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Brief report

A molecular biological protocol to distinguish potentially human pathogenic *Stenotrophomonas maltophilia* from plant-associated *Stenotrophomonas rhizophila*

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Summary

In recent years, the importance of the Gram-negative bacterium *Stenotrophomonas* as an opportunistic pathogen as well as in biotechnology has increased. The aim of the present study was to develop new methods for distinguishing between strains closely related to the potentially human pathogenic *Stenotrophomonas maltophilia* and those closely related to the plant-associated *Stenotrophomonas rhizophila*. To accomplish this, 58 strains were characterized by 16S rDNA sequencing and amplified ribosomal DNA restriction analysis (ARDRA), and the occurrence of specific functional genes. Based on 16S rDNA sequences, an ARDRA protocol was developed which allowed differentiation between strains of the *S. maltophilia* and the *S. rhizophila* group. As it was known that only salt-treated cells of *S. rhizophila* were able to synthesize the compatible solute glucosylglycerol (GG), the *ggpS* gene responsible for GG synthesis was used for differentiation between both species and it was confirmed that it only occurred in *S. rhizophila* strains. As a further genetic marker the *smeD* gene, which is part of the genes coding for the multidrug efflux pump SmeDEF from *S. maltophilia*, was used. Based on the results we propose a combination of fingerprinting techniques using the 16S rDNA and the functional genes *ggpS* and *smeD* to distinguish both *Stenotrophomonas* species.

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Introduction

In the last decade, multidrug-resistant *Stenotrophomonas maltophilia* has become increasingly significant causing case/fatality ratios associated with a broad spectrum of clinical syndromes like bacteraemia, endocarditis, respiratory tract infections, etc. in certain patient populations, particularly in those who are severely debilitated, immunosuppressed or suffering from cystic fibrosis (for a review, see Denton and Kerr, 1998). Besides being a relevant opportunistic pathogen, *Stenotrophomonas* species are also frequently used in applied microbiology and biotechnology, e.g. for plant growth promotion as well as in biological control of plant fungal diseases (Berg *et al*., 1994; Dunne *et al*., 2000) and in bioremediation (Lee *et al*., 2002). *Stenotrophomonas* strains are ubiquitous, but they have been preferentially recovered from rhizospheres all over the world, and from a wide range of nosocomial sources (Denton and Kerr, 1998).

With the aim of differentiating clinically relevant strains from environmental ones, we applied diverse typing strategies (Berg *et al*., 1999; Minkwitz and Berg, 2001). From the 16S rDNA sequencing analysis, the isolates could be separated into three genomovars: two of which consisted of isolates originating from the environment, especially rhizosphere isolates (E1 and E2), and one contained clinical and aquatic strains (C). In contrast to recent investigations (Denton and Kerr, 1998), most of the strains could be grouped based on their source of isolation (Minkwitz and Berg, 2001), in spite of the fact that the antibiotic resistance profile of *S. maltophilia* isolates, and the ability

to colonize plant roots did not correlate with their origin (Suckstorff and Berg, 2003). However, it was possible to establish a new, clearly plant-associated species *Stenotrophomonas rhizophila* (Wolf *et al*., 2002) that comprised the isolates of the E1 cluster, and could be further distinguished from *S. maltophilia* isolates by (i) growth temperatures, (ii) xylose assimilation and (iii) osmolyte production. In contrast to the potentially human pathogenic *S. maltophilia*, no pathogenicity to humans is known for plant-associated *S. rhizophila*.

The objective of our study was to develop new techniques to differentiate between the species *S. maltophilia* and *S. rhizophila*, which can both occur in the rhizosphere, because some of them can be used in biotechnology for stimulation of plant growth or protection against plant pathogens whereas some others might be virulent.

Results and discussion

On the basis of 50 already characterized isolates obtained from different origins including clinical and environmental sources (Minkwitz and Berg, 2001), several methods were systematically compared. To verify our techniques and hypotheses, eight new strains isolated from the rhizosphere of marram grass from Dutch dunes were used (De Boer *et al*., 2001). Although all investigations were carried out for all 58 strains, the results are shown for 17 strains including nine strains representing well-characterized *Stenotrophomonas* strains, the type strains of *S. maltophilia* and *S. rhizophila*, and eight new marram grass-associated strains (Table 1).

By using primers annealing at the 9–27 bp forward and 1525–1545 bp reverse positions of the 16S rDNA, nearly the complete gene could be sequenced (deposited in the EMBL database under the Accession No. AJ293461–AJ293474 and AJ884480–AJ884487). According to the sequences, a separation in two clusters one containing *S. maltophilia* and the other containing *S. rhizophila* was obtained (Fig. 1). From the dune isolates four clustered among *S. maltophilia* strains, whereas four others were found in the *S. rhizophila* cluster. These two clusters were also clearly separated from the 16S rDNA sequences of the two other *Stenotrophomonas* species, *S. acidaminiphila* and *S. nitritireducens* (Fig. 1).

Sequencing of 16S rRNA gene is rather expensive and time-consuming therefore amplified ribosomal DNA restriction analysis (ARDRA) of 16S rRNA fragments was used for *Stenotrophomonas* differentiation. Based on the known sequences two enzymes were selected. The restriction by HhaI led to five fragments with 16S rDNA of both species, which was performed to confirm the genus. PstI was expected to allow distinguishing *S. maltophilia*

Table 1. Features of all investigated *Stenotrophomonas* strains.

		Detection of[a]		ARDRA[b]		Osmolytes[c]		
No.	Isolate No.	*smeD*	*ggpS*	HhaI	PstI	Trehalose	GG	References
Stenotrophomonas maltophilia C cluster								
1.	c5	+	–	5	2	+	–	Minkwitz and Berg (2001)
2.	c23	+	–	5	2	+	–	Minkwitz and Berg (2001)
3.	e-a2	+	–	5	2	+	–	Minkwitz and Berg (2001)
4.	DSM 50170[d]	+	–	5	2	+	–	Hugh and Ryschenko (1961)
Stenotrophomonas maltophilia E2 cluster								
5.	e-p3	+	–	5	2	+	–	Minkwitz and Berg (2001)
6.	e-p20	+	–	5	2	+	–	Minkwitz and Berg (2001)
Stenotrophomonas rhizophila								
7.	e-p7	–	+	5	1	+	+	Minkwitz and Berg (2001)
8.	e-p14	–	+	5	1	+	+	Minkwitz and Berg (2001)
9.	DSM 14405[d]	–	+	5	1	+	+	Wolf *et al*. (2002)
Stenotrophomonas isolates from Dutch dune rhizospheres								
10.	68	–	+	5	1	+	+	De Boer *et al*. (2001)
11.	274	–	+	5	1	+	+	De Boer *et al*. (2001)
12.	276	+	–	5	2	+	–	De Boer *et al*. (2001)
13.	296	+	–	5	2	+	–	De Boer *et al*. (2001)
14.	J1-5	+	–	5	2	+	–	De Boer *et al*. (2001)
15.	Ns2-6	+	–	5	2	+	–	De Boer *et al*. (2001)
16.	M2-2	–	+	5	1	+	+	De Boer *et al*. (2001)
17.	M10-4	–	+	5	1	+	+	De Boer *et al*. (2001)

a. Polymerase chain reaction product of the *smeD* or *ggpS* genes (see, for examples, Fig. 3).
b. Amplified ribosomal DNA restriction analysis (ARDRA) pattern of an internal 16S rDNA fragment cut by HhaI (five bands) or PstI (one or two bands; see, for examples, Fig. 2).
c. The ability to synthesize the osmolytes trehalose or glucosylglycerol (GG) was analysed according to Roder and colleagues (2005). +, DNA fragment or osmolyte present; –, DNA fragment or osmolyte absent.
d. Type strains of *S. maltophilia* or *S. rhizophila* group.

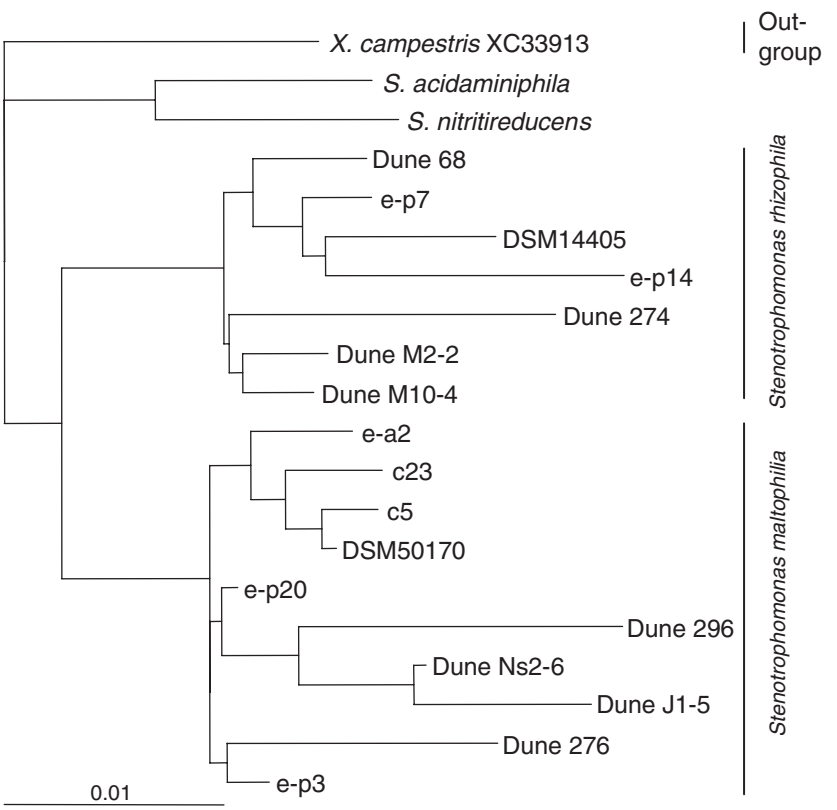

Fig. 1. Phylogenetic tree (CLUSTAL X; Thompson *et al*., 1994) of partial 16S rDNA sequences of selected isolates of *Stenotrophomonas maltophilia* and *S. rhizophila* obtained from clinical and environmental habitats including the strains from Dutch dunes (see Table 1) using the neighbour-joining method. 16S rDNA sequences from *S. acidaminiphila* (Accession No. AF273079) and from *S. nitritireducens* (Accession No. AJ012230) were included. A corresponding sequence from *Xanthomonas campestris* (XC33913) was taken as outgroup. Dendrograms were constructed with TREEVIEW v. 1.5 software programs (Page, 1996).

strains (one site, two fragments) from *S. rhizophila* strains (no site, one fragment). This assumption was fully supported by the experimental data. The 16S rDNA fragment from all strains classified as *S. maltophilia* was cut by PstI, while the *S. rhizophila* DNA fragment remained uncut (Table 1). Results are shown for selected representatives in Fig. 2A. In addition, the grouping of the Dutch dune isolates in the two *Stenotrophomas* species was also reproduced by the ARDRA analysis (Fig. 2B). The results obtained by ARDRA confirmed those of sequencing but were obtained with considerably less time and costs.

Besides 16S rRNA gene two other genes were included in the study to distinguish the *Stenotrophomonas* isolates. It was shown that salt-treated cells of *S. rhizophila* were able to synthesize the compatible solutes GG and trehalose, while strains of *S. maltophilia* were only able to accumulate trehalose (Wolf *et al*., 2002). This ability was tested for all strains and the resulting pattern corresponded with the 16S rDNA results, i.e. GG was only detected in cells of *S. rhizophila* (Table 1), whereas trehalose was extracted from all isolates. Among the Dutch dune isolates, four GG-producing strains were identified, namely those that showed 16S rDNA sequence and ARDRA pattern characteristic for *S. rhizophila*. The gene encoding for one biosynthetic enzyme of GG, the glucosylglycerol-phosphate synthase (GgpS), is named *ggpS*. Based on the sequence of the *ggpS* gene obtained for the type strain DSM 14405 (sequence Accession No. AJ878740), primers were derived to detect the gene by a polymerase chain reaction (PCR) approach. A PCR fragment of the expected size (882 bp) was only detectable in *S. rhizophila* strains (Fig. 3A) and in GG-producing Dutch dune isolates (Fig. 3B). Additionally, the occurrence of *ggpS* in *S. rhizophila* and its absence from all *S. maltophilia* strains was confirmed by Southern blot experiments with DNA isolated from selected strains of both species including all PCR product negative ones and type strains, and by partly sequencing of genes (not shown here). Thus, a full correspondence between the phenotypical feature GG accumulation and the genotypic marker (*ggpS* gene fragment) was observed. Moreover, an internal fragment of the *tps* gene, which is responsible for trehalose synthesis, could be amplified for all investigated *Stenotrophomonas* isolates. This corresponded to the trehalose accumulation by all strains (data not shown).

As a further genetic marker the occurrence of the multidrug resistance (MDR) efflux pump SmeDEF, recently found in clinical isolates of *S. maltophilia* (Alonso and Martinez, 2001), was studied. Specific primers to amplify a 150 bp fragment of the *smeD* gene (Alonso and Martinez, 2001) were chosen and applied to DNA isolated from all strains. A pattern complementary to the occurrence of the *ggpS* gene was found; all *S. maltophilia* strains possessed a *smeD* fragment, while it was absent

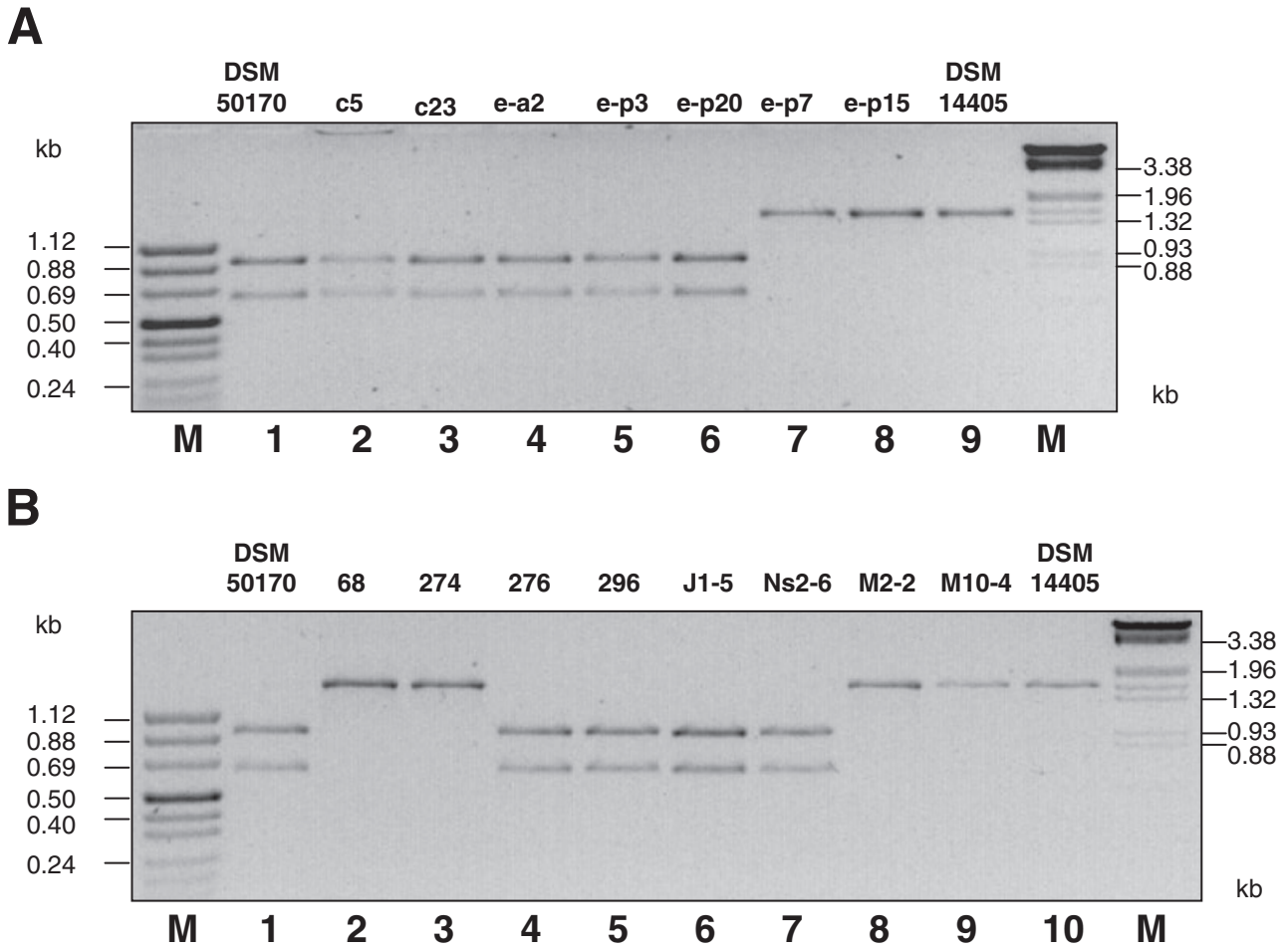

Fig. 2. Amplified ribosomal DNA restriction analysis (ARDRA) patterns of 16S rDNA fragments amplified by PCR using the primer EubI-forward and 1 μl EubII-reverse and cut by PstI analysed by gel electrophoresis on ethidium-bromide-stained 1.5% agarose gels. Patterns of selected isolates of *Stenotrophomonas maltophilia* and *S. rhizophila* (A, see Table 1) and patterns of non-characterized isolates from Dutch dunes (B). M, molecular mass marker.

in all strains belonging to *S. rhizophila* (Table 1, Fig. 3A). A corresponding picture was obtained with DNA from the Dutch dune isolates. Again, strains missing the *ggpS* fragment gave a *smeD* fragment and vice versa (Fig. 3B). Therefore, the detection of the *smeD* fragment seems to be specific for *S. maltophilia* strains and its absence is an indication for an *S. rhizophila* strain. The presence of the same MDR pumps in clinical and environmental isolates of a given species is not completely unexpected, as these are ancient genes (Saier *et al*., 1998), present in the chromosomes of all cells, from bacteria to humans, and might also play a relevant role for bacterial adaptation to non-antibiotic but toxic compounds (Alonso *et al*., 2001; Sanchez *et al*., 2004). However, we only detect the PCR product, functional evidence on whether the level of expression in clinical and environmental isolates is different is still missing. Experiments in one of our laboratories have shown that the *smeD* gene is absent in a battery of clinically relevant bacteria (J.L. Martínez *et al*., unpublished), indicating that this gene might be a good marker for *S. maltophilia*, so that we tested its presence in *S. rhizophila*. Sequencing analysis (Gould *et al*., 2004) has demonstrated that *smeDEF* sequence is well conserved in clinical *S. maltophilia* strains. However, the intergenic *smeT-smeD* region has a high degree of variation, to that point that it was possible to group clinical isolates based on their sequences (Gould *et al*., 2004; Sanchez *et al*., 2004). Thus, the fact that the *S. rhizophila* strains do not render a specific *smeD* PCR fragment is not complete evidence that *smeDEF* genes are not present in *S. rhizophila* genome. Nevertheless, the occurrence of *smeD* in *S. maltophilia* and its absence from *S. rhizophila* were confirmed by Southern blot experiments with DNA isolated from all *smeD*$^-$ strains and type strains of both species using a probe with the same sequence as for PCR and highly stringent conditions (42°C with 50% formamide for hybridization and washing in 0.01% SDS and 68°C, not shown here).

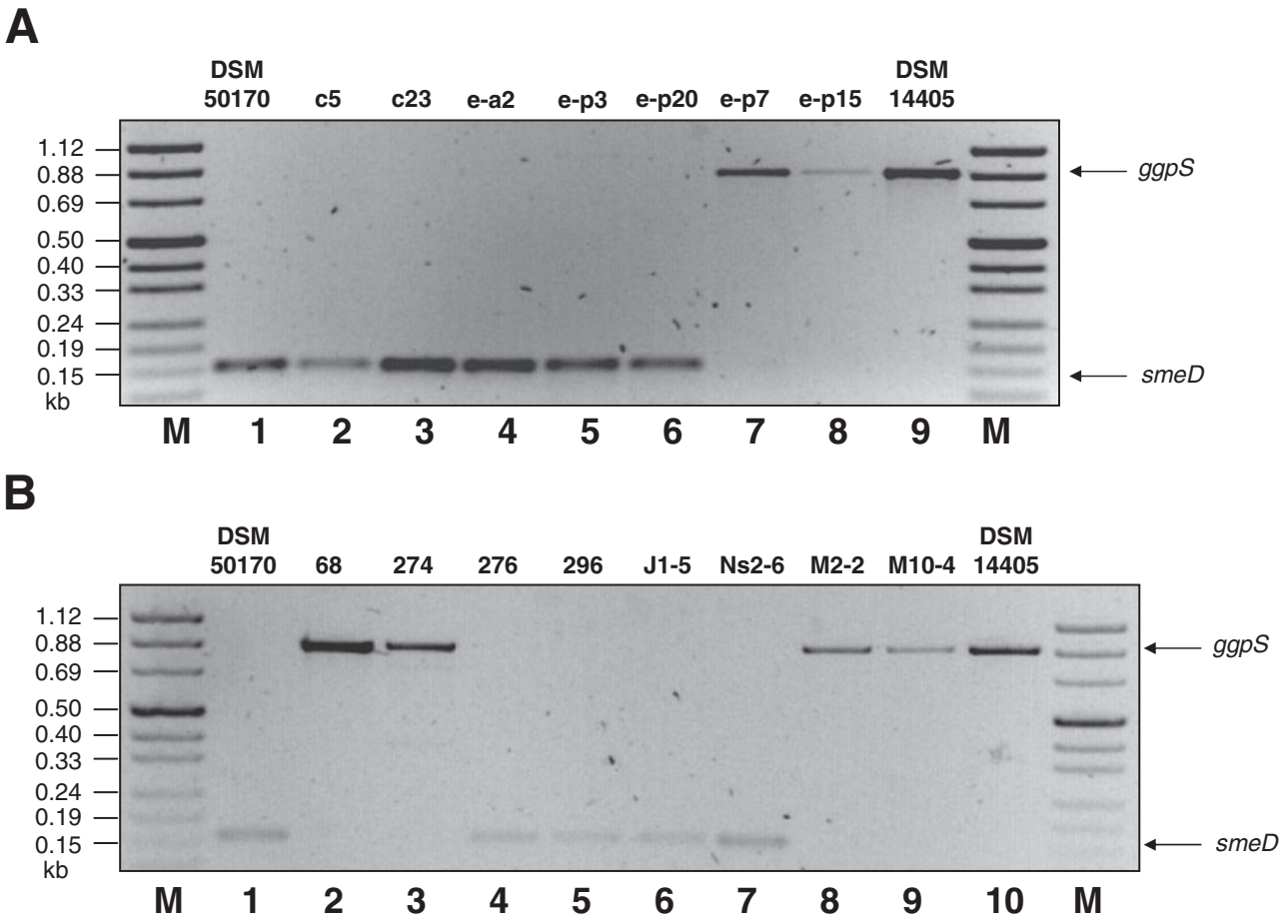

Fig. 3. Multiplex PCR to amplify partial gene sequences of *ggpS* (885 bp, upper fragment, marked by an arrow) and *smeD* (150 bp, lower fragment, marked by an arrow) from selected isolates of *Stenotrophomonas maltophilia* and *S. rhizophila* (A, see Table 1) and from non-characterized isolates from Dutch dunes (B). For the amplification of internal *ggpS* or *smeD* (Alonso and Martínez, 2001) fragments gene-specific primers were used (ggpSG3i: 5′-CAA CGG CGT GAT CCA CAT-3′; ggpSG5i: 5′-GAT GCT GCA TGT GTT CTG-3′, smeD3: 5′-CCA AGA GCC TTT CCG TCA T-3′; smeD5: 5′-TCT CGG ACT TCA GCG TGA C-3′). The PCR products were separated on 1.0% agarose gels.
M, molecular mass marker.

The developed methods enabled us to differentiate between *S. maltophilia* and *S. rhizophila*. Using our protocol, for all 58 strains that were included in this study, a clear classification was possible. Interestingly, four marram grass-associated isolates belong to *S. rhizophila*, while the other four isolates could be identified as *S. maltophilia.* This was confirmed by all applied methods. This underlines again that strains of *S. maltophilia* and *S. rhizophila* groups can be present in the rhizosphere of the same plant species, in this case marram grass. Until now, for plant-associated *S. rhizophila* no pathogenicity to humans is known. To develop methods to assess strains for their pathogenicity is an important task for the future. However, among *S. maltophilia* strains human pathogens have been detected and, so far, it is not possible to distinguish, with certainty, potential human pathogens from non-pathogens. Therefore, virulence determinants and/or pathogenicity islands might be good markers for differentiating both types of populations. Unfortunately, the mechanisms involved in the beneficial/pathogenic interactions between associated bacteria and their host plants are similar with those responsible for bacterial virulence in humans (Berg *et al.*, 2005). It seems that, at least for some bacterial species, the differences between environmental strains and those causing infections may occur at the level of gene regulation, rather than because of the presence or absence of genes (Alonso *et al.*, 1999; Parke and Gurian-Sherman, 2001). Therefore, the analysis of functional genes involved in interaction with eukaryotes, plants as well as humans, and their regulation is important to understand the ecological behaviour of bacteria with an environmental habitat and also causing human infections such as *Stenotrophomonas*.

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