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Delineation of *Stenotrophomonas* spp. by multi-locus sequence analysis and MALDI-TOF mass spectrometry

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ABSTRACT

The genus *Stenotrophomonas* is genetically and phenotypically heterogeneous. Of the nine species now accepted, only *S. maltophilia* is of clinical importance. Based on DNA-sequences of seven house keeping genes, it encompasses genogroups of DNA-similarity below 97% that predominantly comprise strains of environmental origin. Therefore, in order to unravel the uneven distribution of environmental isolates within genogroups and reveal genetic relationships within the genus, there is need for an easy and reliable approach for the identification and delineation of *Stenotrophomonas* spp. In this first study, a multi-locus sequence analysis (MLSA) with seven housekeeping genes (*atpD*, *gapA*, *guaA*, *mutM*, *nuoD*, *ppsA* and *recA*) was applied for analysis of 21 *S. maltophilia* of environmental origin, *Stenotrophomonas* spp. and related genera. The genotypic findings were compared with the results of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analyses. Our MLSA provided reliable inter- and intra-species discrimination of all tested isolates that correlated with the MALDI-TOF mass spectrometry data. One distantly related genogroup of environmental *S. maltophilia* strains needs to be reclassified as *S. rhizophila*. However, there are still remaining delineated *S. maltophilia* genogroups of predominantly environmental origin. Our data provide further evidence that '*Pseudomonas*' *beteli* is a heterotypic synonym of *S. maltophilia*. Based on MLSA and MALDI-TOF data, *Stenotrophomonas* sp. (DSM 2408) belongs to *S. koreensis*.

Introduction

Stenotrophomonas spp. have growing significance in applied microbiology and biotechnology, e.g., for stimulation of plant growth and biological control of plant fungal diseases, but also as relevant opportunistic pathogens [4,25]. *S. maltophilia* is associated with an ever-expanding spectrum of clinical syndromes, such as septicaemia, respiratory and urinary tract infections as well as, endocarditis, particularly in immune-compromised patients. Therefore, the clinical significance of this species should not be underestimated.

The taxonomy of the genus *Stenotrophomonas* and its type species *S. maltophilia* has been subject to considerable revision over the years. Initially described as *Pseudomonas maltophilia* [15], the species was later grouped in the genus *Xanthomonas* [27] before eventually becoming the representative of the genus *Stenotrophomonas* in 1993 [24]. The introduction of polyphasic taxonomic studies, which combine phenotypic and genotypic approaches, and development of new genotyping methods led to rapid expansion of knowledge of genus diversity. Nine distinct species of the genus *Stenotrophomonas* are validated in the List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/s/stenotrophomonas.html accessed August 2010): *S. maltophilia* [24], *S. rhizophila* [29], *S. koreensis* [30], *S. acidaminiphila* [3], *S. nitritireducens* [7], *S. terrae* [13], *S. humi* [13], *S. chelatiphaga* [17]. Another strain, *Stenotrophomonas* sp. (DSM 2408), has not been assigned a species name [23]. Recently, a new strain isolated from a soil sample taken from a ginseng field in South Korea was described as *S. ginsengisoli* [18]. The taxonomy of two other *Stenotrophomonas* species was changed. *Stenotrophomonas dokdonensis* was transferred to the genus *Pseudoxanthomonas* as *P. dokdonensis* [20,31] and *S. africana* is now described as a heterosynonym of *S. maltophilia* [5].

In our previous work, we developed and evaluated a multi-locus sequence typing scheme for *S. maltophilia* [16]. Sixty-eight *Stenotrophomonas* isolates obtained as *S. maltophilia* from five different collections were assigned to 15 MLSA genogroups. Remarkably, four of these (groups 5, 8, 9 and 10) contained isolates of predominantly or strictly environmental origin, as reported earlier by use of Amplified Fragment Length Polymorphism (AFLP)

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[12]. Moreover, MLSA genogroups 8 and 10 featured high DNA-sequence distances to other MLSA genogroups, including groups 5 and 6 with currently sequenced environmental R551-3 and clinical K279a strains. To unravel the uneven distribution of environmental isolates and reveal phylogenetic relationships in the genus *Stenotrophomonas,* we applied MLSA to the 13 *S. maltophilia* strains previously assigned to the MLSA genogroups 5, 8, 9 and 10 described by Hauben and colleagues [12]. In addition, 8 closely related non-*maltophilia* species were analyzed.

Our MLSA results were supplemented with the matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) of 21 strains and species. This method has been applied successfully by others for species identification, based on the protein compositions of bacterial cells [6,14,19]. The reference database for MALDI-TOF MS-based non-fermenter identification, including *S. maltophilia*, *S. acidaminiphila*, *S. africana*, *S. nitritireducens*, *S. rhizophila*, '*Pseudomonas*' *beteli* has been reported previously [22].

The aims of this study were to apply MLSA for inter- and intra-species delineation of *Stenotrophomonas* spp., in order to unravel the uneven distribution of environmental and anthropogenic isolates within the species *S. maltophilia*, and to compare the results of MALDI-TOF MS analyses with the genotypic findings.

Materials and methods

Bacterial strains and growth conditions

A total of 21 bacterial strains and species belonging to the genus *Stenotrophomonas* and type strains of related genera '*Pseudomonas*' *beteli* (964-T) and *Pseudoxanthomonas dokdonensis* (963-T) were analyzed in the present study, in which a T following the strain number indicates a type strain. Data on the investigated strains are depicted in Table 1. Bacteria were maintained at -70 °C in defibrinated horse blood and cultured on 5% Columbia sheep blood. Clinical strains were grown at 36 °C, environmental strains and species at 22 °C.

Multi-locus sequence analysis (MLSA)

The detailed MLSA procedure and set of primers used are posted at http://pubmlst.org/smaltophilia/info/primers.shtml and in supplementary material (Table S1). Briefly, genomic DNA was extracted with High Pure PCR Template Preparation Kit (Roche, Mannheim). The seven genes *atpD*, *gapA*, *guaA*, *mutM*, *nuoD*, *ppsA*, and *recA* were amplified, using *Taq* DNA polymerase (Ampli *Taq* Gold; Applied Biosystems, Darmstadt, Germany), as follows: initial denaturation at 95 °C for 9 min; followed by 30 cycles of denaturation at 94 °C for 20 s; annealing at the appropriate T_{ann} for 1 min; and extension at 72 °C for 50 s; followed by a final elongation step at 72 °C for 5 min. We were able to amplify all seven gene loci with the primer sets described in previous work [16], except for *ppsA* in the case of *Stenotrophomonas* sp. (957) and *S. koreensis* (959-T) and *gapA* in the case of '*P*'. *beteli* (964-T) and *P. dokdonensis* (963-T). To examine these strains, new primers were designed for the following genes: ppsA forw (2f), ppsA rev (2r), gapA forw (2fwd) and gapA rev (2r). Although the *nuoD* gene was amplifiable from all strains with the primers described previously, we used a new primer set for the optimization of PCR quality and yield (nuoD forw (2f) and nuoD rev (2r). For all genes, two separately generated amplicons for forward and reverse sequencing were purified from unincorporated nucleotides, using exonuclease I-phosphatase (USB, Staufen, Germany), according to the manufacturer's protocol. The purified template was quantified by using a Nanodrop ND-1000 spectral photometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). The sequencing reaction was performed with 20 ng DNA and the BigDye Terminator Ready Reaction Mix (version 1.1; Applied Biosystems). The air-dried reaction product was resuspended in 20 μl of Hi-Di formamide, separated and detected on an ABI Prism 310 genetic analyzer using POP-6 polymer and a 61-cm genetic analysis capillary (Applied Biosystems).

Sequence data processing and phylogenetic analysis

The sequence data were assembled from the resultant chromatograms with the Staden suite (version 1.7.0) and analyzed by use of BioNumerics analysis software (version 4.6; Applied Maths BV, Kortrijk, Belgium). GenBank accession numbers for the sequences reported in this study are HM370131–HM370277. Pairwise similarities were calculated on the basis of the concatenated seven gene sequences without further corrections. Cluster analysis was performed with the unweighted pair group method, using arithmetic averages (UPGMA). Bootstrap analysis was done with 1000 simulations to run. The dendrogram of 55 *Stenotrophomonas* strains described previously [16] was constructed by use of BioNumerics analysis software (Fig. 1), together with 20 newly analyzed strains and species with unique allelic profiles. Strain 967 was omitted from this analysis because of its identical allelic profile to strain 966.

Sample preparation for MALDI-TOF MS, spectrum generation and data analysis

Sample preparation for MALDI-TOF MS bacterial profiling was done as described previously [22]. Measurements were performed with a microflex LT (Bruker Daltonik GmbH, Leipzig, Germany) bench-top mass spectrometer equipped with a 60-Hz nitrogen laser (parameter settings: ion source 1 (IS1), 20 kV; IS2, 18.5 kV; lens, 8.5 kV; detector gain, 2.650 V; and gating, none). Spectra were recorded in the positive linear mode for the mass range of 2000–20,000 Da at maximum laser frequency. Raw spectra of bacteria were analyzed, using the MALDI Biotyper 2.0 software package (Bruker Daltonik GmbH, Bremen) with default settings. The whole process, from MALDI-TOF MS measurement to identification was performed automatically, without any user intervention. Representative spectra of four different species are shown as curves and a so-called pseudo-"gel"-view in Fig S1. For microorganism identification, the raw spectra were imported into the MALDI BioTyper software and analyzed in a range of 3000–15,000 Da by standard pattern matching (with default parameter settings) against the main spectra of 2506 microorganisms used as reference data in the BioTyper database (these spectra are an integrated part). The algorithm computes and combines three individual scores for three fundamental characteristics where the unknown spectrum and the reference spectrum are compared. First, the number of signals in the reference spectrum that have a matching partner in the unknown spectrum is determined. Next, the number of signals in the unknown spectrum that have a matching partner in the reference spectrum is determined. Finally, the symmetry of all ordered matching signal pairs is computed. If the high signals of the unknown spectrum correspond to the high signals of the reference spectrum, and the low signals also correspond, this results in a high symmetry value. For each individual score the maximum value is 1. The three characteristic scores are multiplied and the product is raised by a factor of 1000. The logarithm of the resulting value is taken. The result of this calculation is the log(score) value. The maximum obtainable value for the log(score) is three (log10(1000) = 3).The log(score) value between <2.0 and ≥1.7 indicates prediction to the genus level, a log(score) ≥ 2.0 indicates prediction to the species level. The cut-off values have been defined by the manufacturer on the basis of a pattern database,