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Overexpression of the multidrug efflux pump SmeDEF impairs *Stenotrophomonas maltophilia* physiology

Ana Alonso[1], Gracia Morales[1], Ricardo Escalante[2], Ester Campanario[1], Leandro Sastre[2] and Jose L. Martinez[1]*

[1]Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049-Madrid; [2]Instituto de Investigaciones Biomédicas, CSIC/UAM, C/ Arturo Duperier 4, 28029-Madrid, Spain

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***Objectives*: The use of antibiotics for the treatment of infectious diseases has led to important changes in the structure of pathogenic bacterial populations. However, these changes could be buffered if the expression of antibiotic resistance genes were to lead to the counter-selection of antibiotic-resistant strains in antibiotic-free environments. To test the effect of antibiotic resistance on bacterial fitness, we analysed the effect of the overproduction of the multidrug efflux pump SmeDEF on the physiology of *Stenotrophomonas maltophilia*. SmeDEF confers resistance to antibiotics belonging to different structural families, and its overexpression is associated with an antibiotic resistance phenotype in clinical isolates of *S. maltophilia*.**

***Results*: Two *S. maltophilia* isogenic strains were analysed: the wild-type strain D457 and strain D457R, which is a SmeDEF overproducer. In co-culture experiments, under non-selective pressure the wild-type strain displaced the mutant strain D457R. Metabolic profiling showed that SmeDEF overproduction leads to several changes in *S. maltophilia* metabolism. Using a *Dictyostelium discoideum* model of bacterial virulence, we found overexpression of SmeDEF to be associated with a reduction in *S. maltophilia* virulence.**

***Conclusions*: Together, these data indicate that overexpression of the multidrug efflux pump SmeDEF causes a metabolic burden for *S. maltophilia*.**

Keywords: antibiotic resistance, *S. maltophilia*, MDR, bacterial fitness, metabolic profiling, virulence

Introduction

Stenotrophomonas maltophilia is an increasingly important opportunistic pathogen associated with several human diseases,[1] with treatment proving difficult due to the species' intrinsic antibiotic-resistant phenotype. In Gram-negative bacteria, intrinsic resistance is due to interplay between the permeability of the bacterial envelope and the presence of multidrug resistance (MDR) efflux pumps.[2] MDR determinants are usually down-regulated, at least under laboratory growth conditions, but mutants overexpressing MDR pumps are easily selectable both *in vitro* and *in vivo*. MDR determinants extrude antibiotics from bacterial cytoplasm, as well as a plethora of other compounds,[2] quorum sensing signals included,[3] suggesting that their overexpression could be deleterious. Recent studies indicate that the overproduction of MDR pumps has a dramatic effect on the fitness of *Pseudomonas aeruginosa*, including a lack of quorum-sensing response[3] and a reduction in the potential transmissibility and virulence of MDR mutants (at least in laboratory models).[4,5]

Antibiotic resistance is very important for bacterial growth during antibiotic treatment. Nevertheless, it can cause a metabolic burden that can become onerous once the selective pressure is removed. Under such conditions, antibiotic-resistant bacteria can be displaced by their susceptible counterparts.

In earlier work, we characterized the SmeDEF efflux pump of *S. maltophilia*.[6] This is involved both in intrinsic[7] and acquired[8] antibiotic resistance in this species. The present study was undertaken to establish the effect of the overexpression of SmeDEF on *S. maltophilia* fitness.

Materials and methods

Bacterial strains and growth conditions

The strains used were *S. maltophilia* D457 and its multiresistant derivative, D457R. The latter overexpresses the *smeDEF* operon[6] because it has a point mutation in *smeT*, the gene coding for the repressor of

*Corresponding author. Tel: +34-91-5854542; Fax: +34-91-5854506; E-mail:jlmtnez@cnb.uam.es

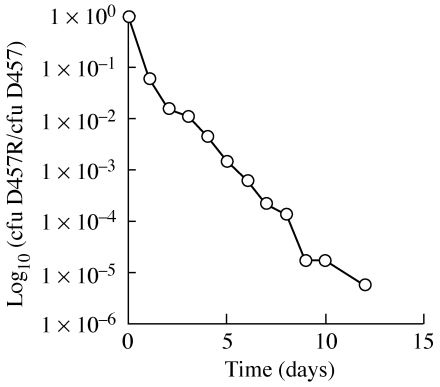

Figure 1. Growth competition between *S. maltophilia* D457 and D457R. The capability of the wild-type strain D457 for displacing the MDR mutant D457R was evaluated in co-culture experiments. Overproduction of the MDR efflux pump led to reduced fitness of *S. maltophilia*; the MDR mutant D457R was readily displaced by the wild-type D457 strain.

smeDEF expression.[9] Both strains were grown in LB medium at 37°C unless otherwise indicated. Strains *P. aeruginosa* PAO1 and *Klebsiella aerogenes* were grown as described in the *Dictyostelium discoideum* assays below.

Growth competition between S. maltophilia D457 and S. maltophilia D457R

Growth competition experiments between D457 and D457R were performed in LB broth by mixing 10^6/mL cells of each strain and incubating them at 37°C with agitation. Every 24 h the bacterial mixture was diluted 1/1000 with pre-warmed LB broth. The representation of the strains in the culture was determined at different times by plating serial dilutions on both non-selective and selective LB agar plates containing 15 mg/L of nalidixic acid (nalidixic acid MIC: D457 = 8 mg/L, D457R = 128 mg/L).[3] The plates with nalidixic acid only allowed the growth of D457R colonies. Both D457 and D457R colonies grew on the non-selective LB agar plates. As controls, the same experimental procedure was undertaken but using pure cultures of D457 and D457R. The number of nalidixic acid-resistant D457 mutants and the number of D457R cells that lost the MDR phenotype was $<10^{-7}$ at all times.

Metabolic profiling

Overnight cultures of D457 and D457R were washed twice with 0.85% NaCl and diluted to OD_{550} 0.1 (79%T) in 0.85% NaCl supplemented with trace elements. Bacterial suspension (150 µL) was poured into each well of a 96-well GN2 plate (Biolog, Hayward, CA, USA) and incubated at 37°C for 24 h. The GN2 plates have different carbon sources in each well and a metabolism reporter based on the reduction of tetrazolium coupled to bacterial respiration.[10] The response of the cells was monitored colorimetrically using a Spectramax 250 (Molecular Devices Corporation, Sunnyvale, CA, USA) at 590 nm. This experiment was performed in triplicate.

Effect of S. maltophilia on D. discoideum growth

D. discoideum cells (strain AX4) were grown in HL5 medium at 22°C. The bacterial strains were grown at 37°C in SM (1% glucose, 1% peptone, 0.1% yeast extract, 0.1% $MgSO_4 \cdot 6H_2O$, 0.19 % KH_2PO_4, 0.06 % K_2HPO_4, pH 6.5) to an optical density of 0.6 at 600 nm before plating.[11]

For quantitative virulence assays, the two *S. maltophilia* test strains, *P. aeruginosa* and *K. aerogenes*, were plated on SM agar and then spotted with serial dilutions of *D. discoideum* cells (4000 cells in drop 1, 2000 cells in drop 2, 1000 cells in drop 3, followed by a consecutive 2× dilution until drop 11 with two cells). The bacterial lawns were formed with a mixture containing *K. aerogenes* as the food source for *D. discoideum* plus the strain to be tested. All plates were incubated at 22°C for 5 days.[11]

Results and discussion

It is generally accepted, although with limited published data, that the expression of antibiotic resistance determinants has a physiological cost for bacteria.[12] Quantification of bacterial colonies showed that the number of bacterial cells within the colonies of the SmeDEF-overexpressing MDR *S. maltophilia* D457R mutant strain was lower ($0.9 \times 10^{10} \pm 0.2 \times 10^{10}$) than in those of the wild-type strain ($3.3 \times 10^{10} \pm 0.3 \times 10^{10}$). This indicates that SmeDEF overproduction is associated with a reduction in colony growth of *S. maltophilia*. We also compared the growth of the wild-type strain *S. maltophilia* D457 and its isogenic MDR mutant D457R on rich liquid culture medium. No major differences were detected between the growth kinetics of these strains during the exponential growth phase. However, after overnight incubation, the wild-type strain reached an A_{600} of 4.7 ± 0.4, whereas the MDR mutant D457R only reached 3.7 ± 0.4, indicating that overproduction of SmeDEF produced a shift in the onset of the stationary phase.

To analyse whether overexpression of SmeDEF affects the fitness of *S. maltophilia*, we studied growth competition, under conditions of feast/famine[13] that bacteria probably encounter in their natural environment. Figure 1 shows that within 2 weeks the wild-type strain progressively displaced the MDR strain to almost undetectable levels, indicating that overexpression of the MDR pump SmeDEF impairs the fitness of *S. maltophilia*.

The fact that the SmeDEF overproducing D457R strain is 'less fit' than the wild-type D457 strain suggests the possibility of general metabolic impairment associated with the MDR phenotype. To confirm this, metabolic profiling[10] of both strains was performed. The data obtained (Table 1) suggest that the MDR strain *S. maltophilia* D457R is impaired in terms of amino acid use, perhaps explaining why it was displaced by the wild-type strain in the co-culture experiments (performed in an amino acid-rich LB medium). In contrast, D457R makes better use of sugars such as dextrin, mannose or gentibiose, and is more efficient in its use of the C1 carbon source formic acid. Together, these data indicate that SmeDEF overexpression is associated with several pleiotropic changes in bacterial physiology, although there appears to be no global, non-specific burden on bacterial metabolism.

To go one step further, we wanted to analyse the effect of SmeDEF overproduction on *S. maltophilia* virulence. Such studies on this species are scarce and limited by the lack of useful models.[14] The non-mammalian *D. discoideum* virulence model has been valuable in analysing the virulence of opportunistic pathogens such as *P. aeruginosa*[5] and *Legionella pneumophila*,[11] and it was therefore used here to study the virulence of *S. maltophilia*. To validate the assays performed, we used a strain of *K. aerogenes* (which allows the growth of *D. discoideum*) as a negative control of virulence, and *P. aeruginosa* PAO1 (non-permissive) as a positive control. A quantitative assay of virulence was performed as previously described.[5] Serial 1/2 dilutions of *D. discoideum* cells (from 4000 cells in drop 1 to two cells in drop 11) were spotted as droplets onto lawns of bacterial mixtures containing *K. aerogenes* and the bacteria to be analysed. Non-virulent bacteria allow the growth of *D. discoideum* at all dilutions, and the degree of virulence correlates with the number

Table 1. Differential use of substrates by *S. maltophilia* D457 and D457R[a]

Substrates better used by D457	D457 (A_{590})	D457 (A_{590})	Ratio D457/ D457R (%)	Substrates better used by D457R	D457 (OD)	D457R (OD)	Ratio D457R/ D457 (%)
L-alanylamide	0.70 ± 0.05	0.41 ± 0.01	170	Dextrin	0.69 ± 0.16	0.99 ± 0.04	143
L-alanine	0.62 ± 0.06	0.38 ± 0.05	163	D-fructose	0.69 ± 0.12	0.85 ± 0.10	123
D-alanine	0.51 ± 0.06	0.30 ± 0.09	170	D-mannose	0.70 ± 0.18	0.96 ± 0.03	137
L-alanyl-glycine	0.98 ± 0.08	0.67 ± 0.01	146	Gentibiose	0.03 ± 0.00	0.19 ± 0.03	633
L-proline	0.68 ± 0.05	0.52 ± 0.02	131	D-psicose	0.16 ± 0.01	0.26 ± 0.05	162
L-serine	0.91 ± 0.08	0.71 ± 0.08	128	Mono-methyl-succinate	0.10 ± 0.03	0.14 ± 0.04	140
N-acetyl-D-galactosamine	0.15 ± 0.06	0.12 ± 0.03	125	Formic acid	0.02 ± 0.02	0.23 ± 0.02	1150
β-hydroxybutyrate	0.21 ± 0.01	0.07 ± 0.03	300	α-ketoglutaric acid	0.38 ± 0.15	0.51 ± 0.17	134
γ-hydroxybutyrate	0.10 ± 0.04	0.05 ± 0.02	200	α-ketovaleric acid	0.17 ± 0.02	0.34 ± 0.01	200

[a]Only the substrates allowing an optical density (OD) of >0.1, and differences between D457 and D457R >15%, are shown.

of *D. discoideum* cells needed for producing a clear growth plaque on the bacterial lawn. These studies showed that the wild-type *S. maltophilia* D457 strain was as virulent as the control virulent strain *P. aeruginosa* PAO1 and only allowed the growth of *D. discoideum* cells on the first and second dilutions (4000 and 2000 cells). However, a clear plaque could be seen with the MDR D457R strain even with dilution eight of the series (15 *D. discoideum* cells). These results clearly indicate that the SmeDEF-overproducing *S. maltophilia* D457R strain is less virulent than its wild-type isogenic parent *S. maltophilia* D457R, at least using the *D. discoideum* model system.

Conclusions

Overexpression of the MDR pump SmeDEF in *S. maltophilia* was associated with decreased fitness as well as a reduction in cell size when these bacteria were grown in rich medium. It was also associated with lower virulence as determined by the non-mammalian *D. discoideum* virulence model. Despite the pleiotropic phenotype observed, these effects are not the consequence of a general, non-specific impairment of bacterial physiology. In fact, metabolic profiling showed that D457R can use formic acid and some sugars more efficiently than the wild-type isogenic D457 strain. It has previously been shown that mutants that compensate for fitness defects of antibiotic-resistant bacteria differ depending on the environment in which they evolved.[15] Our results suggest that the fitness costs associated with antibiotic resistance might also be different depending on the environment in which bacteria grow, at least in the case of SmeDEF-overproducing *S. maltophilia* strains.

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