          LKQAGAQYVMVPNLPDVGITPGFIDRGAAGQAQGTALSTAYNNALYGGLKQAGIEFIPLD 233
OBGTC36          LKQAGAQYVLVPNLPDVGITPQFRGPNAA---AATALSAGYNKALYGGLKQAGIEFIPLD 234
OBGTC17          LKQAGAQYVLVPNLPDVGITPQFRGPNAA---AATAMSVGYNKALYGGLKQAGIEFIPLD 234

AM743169,TC20,32 TFTVLGEVAANPAMYGFTNVTSTACKIDPANSTQSILTCNPTSYVSPDAANTYLFADGVH 288
OBGTC9,10        TFTVLGEVAANPAMYGFTNVTSTACKIDPANSTQSILTCNPTSYVSPDAANTYLFADGVH 288
OBGTC23          TYTLLHEIIANPGMYGFSNVTGRACLVASS------LTCSPLAYVTPDAANSYLFADDVH 287
OBGTC36          TFSILREVTANPAMYGFTNVTSTACKIDPNNATASIIGCNPTSYVSPDAANTYLFADGVH 294
OBGTC17          TFSILREVTANPGMYGFTNVTGTRLQDQPGQHHGQHHRLHPDHLRQPRCVP----DLSVH 290

AM743169,TC20,32 PTTAGHQLLGQYAVSVLEAPRLQQVLSHSAQTIGRSRADQVSMHLGGRP-ADGLSWWGGV 347
OBGTC9,10        PTTAGHQLLAS-------------TRSRCWKPRACSRC*                      313
OBGTC23          PTSAAHQMLGQYAISILEGPRLQQVLSHSAQTIGRSRADQVSLHRSGRP-ADGLSWWGGV 346
OBGTC36          PTTAGHQLLGQYAVSVLEAPRLQQC*                                   319
OBGTC17          RWRASDRYRSPAAGPVRRLGAGGSAPAAGAEPLGTDRRPFACRPGQQPPGRPSGRWPVVV 350

AM743169,TC20,32 RGDLQRYDHADLYDGLAPAGLFGIDWARDGMVVGGFAGFGRLNADFGNSRGDFTQKDTTA 407
OBGTC23          RGDLQRYDHADLYDGMAPAGLFGVDWARDGMVFGGFAGFGRLNADFGNSRGDFTQKDTSA 406
OBGTC17          GWRAR*                                                       355

AM743169,TC20,32 GLFAGWYGDRIWVNGQVSYTWLSYDVNRKVQLGPATREHGGSPDGSNLTAALNAGYEFGT 467
OBGTC23          GLFAGWYGDRIWVNGQVSYTWLSYDVNRKVQLGPATREHSGRPTAAT*             453
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Figure 8. **Multiple alignment of esterases from *S. maltophilia* strains**. Accession number of the protein used for the alignment is AM743169 (*S. maltophilia* K279a locus Smlt3773; Crossmann et al., 2008). The amino acids of the catalytic triad, Ser, Asp, and His are highlighted in gray and the conserved blocks around the catalytic triad residues are underlined. Other highlighted in gray (from residue 367 to the end) indicate putative amphipathic β-strands of autotransporter (Talker-Huiber et al., 2003). Protein regions that significantly differ with the reference sequence AM743169 are in grey; the premature termination of truncated proteins is marked with an asterisk.

2.4 The expression of protease, but not of esterase, appears to be important for *S. maltophilia* virulence in the *G. mellonella* model of infection.

Recently, the larvae of *Galleria mellonella* have been used as non-mammalian hosts to study the virulence of microbial pathogens (Kavanagh and Reeves, 2004; Seed and Dennis, 2008). To assess the contribution of StmPr1 and StmPr2 proteases and of esterase to the virulence of *S. maltophilia*, we infected *G. mellonella* larvae with cultures of different clinical and environmental *S. maltophilia* strains, previously characterized for the presence or the absence of functional genes (Table 1). Virulence displayed towards *G. mellonella* was measured by determining LD_{50} values. Strains used to infect larvae were: OBGTC20, K279a and the environmental strains LMG959 and LMG10879 (as prototypes of protease and esterase producing strains); OBGTC9, (as prototype of strains devoid of esterase activity); OBGTC13, OBGTC23, OBGTC30 and OBGTC42, the four pulsotype 17 strains lacking protease and esterase activity; and OBGTC32 which expressed protease (it lacks *StmPr1* but carries a functional *StmPr2* gene) and esterase (Table 1). As controls we infected larvae with the *P. aeruginosa* strain PA14 (positive control) and with the *E. coli* K-12 strain MG1655 (negative control). Infection of larvae with PA14 produced an LD_{50} of < 30 bacteria (Jander et al., 2000). As shown in Table 3 *S. maltophilia* K279a, OBGTC20 and the esterase-negative strain OBGTC9 displayed the lower LD_{50} values (1.0, 2.2 and 1.0 $x10^4$, respectively) indicating that the expression of esterase did not influence the killing ability of *S. maltophilia* towards wax moths. On the other hand, the four protease-negative strains with pulsotype 17 and the protease-positive (StmPr2) strain OBGTC32 displayed a significant decrease in virulence (about two log difference) compared to OBGTC20, OBGTC9 and K279a, indicating that the major protease StmPr1, rather than StmPr2, may be important for the virulence of *S. maltophilia.* Interestingly, the protease-positive environmental strains (LMG959 and LMG10879) showed the lowest killing ability ($LD_{50} > 10^7$). In this respect it is worth stressing that all reference strains of environmental origin do not produce the virulence-associated type-1 fimbriae (Table 1).

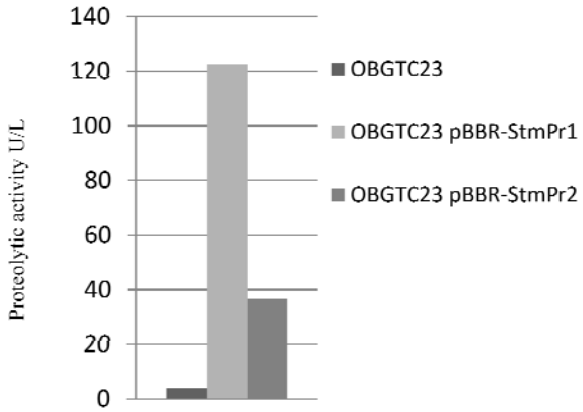
Table 3. Experimental infections of *G. mellonella* with naturally occurring variants of *S. malthopilia* strains.

Strain	Fimbriae	Protease			Esterase	LD_{50} (CFU) in
	sfm-1	Activity	*StmPr1* [c]	*StmPr2* [c]	Activity	*G. mellonella*
K279a	+	+	+	+	+	$1.0x10^4$
OBGTC20	+	+	+	+	+	$2.2x10^4$
OBGTC9	+	+	+	+	-	$1.0x10^4$
OBGTC13[b]	+	-	-	-	-	$4.0x10^6$
OBGTC23[b]	+	-	-	-	-	$3.3x10^6$
OBGTC30[b]	+	-	-	-	-	$5.2x10^6$
OBGTC42[b]	+	-	-	-	-	$8.0x10^6$
OBGTC32	+	+	-	+	+	$3.2x10^6$
LMG959[a], LMG10879 [a]	-	+	+	+	+	$>10^{7d}$

[a] Environmental isolate.
[b] Strains genetically related (pulsotype 17).
[c] Functionality of the genes is indicated.
[d] Mortality never reached 50%, even at the highest dose.

The experiments with the strains lacking of one or both proteases suggest that StmPr1 seems to have a major role. To better understand the relative role of StmPr1 and StmPr2 proteases, the OBGTC23 strain (pulsotypes 17), naturally impaired in protease expression (table 1), was complemented with plasmid pBBR-StmPr1 and pBBR-StmPr2, containing respectively the *StmPr1* or *StmPr2* functional genes of the K279a strain (Figure 9a). The presence of the functional gene *StmPr1* confers higher protease activity to respect the *StmPr2* gene (Figure 9a). Moreover, the expression of the StmPr1 protease, seems to have increased virulence in *G. mellonella* to respect the strain with restored StmPr2 protease (Figure 9b): these data reinforce our hypothesis that the StmPr1 protease may play a major role in *S. maltophilia* pathogenesis.

A



B

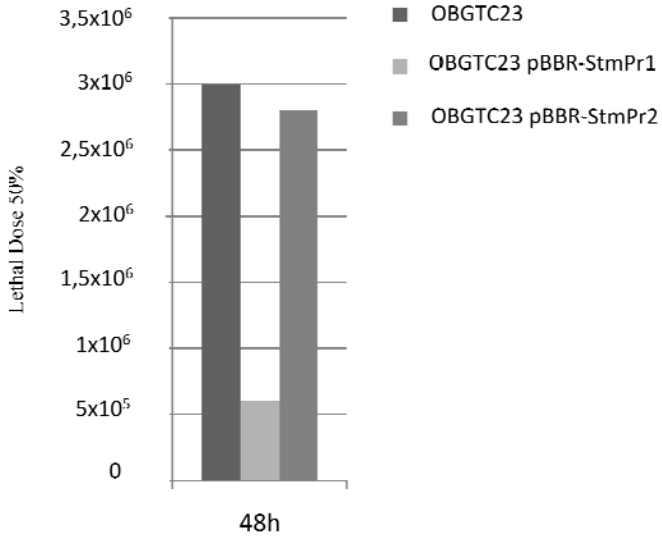


Figure 9. Proteolytic activity (A) and LD_{50} in *Galleria mellonella* virulence assay (B) of the strain OBGTC23 (pulsotype 17) and OBGTC23 complemented with the K279a functional genes coding for StmPr1 or StmPr2.

3) ANALYSIS OF THE ROLE OF *S. maltophilia* VIRULENCE FACTORS BY MUTANTS CONSTRUCTION

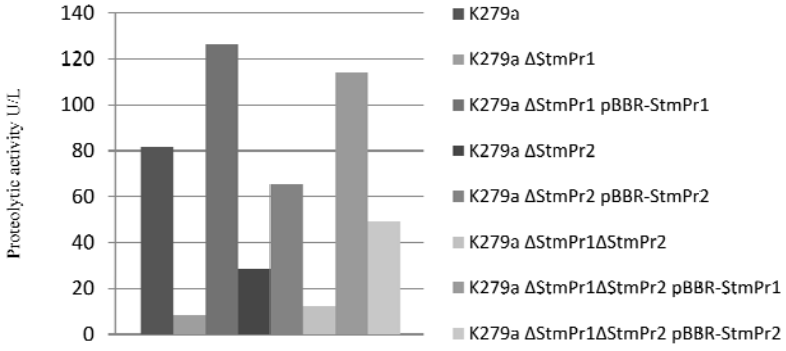
The results that we obtained using different clinical OBGTC strains, impaired in the expression of one or more hypothetical virulence factors, indicates proteases as important virulence determinants for *S. maltophilia*. Nevertheless, these natural mutants strains could lack of many others virulence determinants that may be involved in pathogenesis process. Then, to study the effect of the single factors identified and to confirm their role, deletions encompassing different putative virulence genes are introduced by allelic exchanges (Materials and Methods) in reference strain K279a, whose genome sequence is available.

Using the suicide vector pDM4 (see Material and Method) we constructed deletion mutants in genes coding for StmPr1, StmPr2, DSF (diffusible signal factor) and SMF-1 in order to study the effect of the single factors and clarify their relative importance in *S. maltophilia* pathogenesis.

3.1 Extracellular proteases are important virulence factors for *S. maltophilia* K279a

We obtained single and double mutants in proteases (K279aΔStmPr1, K279aΔStmPr2 and K279aΔStmPr1/ΔStmPr2) of K279a strain. These mutants were then confirmed by PCR for the absence of the gene deleted. A remarkable differences in exoprotease activity, using azoalbumin as substrate, was detected between mutants and parental strains, especially in the *StmPr1* mutant (Figure 10a). The complementation with pBBR-StmPr1 and pBBR-StmPr2, reveals a major activity for StmPr1 proteases (Figure 10a). Then, the virulence of K279a mutants, in one or both proteases, was compared to that of wild type strain in *G. mellonella*. Data obtained show an attenuation of virulence, especially in the first hours post infection (Figure 10b). In fact, after 18 hours, the K279aΔStmPr1, K279aΔStmPr2 and K279aΔStmPr1/ΔStmPr2 strains show LD_{50} value ($1{,}2x10^6$, $1x10^6$, $1{,}4x10^6$ respectively) at least five-fold higher respect K279a (LD_{50} $2x10^5$). After 48 hours the virulence of the protease mutants tends to come back about similar to wild-type, indicating that proteases are important virulence factor during the early stage of colonization, for degrading host tissues and for invasion, while in later stage other virulence determinants become important.

A



B

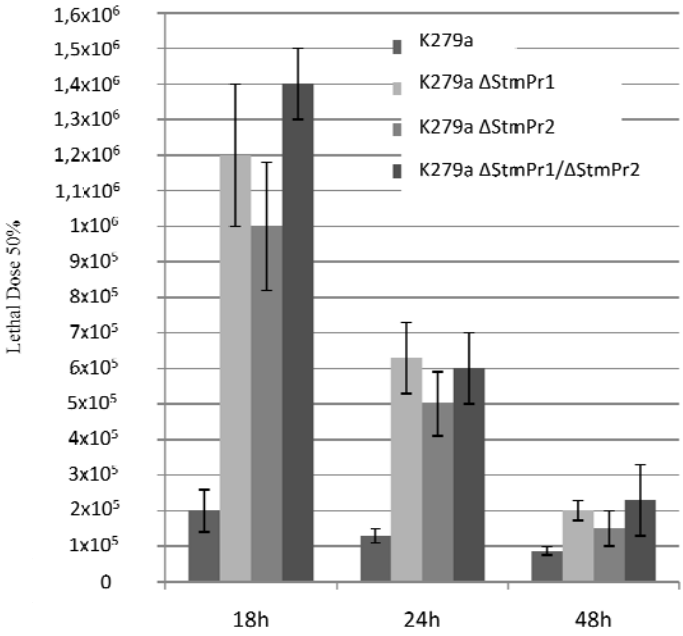
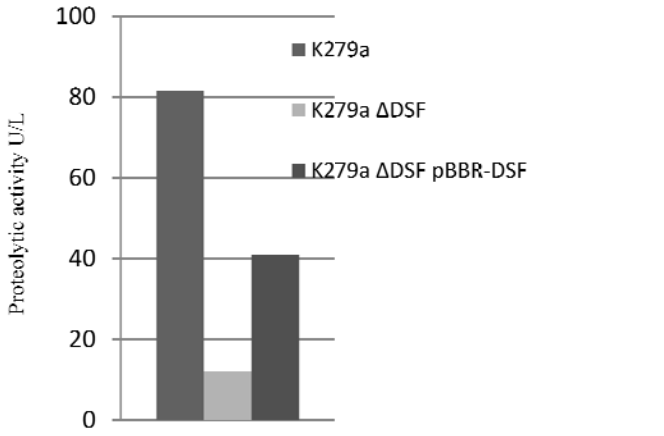


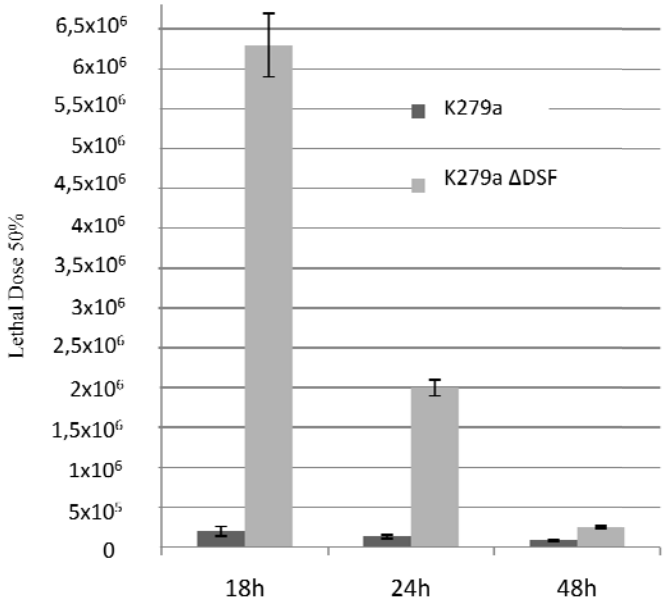
Figure 10. Proteolytic activity (A) and LD_{50} in *Galleria mellonella* virulence assay (B) of the proteases mutants strains and strains complemented with the K279a functional genes coding for StmPr1 or StmPr2, compared to wild type.

3.2 Diffusible Signal Factor controls proteases expression in *S. maltophilia*

Recently, a quorum sensing signal molecule, known as diffusible signal factor (DSF), has been identified in *S. maltophilia* (Fouhy et al., 2007). The DSF controls the expression of several virulence factors, including extracellular enzymes, motility, antibiotic resistance. To assess the role of DSF signaling we constructed a K279a DSF mutant, defective in the synthesis of the signal molecule. The destruction of DSF signaling severely reduces the level of extracellular proteases (Figure 11a). Since the very low level of protease activity is similar to that of *StmPr1/StmPr2* double mutant (Figure 10a), the data observed confirm the crucial role of DSF on proteases expression. The virulence of K279aΔDSF displayed towards *G. mellonella* was measured by determining LD_{50} values. The lower virulence observed for K279a DSF mutant, compared with that of K279a *StmPr1/StmPr2* double mutant (Figure 11b and 10b), confirms that this molecule controls the expression of also other virulence factors in addition to the proteases and also confirms that *S. maltophilia* pathogenesis is a multifactorial process.

A





B

Figure 11. Proteolytic activity (A) and LD_{50} in *Galleria mellonella* virulence assay (B) the DSF mutant strain, compared to wild type and DSF mutant complemented w the functional gene.

3.3 Fimbriae are important for establishment of chronic infection

Adherence to epithelial cells is central to the initiation of colonization or invasion of host tissues by many bacteria. This event is often mediated by fibrillar structures called fimbriae or pili composed of several thousands of monomeric fimbrin or pilin subunits. Fimbrial adhesins may mediate direct binding of the bacteria to the host target cell or indirect binding by forming cross-link liasons between bacteria favouring colonization. In many bacterial species, isogenic mutants defective in fimbriae production are less virulent than the parent strains probably as a result of the lack of efficient interaction of the bacteria with epithelial cells (de Oliveira-Garcia et al., 2003).

All clinical isolates have the type-1 fimbria (SMF-1), a surface structure involved in adherence and in the early stage of biofilm formation: hence, this adhesin represents surely an important virulence factor to colonize the airways of CF patients. We observed (Table 3) that the environmental strains lacking of SMF-1 are not virulent in *G. mellonella* infection model. To analyze the role of SMF-1 fimbria as virulence factor we construct a *S. maltophilia* K279a strain lacking of type-1 fimbria (K279aΔSMF-1). K279aΔSMF-1 seems to be slightly virulent than K279a in *G. mellonella* infection model. Its LD_{50} at 48h is $1{,}4x10^4$, while LD50 of K279a is $8{,}7x10^4$ (table 4). Probably the absence of this structure allows mutants bacteria to spread faster in the hemolymph or escape more easily from the immune system. These data suggest that, despite fimbriae are important for biofilm formation and for establishment of chronic infection in CF patients, their lack may hamper, in *G. mellonella*, the ability to establish chronic infection and enhance the ability to cause acute infection. Probably, the higher LD_{50} that we observed for environmental strains (table 3) indicates that their low virulence could be due to the absence of many other factors, in addition to fimbriae.

Table 4. LD_{50} of K279aΔSMF-1 compared to wild type

Strain	LD_{50} 18h	LD_{50} 24h	LD_{50} 48h
K279a	$2x10^5$	$1{,}3x10^5$	$8{,}7x10^4$
K279a ΔSMF-1	$1x10^5$	$8x10^4$	$1{,}4x10^4$

4) GENOMIC EVOLUTION OF *S. maltophilia* TOWARD A PATHOGENIC LIFESTYLE

Stenotrophomonas maltophilia is a an environmental organism, that in recent years has been isolated with increasing frequency as an opportunistic pathogen in nosocomial infections as well as from the airways of cystic fibrosis (CF) patients. An important virulence factor involved in colonization of CF airways are fimbriae. In fact, we observed that the environmental strains (lacking of fimbriae) show the lowest killing ability ($LD_{50} > 10^7$) in *G. mellonella* infection model (table 3). But the results obtained with K279a SMF-1 mutant (table 4), indicate that the lack of type-1 fimbria alone, could not explain this high LD_{50} value, and also, the ability to express protease activity per se does not account for the virulence of these microorganisms which, being of environmental origin, may lack other important virulence factors likely acquired by clinically-derived strains, during their evolution. Previous works had compared the genome of K279a with that of R551-3, an endophyta able to colonize the poplar *Populus trichocarpa* (Rocco et al., 2009; Ryan et al., 2009). In order to identify the specific genetic determinants acquired by the clinical strains, during the pathogenic lifestyle evolution, we choose to compare the genome of K279a clinical strain with that of LMG959, isolated from a rice paddy, that may represent a true environmental harmless strain, by Suppression Subtractive Hybridization (SSH) (Diatchenko et al., 1996). With this technique, we isolated DNA fragments unique to the clinical strains, that could represent the possible virulence determinants that differentiate the clinical K279a strain from the environmental harmless LMG959 strain.

Subtraction of the genome of LMG959 strain (driver) from that of clinical isolate K279a (tester) produced a library of clones. We obtained 29 clones containing specific fragments (from 199 to 835 bp in length) present only in K279a. Sequencing of the subtracted DNA fragments revealed that the G+C content of the majority of these sequences was considerably lower (average value, 50%) (Table 5) than the average value of total DNA for K279a (66,7%). This result confirms the tendency of SSH in the identification of region different in G+C content (Bernier and Sokol, 2005) and suggests that many of the subtracted DNA fragments may have been acquired from other organisms by horizontal gene transfers. 6 of the 29 sequences were IS elements, transposases, phages integrases, reinforcing the hypothesis that *Stenotrophomonas* genomes have been subjected to numerous horizontal transfer events during evolution and sometimes from phylogenetically distant bacteria.

Particularly interesting was the identification of four genes coding for specific factors, which are known to be important for virulence in many bacteria (Table 5):
i) a putative cell surface filamentous haemagglutinin protein, that in *Bordetella pertussis*, is involved in adhesion to respiratory tract (Locht et al., 1993).
ii) a putative TonB dependent receptor protein, that is an outer membrane protein known mainly for the active transport of iron-siderophore complexes in gram-negative bacteria.
iii) a proteolytic subunit of an ATP-dependent Clp protease. Clp dependent proteolysis has been implicated in expression of extracellular virulence factors in several organisms and in Gram-negative pathogens, and is central to several types of secretion processes. ClpP contributes, in many pathogens, to growth at conditions inducing protein unfolding such as high temperature and oxidative stress (Ingmer H. and Brøndsted L., 2009).
iv) a putative ankyrin repeat-containing protein. Some microbial ANK-containing proteins clearly play a role in host-pathogen interaction and in the evolution of infectious diseases. Their functions might involve molecular mimicry of host proteins to modulate key host cell processes to ensure proliferation of the microbe. The ankyrin repeat (ANK) is a 33-residue motif that often occurs in tandem arrays, which cooperatively fold into structures that mediate molecular recognition via protein-protein interactions. It is considered one of the most common protein-protein interaction motifs in nature (Al-Khodor et al., 2010).

Moreover, the majority of the subtracted sequence represent gene involved in metabolism, DNA restriction/modification system, transmembrane proteins, hypothetical proteins and proteins with unknown function: these differences between *S. maltophilia* K279a and *S. maltophilia* LMG959 could be related to niche adaptation or host preference and this accessory genome may represents an advantage for pathogen evolution driven by the need for continuous adaptation to the host in order to evade or suppress coevolving host defense mechanisms, making *S. maltophilia* an emergent opportunistic pathogen in nosocomial infections.

Table 5. Analysis of the 29 unique DNA fragments of *S. maltophilia* K279a

SSH Clone	Size (bp)	% G+C	K279a predicted function	K279a locus
IS elements, phage-related elements				
1	618	47	putative transposon Tn5044/Tn3926 transposase	Smlt2465
2	481	52	putative phage tail fiber protein	Smlt1952
3	199	56	putative phage integrase	Smlt0025
4	255	53	putative prophage integrase	Smlt1336
5	343	51	putative transposase	Smlt0518
6	603	42	putative ISXac3 like transposase	Smlt2490
Hypothetical virulence factors				
7	507	57	Clp protease proteolytic subunit	Smlt0989
8	288	46	putative cell surface haemagglutinin protein	Smlt4452
9	799	43	putative TonB dependent receptor protein	Smlt3444
10	333	51	putative ankyrin repeat-containing protein	Smlt3033
Cell membrane				
11	418	40	putative transmembrane protein	Smlt1331
12	483	46	putative transmembrane protein	Smlt1849
Metabolism, DNA restriction/modification				
13	406	64	putative glucose-1-phosphate thymidylyltransferase	Smlt0648
14	477	51	putative restriction-modification system methyltransferase	Smlt1013
15	835	34	putative modification methylase	Smlt1844
16	591	65	putative nucleoside hydrolase	Smlt3837
17	545	47	putative polysaccharide deacetylase	Smlt0512
18-26	**Hypothetical proteins**			
27-29	**Unknown function**			

DISCUSSION

Stenotrophomonas maltophilia is the only species of *Stenotrophomonas* that is known to cause human infection (Hauben et al., 1999). Despite its prevalence in the environment, *S. maltophilia* appears more and more able to associate with human hosts. In particular, *S. maltophilia* is emerging as one of the most frequently found bacteria in CF patients.

Due to the remarkable diversity among *S. maltophilia* isolates it is extremely important to acquire a better definition of bacterial clones or lineages able to cause disease in CF patients. The application of molecular characterization and typing methods can provide improved opportunities to specifically define bacterial subtypes that differ in their ability to cause human disease. Whereas many current study are based on taxonomic classification schemes, bacterial species definitions do not necessarily correlate with the ability of bacteria to cause human disease. Thus, a critical need exists for development of better scientific definitions of bacterial clones or lineages able to cause human disease. The application of molecular subtyping methods for characterization of human isolates of *S. maltophilia* may be revealed distinct subgroups or lineages, which may differ in their attitude to cause disease on CF patient. These analysis should eventually indicate which pheno-genotypes are most likely to cause disease, and the information obtained could be used in risk assessment models to differentiate clones that represent hazards from avirulent environmental strains. In our study we have evaluated, by PFGE, RFLP of the *gyrB* gene and analysis of the 16S rRNA gene, the genetic relatedness of 52 *S. maltophila* strains: 43 clinical isolates collected at the Paediatric Hospital "Bambino Gesù" of Rome (41 were from CF patients, and two from blood cultures of two non-CF patients), two *S. maltophilia* reference clinical strains (K279a and LMG958) and seven strains of environmental origin (two isolated within the Hospital "Bambino Gesù"). Though most strains (45 out of 52) were isolated from the same hospital, we identified 47 different pulsotypes, with a similarity ranging from 78 to 100%. This indicates a high degree of genetic diversity, a feature already reported for *S. maltophilia* strains (Hauben et al. 1999; Schaumann et al., 2008; Valdezate et al., 2004). Three clusters strain (pulsotypes 1, 17 and 33) were evidenced, indicating either cross-transmission among patients or infection from a common source. Based on the *gyrB* RFLP, the strains were grouped into nine similarity groups, while six allelic groups were identified by the analysis of the hypervariable region of the 16S rRNA gene (Tables 1 and 2) (Gould et al., 2006). The great majority of CF-derived strains (29 out of 41) belonged to RFLP profile 1, as the reference clinical strains K279a and

LMG958. Since the RFLP profile of strain LMG958 has been recently assigned to cluster C (Coenye et al., 2004), a cluster shared by the *S. maltophilia* strains presenting an increased potential for colonization of the airways of CF patients, it is conceivable that our RFLP profile 1 corresponds to that cluster. Moreover, in spite of being less discriminatory, the RFLP analysis distinguished the four pulsotype 17 strains, which were the only ones that grouped into RFLP profile 2. Interestingly, the two CF-derived strains OBGTC6 and OBGTC11 were the only strains presenting unique profiles either by PFGE, *gyrB* RFLP or by analysis of the 16S rRNA gene (Table 1).

To get further insight into the virulence of *S. maltophilia*, the strains were characterized for the presence and expression of some virulence-associated genes. Only the clinically-derived strains, including the two strains isolated from the hospital environment, were positive for the presence of the *smf-1* gene (Table 1). The finding that type-1 fimbriae are exclusively expressed by *S. maltophilia* strains of clinical origin confirms previous reports (de Oliveira-Garcia et al., 2003) indicating type-1 fimbriae as putative virulence factors of *S. maltophilia.* This suggests that hospital strains might represent a reservoir of potentially pathogenic *S. maltophilia* and thus a threat for CF patients.

It has been recently suggested that the release of proteolytic enzymes might contribute to the damage of pulmonary tissues of CF patients infected by *S. maltophilia*. *StmPr1*, the gene encoding an alkaline serine-protease has been biochemically characterized and sequenced (Windhorst et al., 2002). Moreover, BLAST analysis of the K279a genome (Smlt0686 locus) revealed a *StmPr1* gene that shares 79% DNA similarity (70% protein identity) with the *StmPr1* gene described by Windhorst et al. (2002). Thus, the two *StmPr1* genes present some degree of variability at the level of their DNA sequence. Furthermore, *S. maltophilia* carries also a gene, *StmPr2,* coding for a minor extracellular protease which is highly conserved (>99% identity) in *S. maltophilia* strain K279a (Smlt0861 locus).

All the 52 *S. maltophilia* strains amplified a fragment (1764 bp) corresponding to the presence of the *StmPr2* (Table 1). As far *StmPr1*, the most CF-derived strains (29 out of 41) carried only the 1621 bp allelic variant of *SmtPr1* present in the K279a reference genome, while two isolates amplified only a 868 bp fragment, the *SmtPr1* allelic variant described by Windhorst (Windhorst et al., 2002). Five strains (the four pulsotype 17 strains, which lacked protease activity, and OBGTC6, which displayed it) amplified both a 868 bp and a 1621 bp fragment, while five strains were negative for both fragments (Table 1). The sequences of the 1621 bp (*StmPr1*) and 1764 bp (*StmPr2*) fragments of the four pulsotype 17

strains and of OBGTC6 indicate that the five strains encode inactive forms of the *StmPr1* gene, while OBGTC6 carries a functional *StmPr2* gene accounting for the protease activity of this strain (Table 1).

Esterases are a group of hydrolytic enzymes which has been associated, in some cases, to virulence. An ORF presenting a high degree of homology with *estE* of *X. vesicatoria* and coding for a putative outer membrane esterase (Talker-Huiber et al., 2003) was found in *S. maltophilia* strain K279a (Smlt3773 locus). The predicted amino acid sequence of the *S. maltophilia* outer membrane esterase showed homology with esterases of the GDSL family of hydrolases (Figure 8), in particular in the sequence block around its catalytic triad (serine, aspartic acid and histidine), and in the C-terminal β-barrel domain typically found in this group of lipolytic enzymes (Talker-Huiber et al., 2003; Wilhelm et al., 2007). PCR screening of the 52 *S. maltophilia* strains analysed revealed that only three environmental reference strains failed to amplify the expected 1342 bp DNA fragment (Table 1), while eight strains failed to produce esterase activity, due to the presence of frameshift mutations in the coding region. The expression of esterase activity by most CF-derived *S. maltophila* clinical isolates, reinforces the hypothesis that esterase might play some role in the virulence of this microorganism.

Recently, larvae of *G. mellonella* have been used as a convenient non-mammalian model of infection (Kavanagh and Reeves, 2004; Seed and Dennis, 2008). Grounding on the molecular characterization of our *S. maltophila* strains (Table 1), we infected *G. mellonella* larvae with independent CF-derived strains unable to express StmPr1, StmPr2 or esterase, and their effect has compared with that of strains expressing these activities. Although not sensitive as *P. aeruginosa* (Jander et al., 2000), larvae were found to be susceptible to *S. maltophilia* infection. The results reported in Table 3 indicate that the major extracellular protease (StmPr1) may be a relevant virulence factor of *S. maltophilia* (K279a, OBGTC9 and OBGTC20 vs OBGTC32). On the other hand the killing ability of strain OBGTC32, which is impaired only in the expression StmPr1, is low (LD_{50} 3.2 x 10^6) as that of the four strains unable to synthesize both proteases (OBGTC13, OBGTC23, OBGTC30 OBGTC42). Complementation of the OBGTC23 (pulsotype 17) strain, one of the four negative strains for proteases activity, with the functional gene coding for StmPr1 or StmPr2 (Figure 9a), reinforce the hypothesis that StmPr1 protease could play a major role: only the OBGTC23 strain with StmPr1 activity restored showed an increased virulence in *G. mellonella* larvae (Figure 9b).

Esterase appears not to play a significant role in this assay, since strains displaying or lacking esterase activity, have a similar low LD_{50} (K279a or

OBGTC20 vs OBGTC9). On the contrary the environmental strains LMG959 and LMG10879, although able to express both proteases and esterase activities, were the less virulent strains ($LD_{50} > 10^7$), indicating that they probably lack other virulence factors (for instance type-1 fimbriae) necessary to be virulent in this model.

In the complex, our results had shown a considerable phylogenetic and phenotypic variability among *S. maltophilia* isolates, including those from patients attending at the Paediatric Hospital "Bambino Gesù". This is probably the result of the many environmental niches of this bacterium; most infections are likely to reflect contact with separate environmental sources. Despite this, there is evidence that certain phylogenetic groups are better able to cause infection than others. Based on these considerations, to assess the role of the single putative virulence factors identified, we constructed K279a (the reference clinical strain, whose genome has been sequenced) mutants in "hypothetical" virulence genes. The *in vivo* experiments with mutants in StmPr1 and StmPr2 proteases, suggests that proteases, and particularly the StmPr1 protease, are important during the early stage of infection process, when bacteria needs to spread in the host tissue. In the later stages of infection, many other virulence determinants may be used by *S. maltophilia* to express its full virulence.

Many bacteria use cell-cell communication mediated by diffusible signal molecules to monitor their population density. Pathogenic bacteria may use cell-cell signaling to regulate the expression of factors contributing to virulence. The genome of *S. maltophilia* encodes a cell-cell signaling system that is highly related to the diffusible signal factor (DSF)-dependent system of the phytopathogen *Xanthomonas campestris*. In the clinical isolate *S. maltophilia* K279a, DSF signalling seems to control the expression of several virulence factors. The disruption of DSF signaling lead to a strong reduction of proteases activity (Figure 11a), confirming its role in the control of proteases expression. Moreover, the DSF mutant reveals a greater reduction of virulence in *G. mellonella* infection model (Figure 11b), compared to that of proteases mutants, suggesting a DSF involvement in the control of proteases but also of other virulence factors.

The lack of fimbriae in the environmental strains give us indications of their importance for pathogenicity of the clinical strains: type-1 fimbriae are important for adherence and to establish chronic infections in the respiratory tract of CF patients. Nevertheless, the LD_{50} slightly lower that we observed for the K279a fimbria mutant in *G. mellonella* (table 4), suggests that the fimbria could be not important in this specific model, while this surface structure may be important to establish chronic infection in CF patients.

It is widely accepted that the evolution of bacterial pathogens from harmless ancestors, mainly depends on the acquisition of virulence gene clusters by horizontal gene transfer, that had driven the evolution of the microorganism towards a more pathogenic phenotype (Casalino et al., 2010). A deeper knowledge of the genome organization of *S. maltophilia* could contribute to a better understanding of the rapid adaptation of this environmental bacterium to the human host, making it an emergent pathogen in nosocomial infections.

The environmental strains examined in this study show the higher LD_{50} in *G. mellonella* to respect the reference clinical strain K279a; these strains are probably missing of some important virulence factors, which could be acquired by the clinical strains during the evolution toward pathogenic lifestyle. The comparison of the genome of the clinical strain K279a to that of the environmental strain LMG959, confirm that many DNA fragments are absent in the environmental strain. In fact, by Suppression Subtractive Hybridization, has been isolated 29 DNA fragments, present only in K279a (Table 5). The low G+C content of the subtracted fragments, together with the presence of several putative IS elements, suggests that many of the subtracted sequence may have been acquired from other organisms by horizontal genetic transfer. Interestingly, 21 of the 29 sequence are part of the 41 genomic islands (GEI) described in K279a (Rocco et al., 2009). Among the 29 sequence there are 4 genes that in other bacteria are known as important virulence factors (Table 5): a Clp protease, a haemagglutinin, a putative TonB dependent receptor protein and a putative ankyrin repeat-containing protein. All the other subtracted fragments represent genes involved in metabolism, DNA modification/restriction system, transmembrane proteins and unknown proteins. In the complex, the 29 specific sequence could represent the flexible genome, important for the adaptation to human host, acquired during the evolution of the clinical strains. The acquisition of accessory gene may have turned the harmless *S. maltophilia* into an opportunistic pathogen.

In conclusion, data that we obtained in this study stress that *S. maltophilia* pathogenicity is a complex multifactorial process, still very far from being completely understood. In this context, it is crucial to identify the virulence factors used by *S. maltophilia* to colonize the respiratory tissues of patients with CF, so as to develop specific therapies aimed at combating the pathogenic action of this opportunistic pathogen.

REFERENCES

Al-Khodor S., Price C.T., Kalia A., Abu Kwaik Y., 2010. Functional diversity of ankyrin repeats in microbial proteins. Trends Microbiol. 18, 132-139.

Al Naiemi, N., Duim, B. & Bart, A. 2006. A CTX-M extended-spectrum β lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia. J. Med. Microbiol.* 55, 1607-1608.

Avison, M.B., Higgings, C.S., von Heldreich, C.J., Bennett, P.M., Walsh, T.R., 2001. Plasmid location and molecular heterogeneity of the L1 and L2 β-lactamase gens of *Stenotrophomonas maltophilia.* Antimicrob. Agents Chemother. 45, 413-419.

Berg, G., Marten, P. & Ballin, G. 1996. *Stenotrophomonasmaltophilia* in the rhizosphere of oilseed rape occurrence, characterization and interaction with phytopathogenic fungi. *Microbiol. Res.* 151, 19-27.

Berg, G., Roskot, N., Smalla, K., 1999. Genotypic and phenotypic relationships between clinical and environmental isolates of *Stenotrophomonas maltophilia*. J. Clin. Microbiol. 37, 3594-3600.

Berg, G., Egamberdieva, D., Lugtenberg, B. & Hagemann, M. 2010. Symbiotic Plant–Microbe Interactions: Stress Protection, Plant Growth Promotion, and Biocontrol by *Stenotrophomonas. Cellular Origin, Life in Extreme Habitats and Astrobiology, 17, 445-460*

Bernier S.P and Sokol P.A. 2005. Use of suppression-subtractive hybridization to identify genes in the *Burkholderia cepacia* complex that are unique to *Burkholderia cenocepacia*. J. Bacteriol. 187, 5278-5291.

Canton, R., Valdezate, S., Vindel, A., Sanchez Del Saz, B., Maiz, L., Baquero, F., 2003. Antimicrobial susceptibility profile of molecular typed cystic fibrosis *Stenotrophomonas maltophilia* isolates and differences with non cystic fibrosis isolates. Pediatr. Pulmonol. 35, 99-107.

Casalino M., Prosseda G., Barbagallo M. L., Iacobino A., Ceccarini P., Latella M. C., Nicoletti M., Colonna B.. 2010. Interference of the CadC regulator in the expression of the arginine-dependent acid resistance system in *Shigella flexneri*, *Shigella sonnei* and enteroinvasive *Escherichia coli.* International Journal of Medical Microbiology. 300, 289-95

Coenye, T., LiPuma, J.J., 2002. Use of the *gyrB* gene for the identification of Pandoraea species. FEMS Microbiol. Lett. 208, 15-19.

Coenye, T., Vanlaere, E., LiPuma, J.J., Vandamme, P., 2004. Identification of genomic groups in the genus *Stenotrophomonas* using *gyrB* RFLP analysis. FEMS Immunol. Med. Microbiol. 40, 181-185.

Crossman, L.C., Gould, V.C., Dow, J.M., Vernikos, G.S., Okazaki, A., Sebaihia, M., Saunders, D., Arrowsmith, C., Carver, T., Peters, N., Adlem, E., Kerhornou, A., Lord, A., Murphy, L., Seeger, K., Squares, R., Rutter, S., Quail, M.A., Rajandream, M.A., Harris, D., Churcher, C., Bentley, S.D., Parkhill, J., Thomson, N.R., Avison, M.B., 2008. The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. Genome Biol. 9, R74.

de Oliveira-Garcia, D., Dall'Agnol, M., Rosales, M., Azzuz, A.C., Alcantaram, N., Martinez, M.B., Giron, J.A., 2003. Fimbriae and adherence of *Stenotrophomonas maltophilia* to epithelial cells and to abiotic surfaces. Cell. Microbiol. 5, 625-636.

de Oliveira-Garcia, D., Dall'Agnol, M., Rosales, M., Azzuz, A.C., Martinez, M.B., Giron, J.A., 2002. Characterization of flagella produced by clinical strains of *Stenotrophomonas maltophilia*. Emerg. Infect. Dis. 8, 918-923.

Denton, M., Kerr, K.G., 1998. Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clin. Microbiol. Rev. 11, 57-80.

Denton, M., Todd, N.J., Kerr, K.G., Hawkey, P.M., Littlewood, J.M., 1998. Molecular epidemiology of *Stenotrophomonas maltophilia* isolated from clinical specimens from patients with cystic fibrosis and associated environmental samples. J. Clin. Microbiol. 36, 1953-1958.

Diatchenko L., Y. F. C. Lau, A. P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E. D. Sverdlov and P. D. Siebert 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* 93:6025–6030

Di Bonaventura, G., Prosseda, G., Del Chierico, F., Cannavacciuolo, S., Cipriani, P., Petrucca, A., Superti, F., Ammendolia, M.G., Concato, C., Fiscarelli, F., Casalino, M., Nicoletti, M., Colonna, B., 2007. Molecular characterization of virulence determinants of *Stenotrophomonas maltophilia* strains isolated from patients affected by cystic fibrosis. Int. J. Immunopathol. Pharmacol. 20, 529-537.

Di Bonaventura, G., Spedicato, I., D'Antonio, D., Robuffo, I., Piccolomini, R., 2004. Biofilm formation by *Stenotrophomonas maltophilia*: modulation by quinolones, trimethoprim-sulfamethoxazole, and ceftazidime. Antimicrob. Agents Chemother. 48, 151- 160.

Di Bonaventura G, Pompilio A, Zappacosta R, Petrucci F, Fiscarelli E, Rossi C, Piccolomini R. 2010. Role of excessive inflammatory response to *Stenotrophomonas maltophilia* lung infection in DBA/2 mice and implications for cystic fibrosis. Infect Immun. 78, 2466-2476

Figueiredo, P.M., Furumura, M.T., Santos, A.M., Sousa, A.C., Kota, D.J., Levy, C.E., Yano, T., 2006. Cytotoxic activity of clinical *Stenotrophomonas maltophilia*. Lett. Appl. Microbiol. 43, 443-449.

Fouhy, Y. *et al.* 2007. Diffusible signal factor-dependent cell–cell signaling and virulence in the nosocomial pathogen *Stenotrophomonas maltophilia. J. Bacteriol.* 189, 4964–4968.

Gibson, R.L., Burns, J.L., Ramsey, B.W., 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am. J. Respir. Crit. Care Med. 168, 918-951.

Goss, C.H., Mayer-Hamblett, N., Aitken, M.L., Rubenfeld, G.D., Ramsey, B.W., 2004. Association between *Stenotrophomonas maltophilia* and lung function in cystic fibrosis. Thorax. 59, 955-959.

Gould, V.C., Okazaki A., Avison, M.B., 2006. Beta-lactam resistance and beta-lactamase expression in clinical *Stenotrophomonas maltophilia* isolates having defined phylogenetic relationships. J. Antimicrob. Chemother. 57, 199-203.

Hagemann, M., Hasse, D. & Berg, G. 2006 Detection of a phage genome carrying a zonula occludens like toxin gene (*zot*) in clinical isolates of *Stenotrophomonas maltophilia. Arch. Microbiol.* 185, 449–458.

Hauben, L., Vauterin, L., Moore, E.R., Hoste, B., Swings, J., 1999. Genomic diversity of the genus *Stenotrophomonas*. Int. J. Syst. Bacteriol. 49, 1749-1760.

Hugh, R., Leifson, E., 1963. A description of the type strain of *Pseudomonas maltophilia*. Int. Bull. Bacteriol. Nomencl. Taxon. 13, 133-138.

Jander, G., Rahme, L.G., Ausubel, F.M., 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol. 182,3843-3845.

Kavanagh, K., and E. P. Reeves, 2004. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS Microbiol. Rev. 28:101-112.

Ingmer H., Brøndsted L., 2009. Proteases in bacterial pathogenesis. Res Microbiol. 160, 704-710

Locht, C., Bertin, P., Menozzi, F.D., Renauld, G., 1993. The filamentous haemagglutinin, a multifaceted adhesion pro-duced by virulent *Bordetella* spp. Mol. Microbiol. 9, 653–660.

Looney, W.J., Narita, M., Mühlemann, K., 2009. *Stenotrophomonas maltophilia*: an emerging opportunist human pathogen. Lancet Infect. Dis. 9, 312-323.

Lucas, S., Copeland, A., Lapidus, A., Glavinadel Rio, T., Dalin, E., Tice, H., Pitluck, S., Chain, P., Malfatti, ., Shin, M., Vergez, L., Lang, D., Schmutz, J., Larimer, F., Land, M., Hauser, L., Kyrpides, N., Mikhailova, N., Taghavi, S., Monchy, S., Newman, L., Vangronsveld, J., vander Lelie, D., Richardson, P., Complete sequence of Stenotrophomonas maltophilia R551-3. Submitted (JUN-2008) to the EMBL/GenBank/DDBJ databases.

Lyczak, J.B., Cannon, C.L., Pier, G.B., 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. 15, 194-222.

Okazaki A, Avison MB. 2008. Induction of L1 and L2 betalactamase production in *Stenotrophomonas maltophilia* is dependant on an AmpR-type regulator. *Antimicrob Agents Chemother*; 52**,** 1525‑28.

Pages, D. *et al.* 2008. Heavy metal tolerance in *Stenotrophomonas maltophilia*. *PLoS One* 3, 1539.

Palleroni, N.J., Bradbury, J.F., 1993. *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings et al. 1983. Int. J. Syst. Bacteriol. 43, 606-609.

Pompilio A, Crocetta V, Confalone P, Nicoletti M, Petrucca A, Guarnieri S, Fiscarelli E, Savini V, Piccolomini R, Di Bonaventura G. 2010. Adhesion to and biofilm formation on IB3-1 bronchial cells by *Stenotrophomonas maltophilia* isolates from cystic fibrosis patients. BMC Microbiol., 10:102.

Rocco F., De Gregorio E., Colonna B., Di Nocera P.P. 2009. *Stenotrophomonas maltophilia* genomes: a start-up comparison. Int. J. Med. Microbiol. 299, 535-546.

Roscetto, E., Rocco, F., Carlomagno, M.S., Casalino, M., Colonna, B., Zarrilli, R., Di Nocera, P.P., 2008. PCR-based rapid genotyping of *Stenotrophomonas maltophilia* isolates. BMC Microbiol. 8, 202-211.

Rouf R, Karaba SM, Dao J, Cianciotto NP. 2011. *Stenotrophomonas maltophilia* strains replicate and persist in the murine lung, but to significantly different degrees. Microbiology. 157,2133-2142.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van der Lelie, D., Dow, J.M., 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas.* Nat. Rev. Microbiol. 7, 514-525.

Ryan, R. P. & Dow, J. M. 2008. Diffusible signals and interspecies communication in bacteria. *Microbiology* 154, 1845–1858.

Ryan, R. P. *et al.* 2008. Interspecies signalling via the *Stenotrophomonas maltophilia* diffusible signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 68, 75–86.

Schaumann, R., Laurin, F., Rodloff, A.C., 2008. Molecular typing of clinical isolates of *Stenotrophomonas maltophilia* by pulsed-field gel electrophoresis and random primer PCR fingerprinting. Int. J. Hyg. Environ. Health. 211, 292-298.

Schreiber, L. *et al.* 2005. Plant-microbe interactions: identification of epiphytic bacteria and their ability to alter leaf surface permeability. *New Phytol.* 166, 589-594.

Seed, K.D., Dennis J.J., 2008. Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex. Infect. Immun. 76, 1267-1275.

Steinkamp, G., Wiedemann, B., Rietschel, E., Krahl, A., Gielen, J., Barmeier, H., Ratjen, F., 2005. Prospective evaluation of emerging bacteria in cystic fibrosis. J. Cyst. Fibros. 4, 41-48.

Svensson-Stadler LA, Mihaylova SA, Moore ER. 2011. *Stenotrophomonas* inter-species differentiation and identification by *gyrB* sequence analysis. FEMS Microbiol Lett. 1574-6968.

Taghavi, S. *et al.* 2009. Mechanisms underlying the beneficial effects of endophytic bacteria on growth and development of poplar. *Appl. Environ. Microbiol.* 75, 748-757.

Talker-Huiber, D., Jose, J., Glieder, A., Pressnig, M., Stubenrauch, G., Schwab, H., 2003. Esterase EstE from *Xanthomonas vesicatoria* (Xv_EstE) is an outer membrane protein capable of hydrolyzinglong-chain polar esters. Appl. Microbiol. Biotechnol. 61, 479-487.

Valdezate S., Vindel, A., Martín-Dávila, P., Del Saz, B.S., Baquero, F., Cantón, R., 2004. High genetic diversity among *Stenotrophomonas maltophilia* strains despite their originating at a single hospital. J. Clin. Microbiol. 42, 693-699.

Waters, V.J., Gómez, M.I., Soong, G., Amin, S., Ernst, R., Prince, A., 2007. Immunostimulatory properties of the emerging pathogen *Stenotrophomonas maltophilia.* Infect. Immun. 75, 1698-1703.

Wilhelm, S., A. Gdynia, P. Tielen, F. Rosenau, and K. E. Jaeger. 2007. The autotransporter esterase EstA of *Pseudomonas aeruginosa* is required for rhamnolipid production, cell motility, and biofilm formation. J. Bacteriol. 189:6695-6703.

Windhorst, S., Frank, E., Georgieva, D.N., Genov, N., Buck, F., Borowski, P., Weber, W., 2002. The major extracellular protease of the nosocomial pathogen *Stenotrophomonas maltophilia*: characterization

of the protein and molecular cloning of the gene. J. Biol. Chem. 277, 11042-11049.

Zhang L., L. I. Xian-Zhi and K. Poole. 2001. SmeDEF Multidrug Efflux Pump Contributes to Intrinsic Multidrug Resistance in *Stenotrophomonas maltophilia* Antimicrob. Agents Chemother. p. 3497–3503

ROMA TRE
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SCUOLA DOTTORALE IN BIOLOGIA
SEZIONE SCIENZE BIOMOLECOLARI E CELLULARI
CICLO XXIV

Stenotrophomonas maltophilia isolated from patients affected by cystic fibrosis: genotyping analysis and molecular characterization of virulence determinants

Stenotrophomonas maltophilia isolato da pazienti affetti da fibrosi cistica: analisi genotipica e caratterizzazione molecolare di determinanti di virulenza

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Materials and methods

Bacterial strains

Isolates were identified as *S. maltophilia* by the API-20NE (bioMerieux, La Balme Les Grottes, France) system and 5' end sequencing of the 16S rRNA gene. Strains were routinely grown at 37 or 30◦C either on Luria-Bertani broth (LB), Trypticase Soy broth (TSB) or Trypticase Soy agar plates (TSA; Oxoid Ltd. Basingstoke, Hampshire, England).

DNA manipulations

Total genomic and plasmid DNA extraction, restriction digestion, electrophoresis, and DNA fragment purification were carried out as described previously (Casalino et al., 2003). PCR amplifications were performed using either Taq or, when appropriate, high-fidelity Pfu polymerases (Stratagene). The primers used throughout this study are reported in Table 1. Primers were designed mainly on the basis of the available genomic sequence of *S. maltophilia* strain K279a (GeneBank accession No. AM743169). Forward and reverse DNA sequencing were performed at the Synergene Biotech GmbH Company, Zurich, Switzerland

PFGE

All *S. maltophilia* strains studied were characterized by PFGE macrorestriction analysis of chromosomal DNA as previously described (Roscetto et al., 2008). Briefly, preparation of agarose plugs containing chromosomal DNA for PFGE analysis was performed using the PulseNet standardized procedure (http://www.cdc.gov/pulsenet). The DNA plugs were digested with 60 U of *Xba*I (Roche Diagnostics) at 37°C for 16 h. Genomic DNA fragments were separated by PFGE at 14°C on agarose 1.2% w/v gels in a clamped homogeneous field electrophoresis apparatus (CHEF-DRII system; Bio-Rad, Hemel Hempstead, UK), with pulse times ramped from 1 to 20 s over 21 h at 6.0 V/cm in 0.5× TBE. Lambda ladder and *Xba*I restriction fragments of *Salmonella enterica* serovar Braenderup strain H9B12 genomic DNA were used as size standards (Hunter et al., 2005).

RFLP of the *gyrB* gene

Amplification of the *gyrB* gene was performed using primers UP-1 and UP-2r (Table 1), as previously described (Coenye and LiPuma, 2002). The 1.2-kb *gyrB* amplicons were digested with HaeIII restriction endonuclease, and restriction fragments were separated in 10% polyacrylamide gels. A 50-bpDNA ladder (Amersham Bioscience) was included in all gels as size standard. The gyrB RFLP profiles of the different strains were compared with that of reference *S. maltophilia* strain K279a.

Analysis of PFGE and *gyrB* RFLP profiles

PFGE and *gyrB* RFLP profiles were subjected to cluster analysis using the GelCompar II version 3.5 software package (Applied Maths, Sint-Martens-Latem, Belgium). Clustering was based on the unweighted pair group method with arithmetic averages (UPGMA). The Dice correlation coefficient was used to analyse the similarities of the banding patterns with a tolerance of 1%. The molecular sizes of the bands of each PFGE profile were calculated by comparing their migration to those of a lambda ladder and of the size standard *S. enterica* serovar Braenderup strain H9B12, run on each gel. Only *Xba*I-digested DNA fragments in the range of 20-500 kb were considered. Isolates presenting identical PFGE banding patterns (similarity coefficient of 100%) were considered as indistinguishable strains and classified within the same cluster ("pulsotype"). Simpson's index of diversity (DI), a measure of the discriminatory ability of a given typing method, was calculated as described (Hunter and Gaston, 1988). The molecular size of fragments of the different *gyrB* RPFL profiles were determined by comparing their migration rates to that of a 50-bpDNAladder as well as to *Hae*III fragments of the *gyrB* gene of the reference strain K279a (internal standard). Densitometric analysis, normalization and interpolation of the patterns and numerical analysis were performed as described above.

16S rRNA gene sequencing, sequence comparison and analysis of phylogenetic divergences

A 500-bp DNA fragment within the 16S rRNA gene (Gould et al., 2004) was PCR-amplified from total genomic DNA preparations of all the *S. maltophilia* strains analysed in this study. Bacterial DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Amplification was achieved by using the specific primer pair rDNAf and rDNAr (Table 1), which amplifies a DNA fragment encompassing the majority of sequence variations known to

occur in different Gram-negative bacteria (Avison et al., 2001). Amplicons were purified prior to sequencing with the Minielute PCR purification kit (Qiagen, GmbH, Hilden) and forward and reverse sequencing reactions were performed. Phylogenetic diversity was determined by evaluating the 16S rRNA variable sequence regions essentially as described by Gould et al. (2006).

PCR analysis of virulence-associated genes

The presence of virulence-associated genes encoding type-1 fimbriae (smf-1), major and minor extracellular proteases (StmPr1 and StmPr2), and esterase (encoded by the Smlt3773 locus in the genome of strain K279a) was determined by PCR. Genes were amplified with specific primer pairs (Table 1), designed on the basis of the genomic sequence of the *S. maltophilia* strain K279a. Each gene was amplified separately, and amplicons were identified on the basis of their expected fragment size (smf-1, 674 bp; StmPr1, 1621 and/or 868 bp; StmPr2, 1764 bp; Smlt3773 locus, 1342 bp). When required the amplicons were cloned into the pGEM-T plasmid vector (Promega) for sequencing. Sequences were subjected to BLAST analysis and compared to the genome of *S. maltophilia* reference strain K279a.

Assays for protease and esterase activity

Protease activity was evaluated by plating *S. maltophilia* cultures on specific agar plates. Briefly, bacteria were grown in LB broth to OD_{600} = 1. Protease activity was determined using a casein hydrolysis test by spotting 2 µl of bacterial culture on LB agar plates supplemented with 2% skim milk (Fluka) (Figueiredo et al., 2006).

Quantitative extracellular proteolytic activity was determined using the azoalbumin assay (Phillips et al., 1984). Briefly, 500 µl of a 1 mg/ml azoalbumin solution in Tris-HCl, pH 7.7, were added to 500 µl filter-sterilized supernatants from cultures grown in TSB for 14 h, and then incubated at 37°C for 24 h. Trichloroacetic acid was added at 13% final concentration to precipitate the non-degraded protein. Samples were centrifuged at 6000 g for 10 min, and the OD_{440} of the resulting supernatants was measured.

Esterase activity was evaluated by plating *S. maltophilia* cultures on specific agar plates. Briefly, bacteria were grown in LB broth to OD_{600} = 1. Esterase activity was evaluated by spotting 2 µl of bacterial culture on LB agar plates supplemented with an emulsion of tributyrin-gum Arabic (Wilhelm et al., 1999). Plates were incubated overnight. In both protease and esterase assays, the presence of clear halos around bacterial growth was taken as evidence of protease or esterase activity.

Virulence test in *Galleria mellonella* larvae

Larvae of the greater wax moth *G. mellonella* were reared on a natural diet, honeybee nest debris, at 30°C in the dark. Larvae of about 250-300 mg were used in 50% lethal dose (LD_{50}) experiments. Insect infections with *S. maltophilia* were carried out, essentially as previously described (Seed and Dennis, 2008). Briefly, bacterial cultures were centrifuged, resuspended in 10 mM $MgSO_4$ supplemented with 0.15 mg/ml of ampicillin, and serial 10-fold dilutions were prepared. Five-µl aliquots (containing approximately from 10^7 to 0 CFU) were inoculated at the hindmost left proleg of larvae. Ten larvae were injected with each dilution. As controls, larvae were injected either with the *P. aeruginosa* strain PA14 (positive control), the *E. coli* K-12 strain MG1655 (negative control) or with 5 µl of only 10 mM $MgSO_4$ supplemented with ampicillin, in order to measure any potential lethal effects of the injection process. Larvae were incubated in 10-cm plates at 37°C, and the number of dead or alive larvae was scored every 24 h over a 72-h period. Larvae were considered dead when they displayed increased melanization and lack of movements. LD_{50} values were determined for each strain tested using the Systat computer program (Seed and Dennis, 2008). For calculations, the results from at least three independent experiments were combined.

Nucleotide sequence accession numbers

GenBank accession numbers of the sequences determined in this study are as follows: for StmPr1 gene from FN598883 to FN598890, for the StmPr2 gene from FN598891 to FN598898, and for esterase (Smlt3773 locus) from FN598899 to FN598906. Multiple Sequence Alignment of the deduced protein sequences of StmPr1, StmPr2 and esterase, was performed with ClustalW2 (http://www.ebi.ac.uk/clustalw2/index).

Construction of *S. maltophilia* K279a mutants

The deletion mutants of K279a strain (K279aΔStmPr1, K279aΔStmPr2, K279aΔStmPr1/ΔStmPr2, K279aΔDSF and K279aΔSMF-1) were constructed by allelic exchange using the suicide vectors pDM4. Using K279a template DNA, upstream fragments of the *StmPr1, StmPr2, DSF and SMF-1* genes were amplified using the primers Pr1UPf and Pr1UPr (for *StmPr1*), Pr2UPf and Pr2UPr (for *StmPr2*), DSFUPf and *DSF*UPr (for *DSF*), SMFUPf and *SMF*UPr (for *SMF-1*), while downstream fragments were amplified using the primers Pr1DWf and Pr1DWr (for *StmPr1*), Pr2DWf and Pr2DWr (for *StmPr2*), DSFDWf and DSFDWr (for *DSF*) and SMFDWf and SMFDWr (for *SMF-1*). The resulting PCR products were cloned in pBluescript-II KS+, resulting in the plasmids pBSStmPr1UP and pBSStmPr1DW (for *StmPr1*), and pBSStmPr2UP

and pBSStmPr2DW (for *StmPr2*), pBSDSFUP and pBSDSFDW (for *DSF*) and pBSSMFUP and pBSSMFDW (for *SMF-1*). The upstream and downstream fragments of each gene were then excised with the corresponding restriction enzymes and cloned in the suicide vector pDM4 (Milton *et al*., 1996), resulting in the plasmids pDM4Δ*StmPr1*, pDM4Δ*StmPr2, pDM4ΔDSF* and pDM4Δ*SMF-1*. Allelic exchange in *S. maltophilia* K279a following conjugal mating with *E. coli* S17-1λ*pir* donor strains and sucrose counter-selection was performed as described by Logue et al., (2009) (figure 1). The resulting K279a *StmPr1, StmPr2, DSF* and *SMF-1* K279a mutant strains were confirmed by PCR and phenotypic assays.

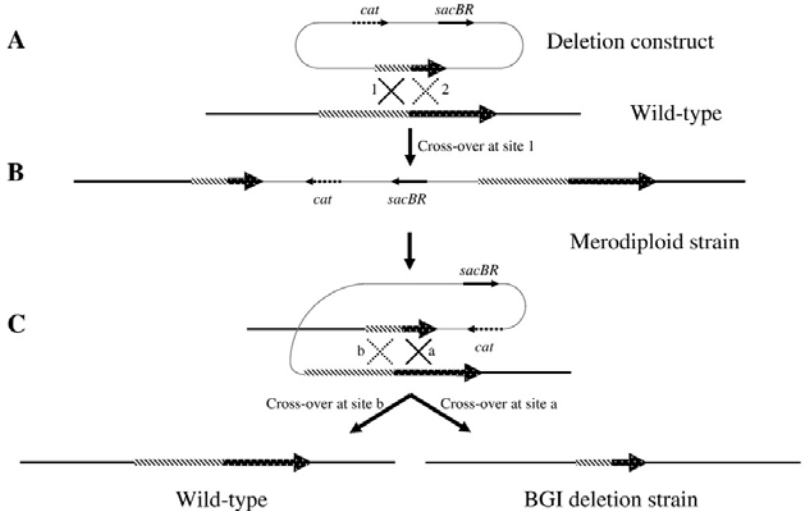

Figure 1. Procedure for allele replacement mutagenesis. A. Generation of a deletion construct by ligation of the deleted allele of the locus into the suicide vector, pDM4. The suicide vector contains the sacBR genes conferring sucrose sensitivity. B. Conjugation of the deletion construct into *S. maltophilia*. The deletion construct integrates into the genome by homologous recombination (depicted in this figure at site 1, although integration can also occur at site 2). The resulting merodiploid strain is selected for chloramphenicol resistance and is sucrose sensitive due to the presence of *sacBR*. C. Excision of the integrated suicide vector (and thus *sacBR*) from the genome results in either: allelic exchange (at site a) and generation of a deletion strain; or conversion back to wild-type (recombination at site b). Strains are selected by sucrose-resistance due to the loss of *sacBR*, followed by screening for chloramphenicol, indicating that the suicide vector has been excised (Logue et al., 2009).

Complementation assay

Complementation assay were performed using the cloning vector pBBR1MCS-5 (Gm^R) (Kovach et al., 1995). The functional genes cloned in pBBR1MCS-5 were amplified from K279a strain using the following primers (table 1): Pr1L2 and Pr1R2 for *StmPr1*, Pr2UPf and Pr2DWr for *StmPr2*, DSFUPf and DSFDWr for DSF. Plasmids with functional gene cloned, were transferred into the mutants strains by elettroporation.

Suppression Subtractive Hybridization (SSH)

Subtractive hybridization is a powerful technique that has been applied to research in many different fields. It was developed for the first time by Diatchenko in 1996 (Diatchenko et al., 1996). Subtraction methods can be used to identify sequences that are present in one bacterial genome, but are absent in another. The genomic DNA sample that contains the sequences of interest is referred to as tester and the reference sample is referred to as driver. Tester and driver DNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized DNAs represent genes that are expressed in the tester yet absent from the driver DNA.

Genomic DNA was isolated from *S. m*altophilia K279a and LMG959 strains as described previously (Casalino et al., 2003). SSH was carried out using the CLONTECH PCR-Select Bacterial Genome Subtraction kit (Clontech) as recommended by the supplier, but with a hybridization temperature of 73°C. In the hybridizations, DNA from the K279a was used as the tester and DNA from strain LMG959 was used as the driver. PCR amplicons obtained following SSH were cloned into pGEM-T Easy (Promega). The subtraction library of *Rsa*I fragments thus constructed was screened by sequencing of plasmid DNA extracted from individual clones. Tester-specific sequences were analysed using BLASTN and BLASTX searches of the general database using the site http://www.ncbi.nlm.nih.gov.

Table 1. Primers used in this work

Primer	Sequence [5´-> 3´]	Target	Reference
UP-1 UP-2r	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAA RTTYGA AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGC RTCNGTAT	*gyrB*	Coenye and LiPuma, 2002
rDNAf rDNAr	TCAGATTTGAACGCTGGCGGCA CGTATTACCGCGGCTGCTGCCAC	16S rDNA	Avison et al., 2001
Fm3L[a] Fm2R[a]	GGAAGGTATGTCCGAGTCCG GCGGGTACGGCTACGATCAGTT	*smf-1*	This study
Pr1aL[a] Pr1aR[a]	GCC GCA GTG TTG GTT CGA TCC A CAG TTC TCG GTG CAC GGC TCT T	*stmPr1*	This study
Pr1L[b] Pr1R[b]	CACGGCGGTCTT GTTGGTCA CGAGAACGACAACGAGTGCTACA	*stmPr1*	This study
Pr2L[c] Pr2R[c]	GCCGATTCCGGCATTCACACC GGTCAGGCCCGAGAAGGTGCT	*stmPr2*	This study
Lp1L[a] Lp2R[a]	CGGTGCCGAACTCGTAACCGG CTTCCGGCCATGGCAGGCGAA	Smlt3773 locus	This study
Pr1UPf[a] Pr1UPr[a]	CCAACACGGTCTAGATGGGCAGCGAA CGTCGAATTCCTCTTCTGGGTCATGG	*StmPr1*UP	This study
Pr1DWf[a] Pr1DWr[a]	GGTGTGAGCGAATTCGCCACGCAGTA CCAGCGCTCGAGCAGATCATCGATCT	*StmPr1*DW	This study
Pr2UPf[a] Pr2UPr[a]	GGGCTTCTAGATGGGCGACATCAACG ACGCGGTTGGAATTCCTGGGACATCG	*StmPr2*UP	This study
Pr2DWf[a] Pr2DWr[a]	GGCCTGGAATTCAACGCCCAGTACTG ACGACAACTCGAGCTACCAGATCGGC	*StmPr2*DW	This study
DSFUPf[a] DSFUPr[a]	CACCCTCTAGACCATCCTGCGTTG GCAATGGGGATCCGGCCTGATCGT	DSF UP	This study
DSFDWf[a] DSFDWr[a]	GGGGATCCGCAGACATGGGAGTT CGACATCTCGAGGATGGACGAGCA	DSF DW	This study
SMFUPf[a] SMFUPr[a]	GGTTCTCTAGACACGTTCTCTGGTTC GCATTCGGAATTCCCTAACCCTACTG	*SMF-1* UP	This study
SMFDWf[a] SMFDWr[a]	GCAGCGTCGAATTCACGATCATCTAC GTAGAAGATCTTCAGGCGCGAACGAA	*SMF-1* DW	This study
Pr1L2[a] Pr1R2[a]	GGAGTCGTCTAGAAATGATGGCCGGA CCCCGTCGACCGCTTTCGTAAA	*StmPr1*	This study

Pr2UPf[a] Pr2DWr[a]	GGGCTTCTAGATGGGCGACATCAACG ACGACAACTCGAGCTACCAGATCGGC	*StmPr2*	This study
DSFUPf[a] DSFDWr[a]	CACCCTCTAGACCATCCTGCGTTG CGACATCTCGAGGATGGACGAGCA	DSF	This study
PCR Primer 1 (SSH)	CTAATACGACTCACTATAGGGC	"Kit Clontech"	
Nested Primer 1 (SSH)	TCGAGCGGCCGCCCGGGCAGGT	"Kit Clontech	
Nested Primer 2R (SSH)	AGCGTGGTCGCGGCCGAGGT	"Kit Clontech	

[a] Primers designed on the basis of K279a genome sequence, accession No. AM743169.
[b] Primers designed on the basis on the *StmPr1* sequence, accession No. AJ291488.
[c] Primers designed on the basis on the *StmPr2* sequence, accession No. AY253983.

REFERENCES

Avison, M.B., Higgings, C.S., von Heldreich, C.J., Bennett, P.M., Walsh, T.R., 2001. Plasmid location and molecular heterogeneity of the L1 and L2 β-lactamase gens of *Stenotrophomonas maltophilia.* Antimicrob. Agents Chemother. 45, 413-419.

Casalino, M., Latella, M.C., Prosseda, G., Colonna, B., 2003. CadC is the preferential target of a convergent evolution driving enteroinvasive *Escherichia coli* toward a lysine decarboxylase-defective phenotype. Infect. Immun. 71, 5472-5479.

Coenye, T., LiPuma, J.J., 2002. Use of the *gyrB* gene for the identification of Pandoraea species. FEMS Microbiol. Lett. 208, 15-19.

Diatchenko L., Y. F. C. Lau, A. P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E. D. Sverdlov and P. D. Siebert 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* 93, 6025-6030.

Figueiredo, P.M., Furumura, M.T., Santos, A.M., Sousa, A.C., Kota, D.J., Levy, C.E., Yano, T., 2006. Cytotoxic activity of clinical *Stenotrophomonas maltophilia*. Lett. Appl. Microbiol. 43, 443-449.

Gould, V.C., Okazaki, A., Howe, R.A., Avison, M.B., 2004. 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. 54, 348-453.

Gould, V.C., Okazaki A., Avison, M.B., 2006. Beta-lactam resistance and beta-lactamase expression in clinical *Stenotrophomonas maltophilia* isolates having defined phylogenetic relationships. J. Antimicrob. Chemother. 57, 199-203.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465-2466.

Hunter, S.B., Vauterin, P., Lambert-Fair, M.A., Van Duyne, M.S., Kubota, K., Graves, L., Wrigley, D., Barrett, T., Ribot, E., 2005. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. J. Clin. Microbiol. 43, 1045-1050.

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM 2nd, Peterson KM. 1995. Four new derivatives of the broad-host-range

cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 166, 175-6.

Logue CA, Peak IR, Beacham IR. 2009 Facile construction of unmarked deletion mutants in *Burkholderia pseudomallei* using sacB counter-selection in sucrose-resistant and sucrose-sensitive isolates. J Microbiol Methods. 763, 320-3.

Milton DL, O'Toole R, Horstedt P, Wolf-Watz H. 1996 . Flagellin A is essential for the virulence of *Vibrio anguillarum.* J Bacteriol. 178, 1310-9.

Phillips P, Prior D, Dawes B (1984) A modified azoalbumin technique for the assay of proteolytic enzymes for use in blood group serology. J Clin Pathol 37,329-331.

Roscetto, E., Rocco, F., Carlomagno, M.S., Casalino, M., Colonna, B., Zarrilli, R., Di Nocera, P.P., 2008. PCR-based rapid genotyping of *Stenotrophomonas maltophilia* isolates. BMC Microbiol. 8, 202-211.

Seed, K.D., Dennis J.J., 2008. Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex. Infect. Immun. 76, 1267-1275.

Wilhelm, S., Tommassen, J., Jaeger, K.E., 1999. A novel lipolytic enzyme located in the outer membrane of *Pseudomonas aeruginosa*. J. Bacteriol. 181, 6977-6986.