

ORIGINAL ARTICLE

Advantage of MALDI-TOF-MS over biochemical-based phenotyping for microbial identification illustrated on industrial applications

S.K. Urwyler and J. Glaubitz

R&D-Microbiology, Omya International AG, Oftringen, Switzerland

Significance and Impact of the Study: MALDI-TOF-MS has revolutionized speed and precision of microbial identification for clinical isolates outperforming conventional methods. In contrast, few performance studies have been published so far focusing on suitability for particularly industrial applications, geomicrobiology and environmental analytics. This study evaluates the performance of this proteomic phenotyping on such industrial isolates in comparison with biochemical-based phenotyping and genotyping. Further the study exemplifies the power of MALDI-TOF-MS to trace cost-efficiently the dominating cultivable bacterial species throughout an industrial paint production process. Vital information can be retrieved to identify the most crucial contaminating source for the final product.

Keywords

BIOTYPER, MALDI-TOF-MS, paint, species identification, VITEK.

CorrespondenceSimon Urwyler, Omya International AG,
Baslerstrasse 42, CH-4665 Oftringen,
Switzerland.
E-mail: simon.urwyler@omya.com2015/1878: received 10 June 2015, revised
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Abstract

Fast microbial identification is becoming increasingly necessary in industry to improve microbial control and reduce biocide consumption. We compared the performances of two systems based on MALDI-TOF MS (VITEK MS and BIOTYPER) and two based on biochemical testing (BIOLOG, VITEK 2 Compact) with genetic methods for the identification of environmental bacteria. At genus level both MALDI-TOF MS-based systems showed the lowest number of false (4%) and approx. 60% correct identifications. In contrast, the biochemical-based systems assigned 25% of the genera incorrectly. The differences were even more apparent at the species level. The BIOTYPER was most conservative, where assigning a species led to the lowest percentage of species identifications (54%) but also to the least wrong assignments (4%). The other three systems showed higher levels of false assignments: 8.7, 40 and 46% respectively. The genus identification performance on four industrial products of the BIOTYPER could be increased up to 94.3% (average 88% of 167 isolates) by evolving the database in a product specific manner. Comparison of the bacterial population in the example of paints, and raw materials used therein, at different production steps demonstrated unequivocally that the contamination of the final paint product originated not from the main raw material.

Introduction

The identification of pathogens is a crucial step of diagnosis to decide on appropriate medical treatment, and time-to-result is important to start the chosen treatment as early as possible. Therefore, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) could establish itself well in the

clinical environment (Seng *et al.* 2009). The interests for such rapid and reliable microbial identification are growing throughout general industry, allowing targeted microbial control, and this is well exemplified by the paint industry. Aqueous-based paints are spoiled easily by micro-organisms, resulting, for example, in impaired rheological parameters, unpleasant odours or discoloration (Dey *et al.* 2004), but the use of biocides is being

curtailed by Governmental regulations (EU 528/2012) and development of microbial resistance limiting the number of permitted biocides (Graves *et al.* 2015). Furthermore, industrial biocides have different specificities against microbes (Karsa and Ashworth 2002), and knowledge of the bacteria present permits the choice of the most effective treatment. Total viable counts provide a good indication for the bacterial burden that challenges the preservative in the paint product. However, a more detailed characterization of the micro-organisms present in raw materials or introduced during production steps helps to trace the origin of the contaminants in spoiled products. This allows cost efficient and effective measures at the source, such as disinfectants, heat treatment and controlled variation of pH.

For identification of bacterial species and genera, the sequencing based on 16S rRNA is most widely used and accepted to classify them phylogenetically (Janda and Abbott 2007). Comprehensive databases are publicly available (e.g. SILVA, RDP and Greengenes). While a similarity between two sequences of <97% calls for a classification into different species, a close similarity (e.g. 99%) does not call of necessity for the same species (Janda and Abbott 2007). *Pseudomonas* spp. are particularly difficult to identify by 16S rRNA, which requires sequence comparison of less conserved genes within the interrogated genus, such as the RNA polymerase sigma factor *rpoD* gene (Mulet *et al.* 2011).

Since sequencing remains laborious and time consuming, faster identification systems have been developed designed for medical purposes. All identification methods rely on the quality of the underlying database, and perform very well on bacteria studied from a medical application background (Xiao *et al.* 2014).

The objective of the study was to compare unprejudiced the performance of the four commercially available identification systems (BIOTYPER, VITEK MS, VITEK 2 Compact and GEN III MICROPLATE) with sequence identification exemplified on 24 environmental bacterial isolates. Further, we analysed the performance of the superior MALDI-TOF-MS technique for bacterial classification in mineral dispersions and paints, and exemplified its power to evolve the existing database avoiding laborious techniques to trace population dynamics in industrial processes.

Results and discussion

Instrumental principles

All four tested systems require cultivable bacteria and a reference database for identification.

Two systems, BIOTYPER (Bruker Daltonik GmbH, Bremen, Germany) and VITEK MS (bioMérieux SA, Marcy-l'Étoile, France), use MALDI-TOF-MS to generate a mass spectrum of the bacterial proteins and compare the spectrum (Mass/charge and peak intensity) with underlying databases. The specimen preparation of the two MALDI-TOF-MS-based identification systems are very similar and needed little preparation time. The BIOTYPER reports the best two hits upon comparison with the database and rates their identification probability via a matching scoring system (0.000–3.000) and a consistency rating (A, B, C). The VITEK MS system uses a probability score as percentage or indicate no identification. This method and database have been used successfully in clinics with identification rates of 95.4% at genus and 84.1% at species level (Seng *et al.* 2009).

The other two identification tools are based on biochemical phenotyping of bacterial strains. The VITEK 2 Compact system uses 64 substrates for biochemical phenotyping, such as alkalization, enzyme hydrolysis and growth in the presence of inhibitory substances. Depending on the class of bacteria (e.g. spore-forming Gram-positive bacilli, Gram-negatives) a different set of substrates is tested (Pincus 2006). The VITEK 2 needs special materials (cartridges) depending which kind of bacteria are to be identified (e.g. BCL: spore-forming Gram-positive bacilli, GN: Gram-negative, GP: Gram-positive, CBC: Corynebacteria, NH: Neisseria and Haemophilus spp), thus requiring a preclassification, Gram staining etc. Already among the 24 isolates that were analysed, five different cartridges were required (GN: 13, GP: 2, BCL: 4, CBC: 2, NH: 1). Four of the environmental isolates were not classified, due to consumables supply issues, such as the unavailability of NH or CBC cartridges from the vendor within the timescale of the study.

Similarly, the BIOLOG GEN III MICROPLATE identification system (Biolog Inc., Hayward, CA) analyses bacteria in 94 biochemical tests using different carbon sources and inhibitors. This metabolic fingerprint is then compared with a database consisting of characteristic phenotypes (Bochner 2009). Beside the taxonomy information, the results further provide information regarding the metabolism. The BIOLOG system did not require a preclassification of the isolates and required only one identification plate (Gen III). However, for a few bacteria, the system demanded a retesting using different growth media (Table S2), increasing labour and material costs and delaying the eventual identification. Compared with the MALDI-TOF-MS systems, both biochemical-based methods lead to higher material costs and a technically more demanding preparation.

Identification of the reference environmental isolates by sequencing

For the evaluation of the microbial identification methods, 24 environmental isolates (Table S1) were isolated from different sources, namely, industrial bulk 50% (w/w) urea solution, fish faeces and biocide-preserved and unpreserved calcium carbonate slurries.

For the initial microbial identification, the 16S rRNA and, in addition for the *Pseudomonas* species, the *rpoD* genes were sequenced (Table S1). The 24 environmental isolates consisted of 10 Gram-positive and 14 Gram-negative bacteria from 17 different families and five different bacterial classes, including Bacilli, Actinobacteria, alpha-, beta- and gamma-Proteobacteria. All genera commonly described in paints (*Bacillus*, *Lactobacillus*, *Pseudomonas*, *Enterobacter*, *Escherichia*, *Micrococcus*, *Aeromonas*, *Proteus* and *Serratia*) belong to these classes (Obidi *et al.* 2009). The selection is evidently not representative for all industrial applications, but stands exemplary for a diverse set of frequently occurring bacterial species, isolated distantly from a medical background.

The 16S rRNA sequencing was not able to identify unambiguously all bacteria down to the species level. For 12 isolates (50%), 16S rRNA sequencing allowed identification down to a single (6) or at least two (6) possible species. It has been demonstrated previously that 16S rRNA in general is able to reveal the species only in 65–83% of the cases (Janda and Abbott 2007). The high numbers of *Pseudomonas* spp., which are unable to be classified accurately by 16S rRNA (Mulet *et al.* 2011), account for the lower rate. Thus, the *rpoD* gene was sequenced for *Pseudomonas* spp. (Mulet *et al.* 2011). Nevertheless, in a third of the cases, the species level remained unknown and even one genus could not be identified unambiguously (Fig. 1).

The same bacterial isolates were identified by the four alternative identification systems (Table 1, Table S2).

Identification by MALDI-TOF-MS

Both MALDI-TOF-MS-based methods performed equally well at the Genus level with one false identification (4%) and approx. 60% correct identifications (Fig. 1). In one case, the BIOTYPER was able to identify the environmental isolate, *Sphingomonas koreensis*, while the VITEK MS was unable to decipher the genus although both databases contained *S. koreensis* and multiple further *Sphingomonas* spp. For all other isolates, both systems performed equally.

The bacterium *Morococcus cerebrosus* was classified wrongly as *Neisseria* sp. and *Neisseria mucosa/sicca* by the BIOTYPER and the VITEK MS respectively. Interestingly,

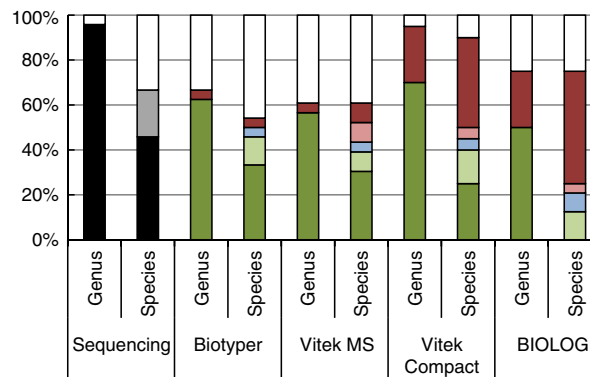


Figure 1 Relative identification performance at genus and species level among different species identification tools of Table 1. Identification rating: (Black) Identified based on 16S rRNA, (grey) identified based on *rpoD*, (dark green) correct assignment, (light green) presumably correct assignment, (blue) undecidable, (light red) presumably incorrect assignment, (dark red) incorrect assignment, (empty) No identification.

the species *M. cerebrosus* was reclassified as a new genus in the family *Neisseriaceae*, and the species was shown to be closely related to *N. mucosa* (Long *et al.* 1981). It is encouraging that the identified genus is at least very closely related, but nonetheless exemplifies the difficulties in microbial identification using methods that rely on a constricted database. In this case, *Morococcus* spp. were not present in any of the databases.

The main differences between the Biotyper and the VITEK MS became apparent at the species level. The VITEK MS claimed for 61% of the bacteria (12) an identified species with very high probabilities, for 8.7% (2) the species were identified with low probabilities (Table S2), and for 39% (9) the bacteria remained unidentified. In contrast, the BIOTYPER claimed consistent species identification only for 21% (5), while for 33% (8) the species were identified with a low consistency, that is, the spectra of other species of the same genus matched, though at lower scores, and in 12.5% (3) the scoring justified identification only at the genus level. No identification was possible for 33% (8).

To determine the plausibility of a true species identification, the results were compared with the sequencing results (Table S1), and classified as being a correct or incorrect identification. For the cases where sequencing was unable to identify the species unambiguously, all results were compared, and the plausibility was rated as presumably correct, presumably incorrect and undecidable (Table 1, Fig. 1). Of the 13 claimed species, including those with low consistency, the BIOTYPER identified all except *M. cerebrosus* correctly. On the other hand, the VITEK MS system classified 5 of 14 species incorrect or presumably incorrect.

Table 1 Comparison of alternative species identification methods with classical DNA sequencing methods

ID	Sequence identification	BIOTYPER		VITEK MS		VITEK Compact		BIOLOG	
1	<i>Aeromonas</i> sp.	+	(+)	+	(-)	+	(-)	+	(+)
2	<i>Aeromonas</i> sp. (<i>Aer. salmonicida/bestiarum</i>)	+	(?)	+	(?)	+	0	+	(-)
3	<i>Chelatococcus</i> sp. (<i>C. daeguensis/sambhunathii</i>)	0	0	0	0	-	-	0	0
4	<i>Chryseomicrobium amylolyticum</i>	0	0	0	0	-	-	-	-
5	<i>Citrobacter</i> sp.	+	(+)	+	(+)	+	(+)	+	(+)
6	<i>Delftia</i> sp.	+	(+)	+	(+)	+	(+)	+	(-)
7	<i>Enterobacter</i> sp./ <i>Citrobacter murlinae</i> / <i>Pantoea agglomerans</i> / <i>Leclercia adecarboxylata</i>	+	(?)	+	(?)	+	(?)	+	(?)
8	<i>Gordonia terrae</i>	0	0	0	0	nd		-	-
9	<i>Halobacillus</i> sp.	0	0	0	0	-	-	0	0
10	<i>Lysinibacillus</i> sp. (<i>L. fusiformis/sphaericus</i>)	+	(+)	+	(+)	+	(+)	-	-
11	<i>Methylobacterium</i> (<i>M. populi/thiocyanatum</i>)	+	0	nd		nd		0	0
12	<i>Microbacterium</i> sp.	0	0	0	0	nd		+	(?)
13	<i>Morococcus cerebrosus</i>	-	-	-	-	nd		0	0
14	<i>Ochrobactrum intermedium</i>	+	+	+	+	+	-	+	-
15	<i>Paenibacillus</i> sp. (<i>P. camelliae/granivorans</i>)	0	0	0	0	0	0	-	-
16	<i>Pseudomonas mendocina</i>	+	0	+	+	+	+	-	-
17	<i>Pseudomonas</i> sp. (<i>Ps. pseudoalcaligenes/oleovorans</i>)	+	(+)	+	(+)	+	(-)	+	-
18	<i>Pseudomonas</i> sp. (<i>Ps. pseudoalcaligenes/oleovorans</i>)	+	(+)	+	(-)	+	(+)	+	-
19	<i>Pseudomonas mosselii</i>	+	+	+	-	+	-	+	-
20	<i>Pseudomonas</i> sp.	+	0	+	(-)	+	(+)	+	(+)
21	<i>Sphingomonas koreensis</i>	+	0	0	0	+	-	0	0
22	<i>Sporosarcina</i> sp.	0	0	0	0	-	-	-	-
23	<i>Sporosarcina</i> sp. (<i>S. luteola/koreensis</i>)	0	0	0	0	-	-	0	0
24	<i>Staphylococcus aureus</i>	+	+	+	+	+	+	+	-
	Summed identifications*	14	10	13	3	9	-3	6	-9

+: correct assignment of Genera (left column) or Species (right column), -: incorrect assignment compared to sequence identification (Table S1). 0: species or genera were not identified. nd, not determined. *Brackets*: In cases where the sequencing result was ambiguously (e.g. multiple species or genera), the identification was compared with all results available and classified as presumably correct (+), incorrect (-) or undecided (?) considering the species identified by most identification methods as correct.

*Sum of genus (left column) and species (right column) identifications: +1 for '+' and '(+)', -1 for '-' and '(-)' and 0 for all others. Isolates 8, 11, 12, 13 were not considered as not determined by all systems.

A result requiring little interpretation, such as disclosed by the VITEK MS, is of advantage for routine classification and fast decisions, but bears inherently an increased risk for conflicting analyses compared with other classification methods, and higher risks for false identification, as is shown here. We, therefore, considered a more detailed interpretation of the results for environmental isolates, as used in this study, with a lower risk for incorrect assigned species being of distinct advantage.

Identification by biochemical phenotyping and comparison of performance

The VITEK 2 showed the highest classification rate at genus level among all four systems, however, 25% (5) were wrongly assigned (Table 1, Fig. 1). The biochemical phenotyping system by BIOLOG performed comparably regarding wrong genus classification, but was further

unable to claim genus identification in an additional 25% of the cases (6). Both appeared to have difficulties with the same bacterial isolates. A similar picture was apparent at the species level. Both systems assigned a species to the majority of the environmental isolates, but a high degree of the assigned species (50 and 54%) differed from the sequencing results and from the results of the other 3 classification systems (Fig. 1).

The biochemical phenotyping systems show advantages over the MALDI-TOF-MS systems, in that they may provide additional information such as antibiotic resistance, nutrient and growth requirements. For the mere identification of the environmental isolates used in this study, neither the VITEK 2 nor the BIOLOG appear to be the optimal choice for routine classification. Sequencing will of course remain the gold standard for phylogenetic classification, but both MALDI-TOF-MS systems came very close for those isolates that showed a matching database entry.

Furthermore, the MALDI-TOF-MS systems are much faster (approx. 15 min from colony to reported classification) compared to the biochemical phenotyping systems (many hours to several days). The low costs of the MALDI-TOF-MS analysis in respect to preparation time (as low as 15 min total bench time for 96 samples) and consumables (less than \$1 per sample) allows a high throughput and, with it, a more thorough analysis of the cultivable bacterial spectrum occurring in industrial samples. In contrast, the reagents costs per sample of the VITEK 2 and BIOLOG system were much higher (>\$10 per analysis), and for our purposes were not compensated by the additional information generated from the biochemical phenotyping. It has to be noted that the equipment costs for the MALDI-TOF-MS systems are relatively high. The MALDI-TOF-MS systems, however, had the further advantage that the mass spectra of unidentified species could be added to the database after identification by sequencing for future reference. To avoid redundant sequencing of colonies from identical species, multiple colonies can be screened by the MS and recognized as to be the same.

It has to be kept in mind that the set of bacterial isolates used for comparison is limited and thus not representative for all applications. Bacterial populations from other industrial applications, such as the food industry, might produce a different picture.

BIOTYPER: from evaluation to practice

Due to the lower error rate at species level we chose the BIOTYPER to characterize the identification performance of isolates from four industrial products where biocide preservation failed. Three products were calcium carbonate slurries with different particle sizes (weight median size, d_{50} : 0.9, 1.5 and 3.8 μm) and the fourth product was a paint sample (Fig. 2). In total 167 bacterial colonies, grown on TSA were analysed. In 63.5% of the cases (106) the system reported reliable genus identification based on the *In Vitro* Diagnostics (IVD) database. For 24.6% of the colonies (41), identification was based on new database entries generated from spectra of initially unidentified species. In 12%, sequencing remained necessary to identify all bacteria, which, however, will extend the database for future uses.

The colonies from the calcium carbonate slurry were identified by the BIOTYPER at a higher rate than from paint. This is particularly obvious at the percentage that remained unidentified (Fig. 2). One reason is that the calcium carbonate slurries tend to have a less diverse bacterial population than paint samples (Table 2). In addition, due to the routine use of the BIOTYPER with mineral slurries, the new database entries are biased to represent such bacteria.

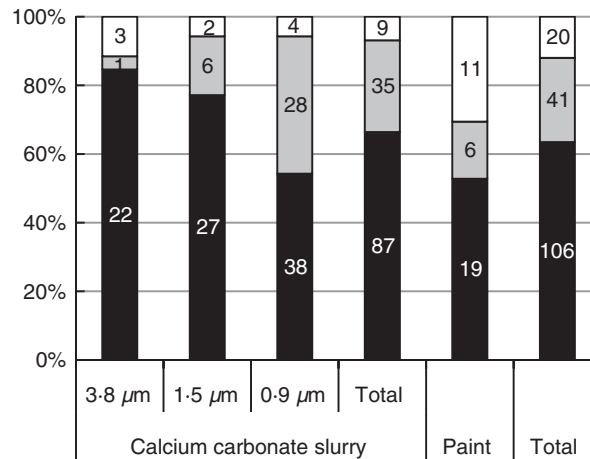


Figure 2 Relative identification performance at genus level of MALDI-TOF-MS BIOTYPER on bacteria from three calcium carbonate slurries with different weight median calcium carbonate particle sizes and paint. (black) Identified by Bruker *In Vitro* Diagnostics database, (grey) identified by main spectra (MSP) added to the extended database, (empty) not identified by BIOTYPER. Numbers refer to absolute values.

The findings above support the use of MALDI-TOF-MS as an identification system for industrial application, as the database evolves quickly in a product and in an application-specific manner. For mineral slurries, the adapted database already identified 93% of the bacterial isolates at the genus level, which is very close to published identification rates of 94.3 and 94.7% from the clinical origin and prior intended use of the IVD database (Harris *et al.* 2012; Lee *et al.* 2015).

Tracing microbial populations

Identification of bacterial colonies is helpful to evaluate potential contamination sources. The low material and labour cost of MALDI-TOF-MS allows a more thorough identification of the cultivated bacterial colonies. For a spoiled paint product, we followed 15 samples from one of the initial raw materials, suspected to be a source of contamination, to the final paint, and classified 83 bacterial colonies with the BIOTYPER (Table 2). This consisted of samples from the storage tank (ST), retain samples (RS), receiving controls (RC) and product rejections (PR) from the raw material supplier and the paint manufacturer. Since the raw material was freshly produced and unpreserved, bacteria could be isolated several days after sampling. Among the cultivated bacteria, only four different genera and seven different species could be identified in the raw material. In contrast, the paint samples were colonized with a more diverse bacterial spectrum, consisting of 12 different genera. Interestingly, none of the isolates identified in the paint samples matched those from the investigated raw

Table 2 MALDI-TOF-MS tracking of the microbial population in paint and one slurry raw material during paint production; from the raw material producer to the final product

Product Sample point	Raw material							Paint						
	T	R	R	R	C	C	T	T	T	T	T	T	R	P
<i>Bacillus</i> sp.	X	X	X	X	X	X	X							
<i>Bacillus cohnii</i>						X		X						
<i>Bacillus halodurans</i> or <i>B. okuhidensis</i>	X			X	X		X							
<i>Chryseomicrobium amylolyticum</i>	X	X	X		X		X	X						
<i>Methylobacterium</i> sp.	X	X	X	X	X									
<i>Methylobacterium populi</i>	X													
<i>Brevundimonas</i> sp.		X	X	X										
<i>Brevundimonas diminuta</i>														X
<i>Alcaligenes</i> sp.														X
<i>Alcaligenes</i> sp. or <i>Wautersiella</i> sp.														X
<i>Arcanobacterium</i> sp. or <i>Trueperella</i> sp. or <i>Georgenia</i> sp.								X	X					
<i>Corynebacterium</i> sp.								X						
<i>Corynebacterium glutamicum</i>										X			X	X
<i>Corynebacterium lubricantis</i>								X	X		X			
<i>Erysipelothrix</i> sp.														X
<i>Leucobacter</i> sp.											X			
<i>Leucobacter aridicollis</i>								X	X					
<i>Lysinibacillus</i> sp.											X			
<i>Lysinibacillus sphaericus</i>								X					X	
<i>Proteiniphilum</i> sp.											X			
<i>Proteiniphilum acetatigenes</i>													X	
<i>Pseudomonas</i> sp.											X			
<i>Sporosarcina koreensis</i> , <i>S. luteola</i> or <i>S. soli</i>											X			
<i>Staphylococcus</i> sp.												X		
<i>Vagococcus fluvialis</i>								X	X		X			X

Sample points: T, storage tank; R, retain sample; C, receiving control; P, product rejection. Raw Material samples were taken at the production site (From left, T, R, R, R) and at the Paint manufacturer (C, C, T, T). All Paint samples were provided by the Paint manufacturer.

material, and only one genera matched between them. For this one genera the comparison of the main spectra (MSP) of the species *Brevundimonas diminuta* identified in the paint with the identified isolate in the raw material clearly showed that they represent different bacterial species.

The complete divergence of species in the final paint and the investigated raw material (Table 2) indicate strongly that other factors during the production process or other additives to the final paint are responsible for the contamination in the final paint product. A whole process analysis of all the raw materials and intermediate production steps would allow the source of contamination to be traced, and to direct focused treatment of the materials. This would reduce the bacterial burden of the final product and reduce the need for preservatives.

Materials and methods

Isolation of bacteria

Bacteria from calcium carbonate slurries were isolated by a 10-fold dilution in Phosphate-buffered saline (PBS) and

plating 0.1 ml on TSA plates (Cat. No. 43011, bioMerieux). Bacteria from bulk-quality 50% w/w urea solution were concentrated by centrifugation of 50 ml at 4000 g for 10 min. The pellet was directly transferred on TSA plate and diluted by streaking. For the isolation of bacteria from fish faeces, excrements were pipetted from the aquarium floor and frozen at -80°C . Bacteria were cloned from the frozen fish faeces by plating dilutions in PBS onto TSA plates. TSA plates were incubated at 30°C for 2–5 days until colonies became visible. Colonies were transferred to fresh TSA plates and subcultured again by streaking method and cultivation on TSA plates. Bacteria were stored long term at -80°C in 50% glycerol or on Microbank™ beads (Pro-Lab Diagnostics, Toronto, Canada) or short term at 4°C on TSA plates.

Identification of bacteria by sequencing

For genomic DNA single colonies were cultivated in 3 ml TSB (Cat. No. 22092, Sigma-Aldrich, St. Louis, MO) over night at 30°C and 200 rev min⁻¹ agitation. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufac-

tures instructions. The primer pairs for 16S rRNA gene amplification (8F: AGAGTTTGATCMTGGCTCAG, 1492R: GGTTACCTTGTTACGACTT), and the primers for full-length sequencing (907R: CCGTCAATTCCTT TRAGTTT, 785F: GGATTAGATACCCTGGTA), were described previously (Turner *et al.* 1999). The PCR was carried out in a 25 μ l final volume using KAPA2G Fast HotStart ReadyMix (KK5601; Axonlab, Baden, Switzerland) according to manufacturer's instruction and the thermocycling conditions 1 \times (95°C 120 s), 40 \times (95°C 15 s, 50°C 15 s, 72°C 8 s). For *Pseudomonas* spp. the *rpoD* gene was additionally amplified and sequenced using the primer pair 30F: ATYGAAATCGCCAARCG and 790R: CGGTTGATKTCCTTGA (Mulet *et al.* 2011) and the same PCR conditions as above, except an annealing temperature of 52°C. PCRs were sequenced at Microsynth (Balgach, Switzerland) by Sanger sequencing and manually assembled. Accession numbers of the nucleotide sequences deposited in GenBank (Benson *et al.* 2013) are listed in Table S1.

Sequences were compared with the 16S ribosomal RNA sequences (Bacteria and Archaea) database or the nucleotide collection (for *rpoD*) using MEGABLAST optimized for highly similar sequences (Altschul *et al.* 1997; Blast.ncbi.nlm.nih.gov, 2014). Matches with query coverages of at least 98% and the highest percentage of identity were reported (Table S1). If more than three species showed the same coverage and identity, only the genera were assigned.

Identification of bacteria by MALDI-TOF-MS, BIOLOG GEN III MICROPLATE and VITEK 2 Compact

Fresh bacterial colonies were prepared by dilution streaking of bacteria from frozen stocks or refrigerated cultures onto TSA plates and incubation at 30°C until colonies became visible (24–48 h).

For sample preparation for MALDI-TOF-MS analysis, the bacterial colonies were processed according to the manufacturer's protocols. Briefly, the colonies were resuspended in 0.3 ml deionized water and supplemented with 0.9 ml ethanol. This preparation was either immediately processed for analysis with the Biotyper and database entry or stored up to 1 week prior to processing and analysis by VITEK MS. The bacteria were then centrifuged >10 000 g for 2 min and the bacterial pellet was dried for a few minutes. Formic acid (70% w/w) was added depending on the size of the pellet (small, just visible pellets: 5 μ l, pellets with diameter of 1–2 mm: 10 μ l) and resuspended by pipetting following an incubation of exactly 2 min at room temperature. An equal volume of pure acetonitrile was added and the samples were centrifuged (13 000 g, 2 min). Of the supernatant 1 μ l was

spotted on the target plate, air-dried (5–15 min), topped with 1 μ l matrix (2-Cyano-3-(4-hydroxyphenyl) acrylic acid) in solvent (Cat. No. 19182, Sigma-Aldrich) and air-dried again. The mass spectra of the dried spots were recorded by MALDI-TOF-MS within 2 h and analysed by the Vitek MS or Biotyper. New species were entered into the extended database using the average spectra of at least two completely independent generated samples and at least two recorded spectra of each independent sample.

Genus and species were reported as provided by the identification tools (Table S2).

For the BIOTYPER, the consistency level (A, B or C) and the identification scores were reported. Species was assigned when the identification score was >1.999. Otherwise only genus (1.999–1.700) or unidentified (<1.7) were reported. For the VITEK MS Genus, species, % probability were reported.

For the VITEK-2, data acquisition and interpretation was performed at an external facility using bacteria from a freshly grown TSA plates, following the manufacturers recommendation. Genus, species, % probability and the reagents used were reported.

Samples were analysed by the BIOLOG according to the manufacturer's instructions. Briefly, bacteria were plated on TSA and incubated at 33°C overnight. From fresh colonies, the Media A provided with the GEN III MicroPlate was inoculated at the desired turbidity and the suspension was used to fill the GEN III MicroPlate with 0.1 ml. The plate was covered and incubated at 33°C for 22 h until the plate was evaluated by the BIOLOG MicroStation. If no identification was achieved, the recommendation by the Software were followed. Either continuous incubation at 33°C or retesting using different inoculation Media (B, C1, C2, all provided with the GEN III MicroPlate). Eventually genus, species, reagents and the similarity score were reported.

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Conflict of Interest

No conflict of interest declared.

References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

- Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Sayers, E.W. (2013) GenBank. *Nucleic Acids Res* **41**, D36–D42.
- Blast.ncbi.nlm.nih.gov (2014) BLAST: *Basic Local Alignment Search Tool*. [online] Available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome [Accessed 15 May 2014]
- Bochner, B.R. (2009) Global phenotypic characterization of bacteria. *FEMS Microbiol Rev* **33**, 191–205.
- Dey, B.K., Hashim, M., Hasan, S. and Sen Gupta, B. (2004) Microfiltration of water-based paint effluents. *Adv Environ Res* **8**, 455–466.
- Graves, J.L. Jr, Tajkarimi, M., Cunningham, Q., Campbell, A., Nonga, H., Harrison, S.H. and Barrick, J.E. (2015) Rapid evolution of silver nanoparticle resistance in *Escherichia coli*. *Front Genet* **6**, 42.
- Harris, P., Winney, I., Ashhurst-Smith, C., O'Brien, M. and Graves, S. (2012) Comparison of Vitek MS (MALDI-TOF) to standard routine identification methods: an advance but no panacea. *Pathology* **44**, 583–585.
- Janda, J.M. and Abbott, S.L. (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* **45**, 2761–2764.
- Karsa, D.R. and Ashworth, D. (2002) *Industrial Biocides: Selection and Application*. Cambridge, UK: Royal Society of Chemistry.
- Lee, M., Chung, H.S., Moon, H.W., Lee, S.H. and Lee, K. (2015) Comparative evaluation of two matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) systems, Vitek MS and Microflex LT, for the identification of Gram-positive cocci routinely isolated in clinical microbiology laboratories. *J Microbiol Methods* **113**, 13–15.
- Long, P.A., Sly, L.I., Pham, A.V. and Davis, G.H.G. (1981) Characterization of *Morococcus cerebrosus* gen. nov., sp. nov. and comparison with *Neisseria mucosa*. *Int J Syst Evol Microbiol* **31**, 294–301.
- Mulet, M., David, Z., Nogales, B., Bosch, R., Lalucat, J. and Garcia-Valdes, E. (2011) *Pseudomonas* diversity in crude-oil-contaminated intertidal sand samples obtained after the Prestige oil spill. *Appl Environ Microbiol* **77**, 1076–1085.
- Obidi, O.F., Aboaba, O.O., Makanjuola, M.S. and Nwachukwu, S.C. (2009) Microbial evaluation and deterioration of paints and paint-products. *J Environ Biol* **30**, 835–840.
- Pincus, D. (2006) Microbial identification using the BIOMÉRIEUX VITEK® 2 system. In *Encyclopedia of rapid microbiological methods* ed Miller, M.J. Vol. II. River Grove, IL: Davis Healthcare International Publishing.
- Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P.E., Rolain, J.M. and Raoult, D. (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Infect Dis* **49**, 543–551.
- Turner, S., Pryer, K.M., Miao, V.P. and Palmer, J.D. (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J Eukaryot Microbiol* **46**, 327–338.
- Xiao, D., Ye, C., Zhang, H., Kan, B., Lu, J., Xu, J., Jiang, X., Zhao, F. *et al.* (2014) The construction and evaluation of reference spectra for the identification of human pathogenic microorganisms by MALDI-TOF MS. *PLoS One* **9**, e106312.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Bacterial isolates used for equipment evaluation.

Table S2 Detailed results from species identification tools.