Delineation of *Stenotrophomonas maltophilia* isolates from cystic fibrosis patients by fatty acid methyl ester profiles and matrix-assisted laser desorption/ionization time-of-flight mass spectra using hierarchical cluster analysis and principal component analysis

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*Stenotrophomonas maltophilia* is an opportunist multidrug-resistant pathogen that causes a wide range of nosocomial infections. Various cystic fibrosis (CF) centres have reported an increasing prevalence of *S. maltophilia* colonization/infection among patients with this disease. The purpose of this study was to assess specific fingerprints of *S. maltophilia* isolates from CF patients (n=71) by investigating fatty acid methyl esters (FAMEs) through gas chromatography (GC) and highly abundant proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and to compare them with isolates obtained from intensive care unit (ICU) patients (n=20) and the environment (n=11). Principal component analysis (PCA) of GC-FAME patterns did not reveal a clustering corresponding to distinct CF, ICU or environmental types. Based on the peak area index, it was observed that *S. maltophilia* isolates from CF patients produced significantly higher amounts of fatty acids in comparison with ICU patients and the environmental isolates. Hierarchical cluster analysis (HCA) based on the MALDI-TOF MS peak profiles of *S. maltophilia* revealed the presence of five large clusters, suggesting a high phenotypic diversity. Although HCA of MALDI-TOF mass spectra did not result in distinct clusters predominantly composed of CF isolates, PCA revealed the presence of a distinct cluster composed of *S. maltophilia* isolates from CF patients. Our data suggest that *S. maltophilia* colonizing CF patients tend to modify not only their fatty acid patterns but also their protein patterns as a response to adaptation in the unfavourable environment of the CF lung.

INTRODUCTION

Cystic fibrosis (CF) or mucoviscidosis is a fatal autosomal recessive disorder that mainly affects the Caucasian population (O’Sullivan & Freedman, 2009). The incidence of CF varies around the world, but estimates show that 1 in 2000–3000 newborns in the European Union is affected by this genetic disorder (WHO, 2014). In recent years, various CF centres worldwide have reported an increasing prevalence of *Stenotrophomonas maltophilia* in CF patients (CFFPR, 2012). *S. maltophilia* commonly colonizes the respiratory tract of CF patients, but its pathogenic importance in the progression of CF lung disease has not yet been completely elucidated. There is an extensive discussion about whether *S. maltophilia* is simply a colonizing organism or whether it actually causes infection and consequently reduces the lung function in CF patients (Waters *et al.* 2011, 2013). In addition, its multidrug resistance coupled with its ability to produce biofilms raises strong concerns in the medical community (Brooke, 2012).

Identification of *S. maltophilia* can be achieved by a wide range of well-established laboratory methods. Gas chromatographic (GC) analysis of whole-cell fatty acid methyl esters (FAMEs) has been widely used to identify a range of organisms (Peltroche-Llacahuanga *et al.*, 2000; Müller *et al.*, 1998; Moore *et al.*, 1994; Sasser, 1991). It is known that the content of fatty acids and the presence of free fatty acids in the bacterial cell may vary qualitatively and/or quantitatively according to genetic composition and other factors such as age, nutrients and oxygen availability.

**Abbreviations:** CF, cystic fibrosis; FAME, fatty acid methyl ester; HCA, hierarchical cluster analysis; ICU, intensive care unit; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAI, peak area index; PCA, principal component analysis.

One supplementary table and two figures are available with the online Supplementary Material.
(Casano et al., 1988). In some bacterial species, it has been demonstrated that certain FAME patterns may be associated with increased pathogenicity; for example, an alteration of the lipid A composition in Pseudomonas aeruginosa was linked with the pathogenesis of CF chronic lung disease (Ernst et al., 1999, 2003; Hajjar et al., 2002).

Currently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is considered a well-established method for the identification of bacteria and fungi in the routine clinical laboratory (Fernández-Olmos et al., 2012; Mellmann et al., 2008; Degand et al., 2008). This technique is based on the detection of molecular masses of highly abundant proteins and some other molecules in the regarded mass range, which produce species-specific mass-spectral fingerprints (Demirev et al., 1999). Characterization of S. maltophilia isolates by means of MALDI-TOF mass spectra, in order to unravel phylogenetic relationships and support genotypic data, is still scarce (Vasileuskaya-Schulz et al., 2011).

The aim of this study was to assess the specific fingerprints of S. maltophilia isolates from CF patients using MALDI-TOF MS and GC methods, and compare them with those derived from intensive care unit (ICU) and environmental isolates.

**METHODS**

**Bacterial strains.** As part of our standard care, bacterial cultures from CF patients admitted to the University Hospital Essen and Ruhrland Clinic (Germany) were collected and stored at −20 °C until use. A total of 102 S. maltophilia isolates (71 isolates from CF patients, 20 isolates from ICU patients and 11 isolates from the environment) were analysed (Table S1, available in the online Supplementary Material). Chronic colonization was defined as the persistent presence of S. maltophilia in two or more sputa from a patient within a period of 12 months (Waters et al., 2011; Gonçalves Vidigal et al., 2013). Isolates that grew on selective agar medium (Gonçalves-Vidigal et al., 2011) were identified by oxidase test and a MicroScan WalkAway system (Siemens). The selective agar was based on Sten medium agar (SMA) with slight modifications. The implementation and validation of this agar for detection of S. maltophilia has been published previously by our group (Gonçalves-Vidigal et al., 2011). The S. maltophilia type strain DSM 50170 from the Deutsche Sammlung von Mikroorganismen (DSM) was used as a quality control. *Stenotrophomonas rhizophila* (DSM 8573) was used as a closely related reference strain.

**FAME pattern analysis by GC.** Isolates were cultured on trypticase soy agar (Becton Dickinson) and incubated at 35 °C for 24 h. Experiments were performed as described previously (Miller & Berger, 1985; Müller et al., 1998). Approximately 100 mg of each culture material was used. A bacterial pellet of each isolate was saponified (15% NaOH in 50%, v/v, methanol), methylated (25% HCl in methanol, 1:1, v/v), extracted (n-hexane in methyl tert-butyl-ether, 1:1, v/v), and cleaned (1.2% NaOH in distilled water) following the instructions of the MIDI system (Microbial Identification System, Operating manual, version 5.0, 1995; MIDI). The FAMES were injected into a GC system series II gas chromatograph equipped with a split inlet and flame-ionization detector, automatic sampler 6890 and fused-silica capillary column (Ultra 2, HP 19091 B-102; 25 m × 0.2 mm with 5.0% cross-linked phenylmethyl silicone) as the stationary phase. Hydrogen was used as a carrier gas, with a sample volume of 2 μl, a split ratio of 1:100, an injector temperature of 250 °C (temperature detector 300) and column temperatures ranging from 170 to 260 °C at 5 °C min⁻¹ and from 260 to 310 °C at 40 °C min⁻¹, followed by an isothermal phase at 310 °C for 4 min. A temperature increase to 310 °C allowed column cleaning during a hold of 2 min. The instrument was coupled with a HP Vectra XU 5/90C computer loaded with 3365 series II Chemstation (version 3.34) software. The identification was performed using a Microbial Identification System (MIS) conformed to the recommended parameters proposed by the MIDI system. FAME peak identification and quantification, FAME profile comparison and data analysis were performed using the system software (part 1300) of the Sherlock software package of MIDI (version 1.06). Cluster analysis by unweighted pair matching (Euclidean distance) and principal component analysis (PCA) of the chromatographic results were carried out using the implemented library generation software (LGS, part 1300). The peak area index (PAI), which indicates the amount of fatty acids produced by the bacteria in the broth culture during growth, was defined as follows: total peak area/solvent peak area × 10,000, as described previously (Müller et al., 1998).

**MALDI-TOF MS fingerprinting.** To carry out the MS analysis, cultures of each isolate previously identified as S. maltophilia were subcultured on Columbia blood agar (Oxoid) and incubated at 35 °C for a period of 24 h. Two CF isolates and the S. rhizophila reference strain (DSM 8573) were not suitable for use in this assay due to sample contamination or reading error. Subsequently, material from a fresh colony of each isolate was placed on a spot on a disposable MALDI-TOF MS target slide (bioMérieux) and overlaid with 1 μl z-cyano-4-hydroxy cinnamic acid as an organic matrix solution (VITEK MS-CHCA; bioMérieux). The matrix was allowed to dry at room temperature. This procedure was performed in duplicate for each isolate.

Measurements were performed using a Shimadzu AXIMA Assurance mass spectrometer and the spectra were analysed with the Vitek MS Plus identification system for research use only (bioMérieux). Acquisition of spectra was carried out in positive-ion mode and within a mass range of 2000 to 20 000 Da. Results from a minimum of 500 shots per sample spot were collected.

To assess intra-laboratory reproducibility and the impact of subculturing, mass spectra of three different colonies of a CF isolate obtained from five repeated subcultures were compared from day 1 to day 5.

In addition, the same spots were analysed with the Vitek MS v.2.0 knowledge base for clinical use. The spectra of the tested samples were matched against the SuperSpectra included in the database. Peak patterns that produced a match with the identification data with a confidence level exceeding 80.0% were considered significant and were displayed. Calibration of the mass spectrometer for every acquisition group was performed using *Escherichia coli* ATCC 8739 from the American Type Culture Collection. For the cluster analysis, the relative peak intensities contained in every peak list derived from the MALDI-TOF mass spectrum were put into a matrix whose columns represent the *m/z* values, ranging from 2000 to 20 000 Da, with a resolution of 5 Da. The rows represent the individual isolates. Based on this parameter matrix, an agglomerative hierarchical cluster analysis (HCA) was performed using Euclidean distance and Ward’s method after standardization in order to calculate the degree of similarity among the isolates in respect of all *m/z* values, using the software DataLab (Epina). In the first step, the two most similar isolates were identified and combined with the first cluster. After the iterative repetition of these calculations, every isolate was a member of a cluster of similar isolates more or less separated from neighbouring clusters. A common visualization of this analysis is a
dichotomous dendrogram. As a second approach, PCA based on the same matrix was performed to identify the first three principal components and also to uncover differences of the isolates in respect of these components. The principal components were the result of an optimization process to find a new coordinate system, along whose axes the variance of the data is maximal. The new coordinates are called the principal components and are linear combinations of the original m/z coordinates. The loading factor of an m/z value tells us how much it contributes to a principal component and therefore how important the presence or absence of a peak with this m/z value is to discriminate between different isolates. In the three-dimensional plot, each isolate is represented as a dot in the PC 123 space. In contrast to HCA, which does not give any hint of the underlying causes of (dis)similarity, PCA helps to identify potentially important mass-spectral components responsible for an observed clustering and therefore possibly suitable candidates for following investigations. Clustering in respect of PCA is not necessarily the same as in HCA because of different mathematical approaches. If, in the extreme, only very few original parameters contribute much to the variance, they will dominate in a PCA over the majority of less important parameters because of their high loading factors. Individuals with differences in these parameters will easily be distinguishable in a plot of the first principal components. In an HCA of the same data, the overwhelming similarity of most of the original parameters, which all contribute with equal weight, would result in a dendrogram with very short last branches and maybe only one large cluster.

**Statistical analysis.** The unpaired non-parametric Mann–Whitney U test (significance set at \( P<0.05 \)) was used to address the fatty acid production differences among CF, ICU and environmental isolates (Prism 5.0; GraphPad Software). DataLab software was used to perform PCA and HCA (version 3.5).

**RESULTS**

Bacterial fatty acid composition is known to be affected by different environmental and physiological factors (Casano et al., 1988). The FAME patterns of *S. maltophilia* isolates from CF and ICU patients, and also from the environment, were therefore used for a PCA (Fig. 1). The principal components had to be extracted from the two-dimensional plots produced by the software Sherlock. The position of 17 CF samples and three ICU samples in the different two-dimensional plots (PC1 vs PC2, and PC1 vs PC3) were superimposed on each other and consequently have been excluded from Fig. 1. According to the PCA, CF isolates tended to cluster together, whereas ICU and environmental strains clustered more separately (Fig. 1).

Furthermore, isolates from the CF chronic colonized group did not show the presence of some fatty acid structural isomers (11:0 anteiso, 12:0 ISO 3OH and 17:0 anteiso), which were present in the ‘CF non-chronic colonized’ group.

PAI provides information about the number of fatty acids present in the broth culture during bacterial growth. When investigating the PAI, isolates of *S. maltophilia* from CF patients, ICU patients and the environment showed mean PAI values of 12.7 (range 4.0–98.8), 9.2 (range 7.2–12.6) and 7.4 (range 5.4–11.2), respectively. Remarkably, specimens from CF patients produced significantly higher amounts of fatty acids in comparison with samples from ICU patients and the environment. It was also noted that there was a significant difference in the amount of fatty acids produced between the ICU patient samples and the environment samples (Fig. 2). *S. maltophilia* isolates from chronic colonized CF patients showed no differences in
relation to the PAI values compared with the ‘CF non-chronic colonized’ group (data not shown).

In order to investigate the variability of fragment patterns, mean mass spectra of strains from different origins were analysed. A visual comparison of mean mass spectra of CF isolates in comparison with those of ICU or environmental samples did not show any overall prominent difference or any difference with respect to single peaks (Fig. S1). The mass spectra of all three colonies from each time point obtained from five repeated subcultures exhibited identical profiles, suggesting reproducibility and subculture stability.

PCA, based on MALDI-TOF mass spectra, was carried out in an attempt to differentiate *S. maltophilia* isolates from different origins (CF and ICU patients and the environment). The score plot of the first three principal components on a set of mass spectra obtained from 100 *S. maltophilia* isolates is shown in Fig. 3. In this plot, the ICU and environmental samples grouped in a mixed manner, but *S. maltophilia* isolates obtained from CF patients formed a cluster with only marginal overlap with the ICU and environmental isolates.

In contrast, the dendrogram generated by HCA, based on the peak lists derived from MALDI-TOF MS, revealed the presence of five distinguished clusters (designated A–E) (Fig. S2). Clusters B (n=21) and D (n=16) were composed only of isolates obtained from CF patients. Cluster analysis using MALDI-TOF MS peak lists demonstrated that *S. maltophilia* isolates also exhibited a high phenotypic diversity.

**DISCUSSION**

The data from the present work suggest that *S. maltophilia* CF isolates (in comparison with non-CF isolates) produced a higher amount of fatty acids. PCA of peak lists derived from MALDI-TOF MS revealed a clustering of CF isolates among all included isolates. To our knowledge, this is the first study to delineate *S. maltophilia* FAME profiles by
LPS is an important pathogenic factor of Gram-negative bacteria (Raetz & Whitfield, 2002). Previous studies have shown that P. aeruginosa is able to synthesize a range of specific lipid A structures that could potentially promote bacterial survival or colonization in the CF airway, as they can exhibit different inflammation-stimulating properties, or can modify its lipid A structure to confer resistance against elements of host innate immunity (Ernst et al., 1999, 2003; Hajjar et al., 2002). An early report addressing the temperature-dependent variation of S. maltophilia clinical isolates (n=38) revealed that their susceptibility to aminoglycoside was significantly linked to changes in the LPS structures (Rahmati-Bahram et al., 1996). In the present study, S. maltophilia isolates from CF individuals produced a significantly higher amount of fatty acids in comparison with isolates obtained from ICU patients and the environment. Additionally, the occurrence of distinct groups of S. maltophilia categorized as ‘CF chronic colonist’ and ‘CF non-chronic colonist’ groups by GC suggests an adaptation mechanism of this microorganism to the CF airway. Fatty acid profiles depend on temperature and pH value. It was shown that membrane fatty acid adaptation is a common mechanism utilized by bacteria to withstand environmental stress (Fozo et al., 2004). We could demonstrate that S. maltophilia isolates from chronic colonized CF patients showed specific adaptation mechanisms to the CF lung environment (Vidigal et al., 2014). Thus, it is reasonable that the amounts of fatty acids of S. maltophilia isolates from CF patients differ compared with isolates from other sources. However, further investigations should be carried out to elucidate whether antibiotic treatment against Gram-negative bacteria during chronic infection will influence fatty acid production. It is possible that S. maltophilia isolates from CF patients have a tendency to produce more fatty acids as a defence mechanism against long-term and repeated treatment with colistin (the mechanism of action relies on its ability to displace bacterial counterions in LPS). Colistin is polycationic and has both hydrophilic and lipophilic moieties. These polycationic regions interact with the bacterial outer membrane by displacing bacterial counterions in the LPS. Hydrophobic/hydrophilic regions interact with the cytoplasmic membrane as a detergent, solubilizing the membrane in an aqueous environment. As a response to colistin treatment, S. maltophilia from the CF lung environment may produce more fatty acids.

PCA is an analytical tool that is used to identify patterns in data and to express the results in a manner to highlight their similarities and differences (Smith, 2002). Although the mean mass spectra did not show significant differences, data derived from PCA demonstrated that S. maltophilia CF isolates exhibited a MALDI-TOF spectra pattern different from the other isolates included in the analysis (ICU and environmental), which allowed them to be clustered close to the first principal component coordinate axis. In this particular case, PCA improved the cluster quality because of its power to separate individuals into different groups based on non-obvious dissimilarities, even in cases of a high degree of overall similarity. In contrast, the calculation of the similarity used to create an HCA is dominated by a high overall similarity and is prone to cover small differences. The cluster behaviour visible in the PCA might suggest a possible adaptation of this microorganism to the CF lung. Conversely, clustering within isolates from ‘CF chronic colonized’ and ‘CF non-chronic colonized’ patients was not observed.

Recently, a German research group showed that mass spectra of nine environmental S. maltophilia isolates exhibited a particular fragment of approximately 6080 Da (Vasileuskaya-Schulz et al., 2011). In the current study, this peak was not found in either the environmental isolates or in the clinical (CF and ICU) isolates.

To the best of our knowledge, this is the first study to characterize S. maltophilia isolates from various sources (CF and ICU patients and the environment) based on FAME fingerprinting and MALDI-TOF MS analysis. Future research should focus on clarifying whether long-term treatment of CF patients will influence the FAME profile of S. maltophilia isolates and on further investigating particular mass spectra, which could explain or be associated with the adaptation of S. maltophilia to the CF lung.

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