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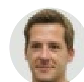
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JCM Accepted Manuscript Posted Online 10 June 2015
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1 # Identification of Highly Pathogenic Microorganisms using MALDI-
2 # TOF Mass Spectrometry – Results of an Inter-Laboratory Ring Trial

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22 **Running title**: Inter-Laboratory MALDI Ring Trial

23 **Keywords**: MALDI-TOF Mass Spectrometry, Highly Pathogenic Bacteria, Identification, External
24 Quality Assurance Exercise, Ring Trial, Microbial Inactivation

25 **Abbreviations**: BSL, biosafety level; CFU, colony-forming units; DHB, 2,5-dihydroxybenzoic acid;
26 EQAE, external quality assurance exercise; FA, formic acid; HCA, heart cysteine agar, HCCA, α-cyano-
27 4-hydroxycinnamic acid; HPB, highly pathogenic bacteria; JA, joint action; MALDI-TOF, Matrix assisted
28 laser desorption/ionization time-of-flight; MLST, multilocus sequence typing; MS, mass spectrometry;
29 MSP, main spectral projections; MW, molecular weight; PAA, peracetic acid; RKI, Robert Koch
30 Institute; SR, security related; TFA, trifluoroacetic acid; TSA, tryptic soy agar; TSB, tryptic soy broth

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38 **1. Abstract**

39 In the case of a release of highly pathogenic bacteria (HPB) there is an urgent need for rapid,
40 accurate and reliable diagnostics. MALDI-TOF mass spectrometry is a rapid, accurate and relatively
41 inexpensive technique which is becoming increasingly important in microbiological diagnostics to
42 complement classical microbiology, PCR and genotyping of HPB. In the present study, the results of a
43 joint exercise with eleven partner institutions from nine European countries are presented. In this
44 exercise ten distinct microbial samples, among them five HPB, *Bacillus anthracis*, *Brucella canis*,
45 *Burkholderia mallei*, *Burkholderia pseudomallei* and *Yersinia pestis* were characterized under blinded
46 conditions. Microbial strains were inactivated by high-dose γ-irradiation before shipment.
47 Preparatory investigations ensured that this type of inactivation induced only subtle spectral changes
48 with negligible influence on the quality of the diagnosis. Furthermore, pilot tests on non-pathogenic
49 strains were systematically conducted to ensure the suitability of sample preparation and to optimize
50 and standardize the workflow for microbial identification.

51 The analysis of the microbial mass spectra was carried out by the individual laboratories on the basis
52 of spectral libraries available on site. All mass spectra were also tested against an in-house HPB
53 library at the Robert Koch Institute (RKI). The average identification accuracy equaled 77% in the first
54 case and improved to > 93% when the spectral diagnoses were obtained on the basis of the RKI
55 library. The compilation of complete and comprehensive databases with spectra from a broad strain
56 collection is therefore considered of paramount importance for accurate microbial identification.

57

58 **2. Introduction**

59 Highly pathogenic bacteria (HPB) are risk group 3 bacteria defined as biological agents that can cause
60 severe human disease and present a serious hazard to workers; it may present a risk of spreading to
61 the community, but there is usually effective prophylaxis or treatment available (1). To this group

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62 belong bacteria such as *Bacillus anthracis* (*B. anthracis*), *Francisella tularensis* (*F. tularensis*) type A,
63 *Yersinia pestis* (*Y. pestis*) and species of the *Brucella melitensis*-group, *Burkholderia mallei* (*B. mallei*),
64 and *Burkholderia pseudomallei* (*B. pseudomallei*). HPB have the potential to be used in bioterrorist
65 attacks (2, 3). The US Centers for Disease Control and Prevention (CDC, Atlanta) has classified *B.*
66 *anthracis*, *F. tularensis*, *Y. pestis* as category A and *Brucella* species, *B. mallei, B. pseudomallei* and *C.*
67 *burnetii* as category B, comprising the main concern for use in bioterrorist attacks (4). These
68 pathogens may cause anthrax, tularemia, plague, brucellosis, glanders, melioidosis and Q-fever,
69 respectively. In most parts of the world the natural prevalence of these agents is low, even though
70 some of these agents cause outbreaks in human and animal populations from time to time (5-8). The
71 intentional release of these agents can however result in severe public health consequences as was
72 shown in the Unites States in 2001 (9, 10). Therefore, accurate assays for microbial identification are
73 important to ensure proper medical intervention, both in the case of a natural outbreak or an
74 intentional release. Such assays must be able to identify unambiguously a broad panel of potential
75 threat microorganisms in different background matrices that may or may not be contaminated with
76 non-HPB bacteria (11).

77 Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is a
78 rapid, accurate, sensitive and cost-effective method that offers an adequate alternative to genome-
79 based approaches and that has been widely used for identification and typing of microorganisms in a
80 clinical routine setup (12-19), but also for HPB (20-27). This method does not depend on exclusive
81 consumables and has revealed high levels of reproducibility in both intra-laboratory and inter-
82 laboratory studies (28, 29). Whole cells, crude cell lysates or bacterial extracts can be utilized to
83 generate taxon-specific fingerprint signatures (30). For safety reasons the application of MALDI-TOF
84 MS for HPB requires complete inactivation of the microbial samples unless the mass spectrometer is
85 operated in a biosafety level (BSL)-3 laboratory. As this is often impossible whole cell preparations, or
86 crude cell lysates cannot be used for MS-based analyses of HPB.

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87 In this paper we describe an international exercise for identification of HPB by MALDI-TOF MS which
88 was carried out in the framework of the EU-funded project "*Quality Assurance Exercises and*
89 *Networking on the Detection of Highly Infectious Pathogens"* (QUANDHIP). The aim of this Joint
90 Action (JA) was to build up a stabilized consortium that links up 37 highly specialized laboratories
91 from 22 European countries and to guarantee universal exchange of the best diagnostic strategies to
92 support a joint European response to outbreaks of highly pathogenic infectious agents. The JA will
93 provide a supportive European infrastructure and strategy for external quality assurance exercises
94 (EQAE), training and biosafety/biosecurity quality management. The aim of this EQAE was (i) to
95 evaluate the current state of the MALDI-TOF MS-based identification technique for highly pathogenic
96 agents in Europe, (ii) to explore opportunities to advance the diagnostic capabilities which includes
97 optimization and standardization of the diagnostic workflow, exchange of standards and protocols
98 (e.g. for verification of MS-compatible inactivation methods) and (iii) to implement measures to
99 improve MALDI-TOF MS-based diagnostics of HPB in Europe (capacity building). The exercise was
100 conducted as a blinded inter-laboratory study with ten different bacterial isolates representing five
101 HPB and five non-HPB test strains and involved in the preparatory phase pilot tests on non-HPB and
102 inactivation tests with γ-irradiated microorganisms . Eleven QUANDHIP project partners from nine
103 European countries participated in this exercise, including three laboratories from Germany and one
104 each from Austria, the Czech Republic, Denmark, Hungary, Italy, Norway, Sweden and Switzerland.

105 **3. Material and Methods**

106 ***Microbial strains and isolates***: All microbial strains originated from the international QUANDHIP
107 strain collection reposited at the unit *Highly Pathogenic Microorganisms* (ZBS 2) at the RKI in Berlin.
108 These strains represent mainly patient isolates sent by the participating laboratories to the
109 QUANDHIP strain collection. All strains were characterized twice, first in laboratories that provided
110 the strains and second at RKI/ZBS 2 by means of a large variety of different methods, including
111 classical microbiological, PCR-based and genotyping methods. An overview of the strains and isolates

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112 used in this study is given in Table 1. All microbial strains and isolates were handled according to the
113 respective biosafety regulations outlined in the TRBA-100 rules (TRBA - protective measures for
114 activities involving biological agents in laboratories) (31). HPB and *F. tularensis* ssp. *holarctica* (Type
115 B; risk group 2) as a very close relative of *F. tularensis* ssp. *tularensis* (Type A; risk group 3) were
116 handled according to TRBA-100 in a BSL- 3 laboratory. The strains were grown under optimal aerobic
117 or microaerophilic conditions on Columbia blood agar plates from Oxoid, Wesel, Germany (*Bacillus*
118 sp., *Yersinia* sp., *Burkholderia* sp., *Brucella* sp., *Ochrobactrum* sp.) or on heart cysteine agar plates
119 (HCA, *Francisella* sp.) for at least 24 h and up to 72 h at 37°C. HCA agar plates were produced in-
120 house from an agar base obtained from Bestbion dx (Cologne, Germany) and sheep blood (Oxoid).
121 Except for *Francisella* sp. isolates, all strains were once transferred onto tryptic soy agar (TSA, VWR,
122 Darmstadt, Germany)/Caso agar (Merck KGaA, Darmstadt, Germany). Cells were harvested from the
123 second passage by resuspending colonies in ddH_2O to an optical density of $OD_{(\lambda=600nm)}$ between 1.0
124 and 1.2.

125 ***Sample preparation/sample inactivation***: The concentration of colony-forming units (cfu) in the
126 microbial suspensions was adjusted to between 10^7 and 10^{10} cfu per mL (cf. Table 1). The
127 suspensions were stored at -75°C until further treatment. Inactivation of microbial samples was
128 carried out by means of high-dose γ-irradiation. For this purpose, microbial suspensions were sent on
129 dry ice from the RKI to Synergy Health Radeberg GmbH (Radeberg/Germany) in accordance with the
130 Dangerous Goods Regulations for category A organisms with UN 2814. Irradiation was carried out
131 according to ISO norm 11137 using a Co-60 γ-ray source. The measured irradiation dose varied
132 between 27.34 and 32.68 kGy. To minimize the possible radiation-associated spectral changes
133 (thermal degradation), the samples were transported and irradiated in the frozen state. For this
134 purpose, all samples were shipped along with a large amount of dry ice. After sample return, it could
135 be verified that a sufficient amount of dry ice was still present and that the samples were not thawed
136 at any time. Tests for sterility after irradiation were conducted by cultivation. In these tests 10% (vol)

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137 of the overall sample solutions were added to tryptic soy broth (TSB) produced in-house using Basis
138 Oxoid (Wesel, Germany). Additionally, 100 µL of sample volume was twice plated onto appropriate
139 media; usually Columbia blood agar or HCA plates (*Francisella*). Incubation for growth in TSB was
140 carried out over a time span of 14 days. Final culturing was performed on Columbia blood agar or
141 HCA plates (*Francisella*), respectively, if visible turbidity of TSB was not observable. All agar plates
142 were incubated under species-specific ideal conditions over a time of 3–7 days. For the EQAE only
143 samples were used which showed no growth after γ-irradiation, neither in TSB, nor on Columbia
144 blood agar/HCA plates.

145 The inactivated microbial samples were aliquoted (1 mL) and stored again at -75°C until shipment.
146 The aliquots were shipped to the eleven partner institutions on dry ice. Before shipment blinded
147 MALDI-TOF MS test measurements were performed at the *Proteomics and Spectroscopy* unit (ZBS 6)
148 to assess the suitability for MALDI-TOF MS.

149 When setting up own spectral databases prior to the ring trial, all partners could choose among a
150 large variety of procedures, protocols and parameters of sample preparation and data acquisition.
151 While some participants routinely utilize the so-called direct transfer method (30, 32) and/or the
152 ethanol/formic acid (FA) protocol recommended by Bruker Daltonics (30, 33), the group at RKI
153 primarily uses the TFA inactivation/sample preparation method (34). A large advantage of
154 inactivation by γ-irradiation is that this method is compatible with all of these sample preparation
155 protocols: Microbial isolates inactivated by γ-irradiation can in principle further processed by utilizing
156 any of the different laboratory-specific methodologies. This allowed optimal usage of in-house
157 spectral databases compiled by the individual partner institutions prior to the ring trial. The specific
158 details and settings of the various experimental protocols were polled as a substantial part of the
159 preparation of the ring trial and are summarized in Table SI-1 of the supplemental information.
160 Furthermore, the preparation of the exercise included systematic MS pilot tests of non-HPB strains
161 by each participating institution. These tests were performed with the aim (i) to identify and

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162 eliminate possible sources of underperformance, such as inadequate procedures of sample
163 preparation or poor parameter selection, and (ii) to standardize - wherever possible - experimental
164 procedures and data acquisition protocols. Within the scope of these pilot tests, MALDI-TOF mass
165 spectra of *Bacillus thuringiensis*, *Burkholderia thailandensis*, *Escherichia coli* and *Yersinia*
166 *enterocolitica* were acquired, shared and jointly analyzed (see also Table 1).

167 ***MALDI-TOF MS***: Details of MALDI-TOF MS measurements can be gathered from Table SI-1 (see
168 supplemental information).

169 ***Identification approach A***: The analysis of mass spectra from blinded microbial samples was carried
170 out first on-site by the ring trial participants themselves. In this approach the participants employed
171 different types of identification software and utilized a variety of distinct mass spectral libraries such
172 as Bruker's commercial database for clinical microbiology, the standard BioTyper® database, the so-
173 called Security Relevant reference library (SR library) from Bruker, the SARAMIS® database and also
174 in-house databases compiled by the institutions themselves (see Table SI-1 for details). During
175 EQAE's preparatory stage, some of the ring trial participants initiated data exchange activities with
176 the purpose of increasing the size and improving the degree of coverage of these in-house libraries.

177 ***Identification approach B***: After submission of the identification results, all mass spectra were
178 collected at the study center (RKI) and subsequently analyzed for a second time using the database
179 of HPB at RKI. This in-house database consists of 1118 entries (main spectral projections, so-called
180 MSPs), each corresponding to a defined microbial strain from the genera *Bacillus*, *Burkholderia*,
181 *Brucella*, *Francisella*, *Vibrio* and *Yersinia* (along with a number of clinically relevant species from the
182 genera *Escherichia*, *Enterococcus*, *Staphylococcus*, *Streptococcus* and others). These MSPs represent
183 database entries of the server component of Bruker's BioTyper® software package which can be
184 queried via BioTyper® software clients (ver. 3.1 built 66, Bruker). Microbial identification was
185 achieved on the basis of the unmodified standard BioTyper® identification method compiled by the
186 manufacturer. Furthermore, identification was conducted by means of logarithmic scores with cut-

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187 off values as specified by Bruker: log score values larger than 2.3 are required for a reliable (highly
188 probable) identification on the species level, and scores between 2.3 and 2.0 represent probable
189 species identification. Score values between 2.0 and 1.7 point towards a reliable genus identification
190 while values below 1.7 are regarded as unreliable (35). Due to the proprietary nature of the spectra
191 data file format, analysis in *identification approach B* was limited to spectra acquired by mass
192 spectrometers produced by Bruker: The BioTyper® client software does not allow importing data in a
193 format other than the Bruker format. As one of the participating institutions employs MS equipment
194 produced by Shimadzu (laboratory XI), *identification approach B* involved analyses of MS data from
195 ten out of eleven participating institutions.

196 ***Identification approach C***: In the third analysis approach the Matlab-based software solution
197 MicrobeMS v. 0.72 (24, 36-39) developed at RKI was used (Matlab, The Mathworks Inc., Natick, MA).
198 MicrobeMS is publicly available as Matlab p-code (free of charge) and provides direct access to
199 Bruker's raw spectral data and to spectra acquired by the VITEK MS® workflow (formerly SARAMIS®,
200 bioMérieux/Shimadzu) via the mzXML data format (40). The software allows spectral preprocessing,
201 such as smoothing, baseline correction, intensity normalization and internal calibration, and can be
202 employed to produce reference peak lists from microbial MALDI-TOF mass spectra (39).
203 Furthermore, MicrobeMS can be used to systematically screen for taxon-specific biomarkers and for
204 visualization of large spectral data sets (via pseudo-gel views). Within the context of the present
205 study the software has been utilized for identification purposes in combination with the mass
206 spectral database for HPB. This allowed cross-platform analysis of microbial mass spectra from
207 partner institutions using instrumentation from two different manufacturers, Bruker and
208 bioMérieux/Shimadzu (see ref. (39) for details).

209

210 **4. Results and Discussion**

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211 ***Gamma inactivation***: Complete inactivation of all pathogens prior to dispatch to the ring trial
212 participants was considered an essential prerequisite for successful implementation of the inter-
213 laboratory ring trial. Although it would in principle have been possible to distribute also viable BSL-3
214 pathogens throughout Europe, the very strict legal provisions would have represented a significant
215 organizational challenge with very high shipment costs. The shipment of viable BSL-3 samples is only
216 allowed as infectious material (class 6.2) category A in accordance with the Dangerous Goods
217 Regulations, whereas inactivated material can be dispatched very easily.

218 As stated earlier, γ-irradiation was selected as the inactivation method of choice. Although the TFA
219 sample preparation protocol has been specifically developed as a MALDI-TOF MS-compatible method
220 for microbial inactivation of HPB, it was decided not to employ this protocol. It is well-known that
221 spectra produced by acid-based methods exhibit systematic changes compared to spectra created by
222 the direct transfer method (41). Differences between spectra obtained by the ethanol/FA and the
223 TFA method, however, are much smaller, since both techniques are ultimately based on acid
224 extraction. Anyway, shipment of γ-inactivated biological material allowed the partners to choose the
225 appropriate preparation protocol, which resulted in a very high degree of compatibility with existing
226 in-house database solutions at the partner institutions.

227 High-dose γ-irradiation is known in the literature as a method suitable for reliably inactivating
228 bacterial pathogens (42, 43) leaving the primary protein structures basically intact. Our comparative
229 measurements of pathogenic and non-pathogenic microbial strains essentially confirmed the
230 literature data: Identification is successful after high-dose γ-irradiation, but irradiation results in
231 slightly lower BioTyper® log score values (data not shown). Under the specific experimental
232 conditions at RKI it was found that the signals relevant for identification remained very marked after
233 γ-irradiation, though with reduced peak intensities. The MALDI-TOF mass spectra of *E. coli* and *B.*
234 *cereus* exemplarily demonstrate the presence of all main peaks in both, the irradiated and the

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235 reference samples (see Figure 1). However, spectra of the γ-inactivated samples, in general, exhibited
236 a lower signal-to-noise ratio due to the slightly reduced peak intensities.

237 ***Pilot tests on non-HPB strains***: These tests were conducted by the partners to identify factors that
238 affect the overall performance of the MS-based identification technique and to standardize
239 experimental procedures, data acquisition protocols and methods of spectral analysis. In the context
240 of the preparation of the pilot tests, experimental methods and parameters were polled (see Table
241 SI-1 for details).

242 The jointly conducted analysis of microbial MALDI-TOF mass spectra from non-HPB revealed a
243 number of peculiarities such as broadened peaks, spectral baseline irregularities (elevated baselines)
244 and the appearance of additional satellite peaks in some of the microbial mass spectra. While peak
245 broadening and baseline elevation effects could be identified relatively easily as a result of the
246 application of excessive laser power (cf. ref. (44)), it was more challenging to identify the sources and
247 causes of additional satellite peaks.

248 ***Satellite peaks***: Figure 2, lower panel, illustrates a first example of satellite peaks in a mass spectrum
249 of *B. thuringiensis*. As shown such additional peaks occurred at 16 Da-intervals at higher molecular
250 weight with respect to the parent peak (cf. peak series at m/z 4,335, 4,351 and 4,367). The spectrum
251 of *B. thuringiensis* obtained by the reference sample preparation method (TFA inactivation) clearly
252 demonstrates the absence of such peaks in the control measurements (Figure 2; upper panel). The
253 observed satellite peaks are caused most likely by the action of sodium hypochlorite (NaClO) which is
254 known as an effective disinfectant and a strong oxidizing agent. NaClO has been applied in the
255 laboratory of one of the partners because of its well-known antimicrobial and sporicidal properties
256 for 15 minutes in a concentration of 10% (vol/vol) for external sterilization of the MALDI-TOF MS
257 sample vials. It seems likely that during this period small amounts of NaClO have entered the tubes,
258 e.g. via incompletely closed lids. In proteins the amino acids methionine and aromatic residues such
259 as tryptophan and tyrosine are potential first oxidation targets (45, 46). In the case of oxidation of

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260 methionine, the experimentally observed mass differences between the parent and satellite peaks of
261 16 Da would fit well with the computed masses of un-oxidized methionine and methionine sulfoxide
262 as the singly oxidized species (47). However, the mentioned mass differences would be also
263 observable in the case of oxidation of other amino acids.

264 Similar oxidation-induced satellite peaks (Δ m/z of +16 Da) were observed when microbial samples
265 were accidentally inoculated with a further sterilizing agent, peracetic acid (PAA). PAA also acts as an
266 oxidizing agent and can cause the oxidation of lipids and amino acid side chains of peptides and small
267 proteins in microbial extracts (data not shown).

268 Satellite peaks were also detected in samples prepared by means of the ethanol/FA sample
269 preparation protocol (30, 33). Using the example of spectra from *B. cereus* ATCC 10987 and *B.*
270 *thuringiensis* DSM 5815, Figure 3 shows the presence of additional peaks at a distance of 28 Da: Black
271 curves denote mass spectra in the m/z 6,250-7,500 region of *Bacillus* samples prepared by the TFA
272 inactivation method, while red spectra were obtained from identical *Bacillus* strains prepared by
273 means of the ethanol/FA sample preparation method which included incubation by 70% FA (vol/vol)
274 for 30 minutes. Both pairs of spectra display parent peaks at m/z 6,695 (*B. cereus*) / 6,711 (*B.*
275 *thuringiensis*) assigned as β–SASP, 6,835 (α–SASP) and 7,082 (α–β SASP, see refs (24, 48) for peak
276 assignments). Apart from these dominating signals, the spectra of FA-treated samples exhibit
277 additional satellite peaks at m/z 6,723 (*B. cereus*) / 6,739 (*B. thuringiensis*) and at m/z 6,863 (both
278 strains). Satellite signals are found at a distance of +28 Da from the parent peaks, typically with
279 intensities of less than 20% of the original signal. A likely explanation for the occurrence of satellite
280 peaks would be chemical modification of the SASPs (formyl esterification) due to prolonged sample
281 treatment by FA. FA treatment has been associated with formylation of proteins in microbial extracts
282 (49) with the specific targets of serine and threonine residues. Furthermore, it is known that
283 formylation is particularly effective when highly concentrated FA is applied to small hydrophobic
284 proteins (50) such as SASPs. Since each additional satellite peak may potentially have a negative

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285 impact on the performance of the identification algorithm, the exposure time to FA should be
286 minimized whenever possible. Taking into account that this note is also given in the BioTyper®
287 manual (see ref. (35)), the reduction of FA incubation time is considered an important measure for
288 improving the accuracy of identification.

289 ***Results of the inter-laboratory ring trial***: Table SI-2 (see supporting information) gives a summary of
290 the identification results in the context of the so-called *identification approach A*. This approach
291 involved data analysis on-site by each partner institution. The table shows not only an overview on
292 the results of the blinded identity tests, but provides also either the logarithmic BioTyper® scores or
293 alternatively the respective SARAMIS® score values. In *approach A* MALDI-TOF mass spectra acquired
294 by laboratory XI were analyzed twice, firstly by using customized in-house algorithms and secondly
295 by an analysis carried out elsewhere by means of the SARAMIS® software and the database solution
296 from Anagnostec. For this reason Table SI-2 includes an additional column designated as "Laboratory
297 XII", which is different from *identification approaches B* and *C*.

298 The color scheme used in Table SI-2 is a traffic light coloring scheme: It uses the colors green for
299 correct, yellow for partially correct and red for false identification results. A correct result was
300 present when the identity was accurately revealed at the genus, species and the subspecies level.
301 Cells colored yellow denote identification results which were either incomplete, for example in cases
302 where the subspecies specification was lacking (see sample 2 – *F. tularensis* ssp. *holarctica*), or where
303 the genus assignment was correct but the species was left unassigned (e.g. in lines 9 and 10,
304 laboratory VIII*, Yersinia* sp., *Bacillus* sp. of Table SI-2). Furthermore, a result was also considered
305 partially correct in cases of contradictory identification results, i.e. if different microbial identities
306 were obtained from spectra of technical replicate measurements. In such cases, however, at least
307 one result had to be correct. An example of contradictory identification results can be found in Table
308 SI-3 for sample 6 from laboratory X. Score values in this or similar instances were indicated by a
309 range of values. Identification results were considered incorrect (red color) if either an HPB was

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310 clearly assigned as a non-HPB (false negative), or alternatively, if a non-HBP was identified as an HPB
311 (false positive). Cases where no false positive / false negative results were obtained, for example if a
312 result was inconsistent or unavailable (no spectrum), were also regarded as partially correct (no
313 confirmation, but also no all-clear). To calculate the overall accuracy index of the entire identification
314 approach, a point system was introduced, giving one point for each correct identification result
315 (green). Furthermore, cells with partially correct results (yellow) received half points while no points
316 were given for incorrect results (red). All points were then summed over the entire table; the sums
317 were subsequently divided by the number of cells of each table. The quotient thus determined was
318 finally multiplied by 100 and expressed in percent. To exclude an undue weighting of the measured
319 data from laboratory XI, the point values from the rows “Laboratory XI” and “Laboratory XII” were
320 averaged before summation in *identification approach A*.

321 The overall identification accuracy of *identification approach A* equaled 77% (see Table SI-2). While
322 the accuracy of identifying samples 1 (*B. mallei*), 4 (*B. anthracis*), 5 (*Ochrobactrum anthropi*), 7 (*B.*
323 *pseudomallei*), 8 (*B. thailandensis*) and 9 (*Y. pestis*) was relatively high, there were major problems
324 when diagnosing samples 2 (*F. tularensis* ssp. *holarctica*), 3 (*B. canis*), 6 (*Y. pseudotuberculosis*) and
325 10 (*B. thuringiensis*). Furthermore, results from laboratory IX were generally difficult to assess. In this
326 laboratory diagnoses were made only on the basis of the standard BioTyper® database for clinical
327 microorganisms; neither an in-house database of HPB nor the SR library from Bruker were available
328 to this partner (cf. Table SI-1 and Table SI-2).

329 The overall identification results improved significantly when spectra of the inter-laboratory exercise
330 were tested against the database of highly pathogenic microorganisms compiled at RKI over the past
331 ten years: The overall identification accuracy improved from 77.0% *in approach A* to 93.5% in
332 *approach B* (see Table SI-3). Improvements were particularly striking in the cases of sample 2 (*F.*
333 *tularensis* ssp. *holarctica*), 3 (*B. canis*) and 10 (*B. thuringiensis*). However, differentiation between
334 samples 6 (*Y. pseudotuberculosis*) and 9 (*Y. pestis*) improved only slightly in *approach B*.

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335 In the third approach, *identification approach C*, the overall picture did not differ much from
336 *approach B* (see Table SI-4 for details). The minor improvement in the overall identification accuracy
337 of 93.7% (compared to 93.5%) is statistically insignificant and not particularly surprising: Although
338 both approaches involved different software implementations with different algorithms, they relied
339 on an identical spectral database. The results given in Table SI-4 demonstrate a decreased
340 identification rate for sample 7 (*B. mallei*) and a slight improvement for sample 6 (*Y.*
341 *pseudotuberculosis*). However, the major advantage of *approach C* over *approach B* consists in the
342 fact that it allows analysis of spectra obtained by means of the bioMérieux/Shimadzu system (cf.
343 rows "Laboratory XI" of Tables 3 and 4). Due to missing support of the Shimadzu-specific spectra
344 format, the data acquired by laboratory XI may be analyzed by *approach C*, yet not using the
345 BioTyper® software employed in *identification approach B*.

346 Table 2 shows a summary of the results of all identification approaches. This table illustrates again
347 the improvements of the identification accuracies in *approaches B* and *C* in comparison to *A*,
348 particularly for the samples 1-4 and 10. With regard to samples 2 (*F. tularensis* ssp. *holarctica*) and 3
349 (*B. canis*) we assume that the relatively high error rates in *approach A* derive from incomplete or
350 missing spectral entries for both subspecies/species in the SR BioTyper® library extension. We have
351 noted that identification of *F. tularensis* ssp. *holarctica* and of *B. canis* was incomplete in cases where
352 identification was made by means of this particular database extension. A closer examination of the
353 SR database content revealed the absence of subspecies information for entries of *F. tularensis*
354 (sample 2) and the lack of spectral entries for *Brucella* species other than *B. melitensis* (sample 3).

355 In contrast, it was interesting to note that the sophisticated software algorithms employed in
356 *approaches B* and *C* can cause problems even in cases where extensive spectral databases are
357 available. To give an example: Differentiation between *Y. pseudotuberculosis* and *Y. pestis* by
358 *approaches B* and *C* is far from being ideal (cf. samples 6 and 9 in Tables SI-3 and SI-4). To a certain
359 extent, this could be caused by the low initial concentration of colony-forming units of *Y. pestis* in the

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360 respective sample solution (1.3 × 10^7, cf. Table 1). Several ring trial participants have indeed reported
361 a relatively poor signal-to-noise ratio in MALDI-TOF mass spectra acquired from aliquots of sample 9.
362 Low spectral quality is certainly a factor which makes differentiation of *Y. pestis* and *Y.*
363 *pseudotuberculosis* difficult. An even more important factor is, however, the very high degree of
364 similarity of spectra from these two very closely related species. In fact, *Y. pestis* is known as a clone
365 of *Y. pseudotuberculosis* which has been only recently evolved from *Y. pseudotuberculosis* (51, 52).
366 Both species share genomic sequences and have identical 16S-rDNA (53). As a consequence, their
367 differentiation by MALDI-TOF MS is challenging; it has been found that differentiation can be carried
368 out only on the basis of one single mass peak at m/z 3,065 (36, 38). This peak has been assigned to a
369 fragment of the plasmid-encoded (pPCP1) Pla protein. Therefore, MS-based differentiation is
370 possible only for strains of *Y. pestis* carrying the pPCP1 plasmid. At this point it should be stressed
371 that visual inspection of the mass spectra would have helped solving the particular problem of
372 differentiating *Y. pseudotuberculosis* and *Y. pestis*. Although the biomarker for *Y. pestis* at m/z 3,065
373 is typically very intense, pattern recognition routines do not always provide reliable results in cases
374 when the outcome of the identification is based on the presence or absence of only one single
375 biomarker. In this line of reasoning, the supervised modelling approach chosen by Laboratory XI,
376 which relies on 15 biomarkers for the discrimination between *Y. pestis*, *Y. pseudotuberculosis* and *Y.*
377 *enterocolitica*, may provide the basis for a more robust typing scheme (54).

378 In the present study problems also occurred when differentiating the closely related members of the
379 *B. cereus* group: *B. anthracis*, *B. cereus* and *B. thuringiensis*. First of all, we have no information on
380 whether MALDI-TOF MS allows reliable differentiation of *B. cereus* and *B. thuringiensis*. Our own
381 observations, however, revealed that strains from both species are frequently identified based on
382 their strain-specific spectral profiles. On the other hand, mass spectra of *B. anthracis* strains exhibit a
383 specific β–SASP- signal at m/z 6,679 (22, 24, 55-58) which is usually not present in spectra of other *B.*
384 *cereus* group members. However, in the recent literature there is increasing evidence that spectra of

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385 certain strains of *B. cereus* and *B. thuringiensis* may also exhibit β–SASP- peaks at m/z 6,679 (59) (cf.
386 also the spectrum of *B. cereus* BW-B of Figure 1). Therefore, this β–SASP- biomarker is not
387 necessarily pathognomonic for *B. anthracis*. Furthermore, we and others have noted that the second
388 published biomarker of *B. anthracis* at m/z 5,413 (24) is often also found in spectra of *B. cereus* and
389 *B. thuringiensis*. Both facts should be considered when assessing the identification results for *B.*
390 *cereus* group members: Results of MALDI-TOF MS should not form the sole basis for potentially far-
391 reaching decisions, for example in the event of suspected intentional release of *B. anthracis*.

392

393 **5. Conclusions**

394 This paper reports on an inter-laboratory external quality assurance exercise (EQAE) conducted by
395 eleven partner institutions from nine European countries. In this ring trial MALDI-TOF MS was used
396 as a means for rapid, reliable and cost-effective identification of highly pathogenic microorganisms.
397 In the preparatory phase of the exercise pilot tests on non-pathogenic strains were carried out in
398 order to optimize and standardize the experimental procedures at the partner institutions and to
399 identify possible sources of underperformance. Irradiation by γ-rays proved to be a MALDI-TOF MS
400 compatible inactivation method which induced only subtle spectral changes with negligible influence
401 on the quality of the diagnosis. In the ring trial, the average identification accuracy equaled 77%
402 when using non-standard mass spectral databases. The accuracy could be improved to > 93% when
403 spectral diagnoses were reached on the basis of an optimized spectral database with a better
404 coverage of highly pathogenic and related species.

405 The present EQAE has highlighted current strengths and weaknesses of the MALDI-TOF MS based
406 approach for identification of HPB and has confirmed the need for high-quality spectral databases to
407 facilitate improved identification accuracy. Experiences gathered from the present international
408 EAQE suggest also that, as long as high-quality and comprehensive spectral databases are available,

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409 different preparative procedures, the degree of user experience as well as the different type of
410 instrumentation and analysis software are not likely to critically affect identification of HPB. The
411 compilation of complete and comprehensive databases is thus considered to be of paramount
412 importance for reaching accurate and reliable spectral diagnoses. Future efforts to improve the
413 diagnostic capabilities should therefore focus on the exchange of validated reference spectra. We are
414 confident that further ring trials will confirm the improvements achieved by such activities.

415 **6. Acknowledgements**

416 The authors wish to thank Dr. T. M. Fuchs (ZIEL, Technical University Munich, Germany), Dr. J. Rau
417 (CVUA, Stuttgart, Germany), Dr. W. Beyer (University of Hohenheim, Stuttgart, Germany), Dr. A.
418 Paauw (TNO, Rijswijk, Netherlands), M. Dybwad (NDRE, Kjeller, Norway), and Dr. N. Schürch (Labor
419 Spiez, BABS, Spiez, Switzerland) for providing strains, samples, or spectra of important microbial
420 pathogens. S. Weil, Dr. S. Zange and Dr. B. Pályi are grateful to Dr. P. Hufnagl (AGES, Vienna, Austria),
421 Dr. B. Thoma (InstMikroBioBw, Munich, Germany) and Dr. M. Iván (Semmelweis University,
422 Budapest, Hungary), respectively. In addition, we like to thank S. Becker, P. Lochau, A. Schneider, S.
423 Howaldt, and R. Andrich (all RKI, Berlin, Germany) for excellent technical assistance. Moreover, we
424 are very grateful to the European Commission and CHAFEA for financially and technically supporting
425 the QUANDHIP Joint Action (CHAFEA Grant Agreement n° 2010 21 02). Parts of this work were
426 supported by the Federal Ministry of Education and Research, BMBF, (Förderkennzeichen / Grant ID:
427 13N11166).

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602 **8. Tables**

603 **Table 1** – Overview on microbial strains and species used in the inter-laboratory ring trial (samples 1-
604 10). * Strains utilized for γ-inactivation test measurements in advance of the ring trial. § Strains used
605 for pilot tests on non-HPB.

606

#	Genus / Species / Strain	Concentration (cfu/mL)
1	*Burkholderia pseudomallei* A101-10	1.1×10^{9}
2	*Francisella tularensis* ssp. *holarctica* Ft 32	1.7×10^{10}
3	*Brucella canis* A183-5	1.9×10^{10}
4	*Bacillus anthracis* AMES	6.4×10^{7}
5	*Ochrobactrum anthropi* A148-11	2.0×10^{10}
6	*Yersinia pseudotuberculosis* type III	1.3×10^{9}
7	*Burkholderia mallei* A106-3	1.0×10^{9}
8	*Burkholderia thailandensis* E125	5.6×10^{10}
9	*Yersinia pestis* A106-2	1.3×10^{7}
10	*Bacillus thuringiensis* DSM350	8.6×10^{8}
11*	*Escherichia coli* RKI A139	
12*	*Bacillus cereus* BW-B	
13 §	*Bacillus cereus* ATCC 10987	
14 §	*Bacillus thuringiensis* DSM 5815	
15 §	*Burkholderia thailandensis* DSM 13276	
16 §	*Yersinia enterocolitica* DSM 4780	

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607 **Table 2 –** Summary of the different identification results of the MALDI-TOF MS ring trial with the number of correct, partly correct and incorrect
608 identifications. The cells contain furthermore a point sum (correct identification: one point, partly correct: half point and incorrect: zero points)
609 and the corresponding identification accuracy (in %). Color scheme, green: the identification accuracy of the given microbial strain is equal or
610 larger than 90%, yellow: accuracy is equal or larger than 75% and below 90% and red: accuracy below 75%.

No.	Sample Identity	Identification Approach A			Identification Approach B			Identification Approach C		
		Correct	Partly correct	Incorrect	Correct	Partly correct	Incorrect	Correct	Partly correct	Incorrect
1	*Burkholderia pseudomallei A101-10*	9	1 9.5 (86%)	1	9	1 9.5 (95%)	0	10	1 10.5 (95%)	0
2	*Francisella tularensis ssp. holarctica Ft 32*	4.5	6.5 7.75 (70%)	0	9	1 9.5 (95%)	0	11	0 11 (100%)	0
3	*Brucella canis A183-5*	3	8 7 (64%)	0	10	0 10 (100%)	0	11	0 11 (100%)	0
4	*Bacillus anthracis AMES*	9	0 9 (82%)	2	9	1 9.5 (95%)	0	11	0 11 (100%)	0
5	*Ochrobactrum anthropi A148-11*	10	1 10.5 (95%)	0	10	0 10 (100%)	0	11	0 11 (100%)	0
6	*Yersinia pseudotuberculosis type III*	8	0 8 (73%)	3	6	3 7.5 (75%)	1	9	1 9.5 (86%)	1
7	*Burkholderia mallei A106-3*	9	0 9 (82%)	2	9	1 9.5 (95%)	0	8	3 9.5 (86%)	0
8	*Burkholderia thailandensis E125*	10	1 10.5 (95%)	0	10	0 10 (100%)	0	11	0 11 (100%)	0
9	*Yersinia pestis A106-2*	7	3 8.5 (77%)	1	6	4 8 (80%)	0	6	5 8.5 (77%)	0
10	*Bacillus thuringiensis DSM350*	4	2 5 (45%)	5	10	0 10 (100%)	0	9	2 10 (91%)	0

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611 **9. Figure Legends**

612 ***Figure 1.*** MALDI-TOF mass spectra of control samples (black traces) and microorganisms inactivated
613 by means of high-dose γ-irradiation (red traces). Irradiated samples of *E. coli* A 139 and *B. cereus* BW-
614 B (spores) were prepared for MALDI-TOF MS in the same way as the retained control samples by
615 means of the TFA inactivation method (34). The spectra (smoothed, baseline corrected) demonstrate
616 only insignificant differences between the irradiated and control samples, suggesting that γ-
617 irradiation is compatible with the routine sample preparation protocols used by the partner
618 institutions (see also text for details).

619 ***Figure 2***. Oxidation of microbial extracts of *Bacillus thuringiensis* by sodium hypochlorite (NaClO).
620 Top panel: Reference mass spectrum of a *B. thuringiensis* sample prepared on the basis of the
621 trifluoroacetic acid (TFA) inactivation technique (34). Lower panel: TFA-treated sample of the same
622 *Bacillus* strain with a likely contamination by sodium hypochlorite. The spectral differences - satellite
623 peaks at +16 Da-intervals – are attributed to a contamination by the oxidant NaClO which was
624 employed for external sterilization of sample vials during outward transfer from a BSL-3 laboratory
625 (spectra were smoothed and baseline corrected, see text for further details).

626 ***Figure 3***. Formylation of spore marker proteins, small acid-soluble proteins (SASP) in test samples of
627 *Bacillus cereus* and *Bacillus thuringiensis* as a possible result of prolonged treatment by highly
628 concentrated (70%) formic acid (FA) (24). [#] peaks at m/z 6,695 or 6,711 corresponding to two
629 possible variants of β–SASP in *B. cereus* and *B. thuringiensis.* [&] peaks at 6,835 (α–SASP, UniProt ID
630 Q73CW6 in *B. cereus* ATCC 10987). All mass spectra were smoothed, baseline corrected and intensity
631 normalized).
632 Black curves: reference MALDI-TOF mass spectra of *Bacillus* samples prepared by the trifluoroacetic
633 acid (TFA) inactivation method (34).

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634 Red curves: Spectra from identical strains processed on the basis of the ethanol/FA method (33).

635 Peaks marked by red number denote additional mass peaks at a distance of +28 Da with reference to

636 the α–SASP (m/z 6,835), or the β–SASP (m/z 6,695/6,711) peaks, respectively.

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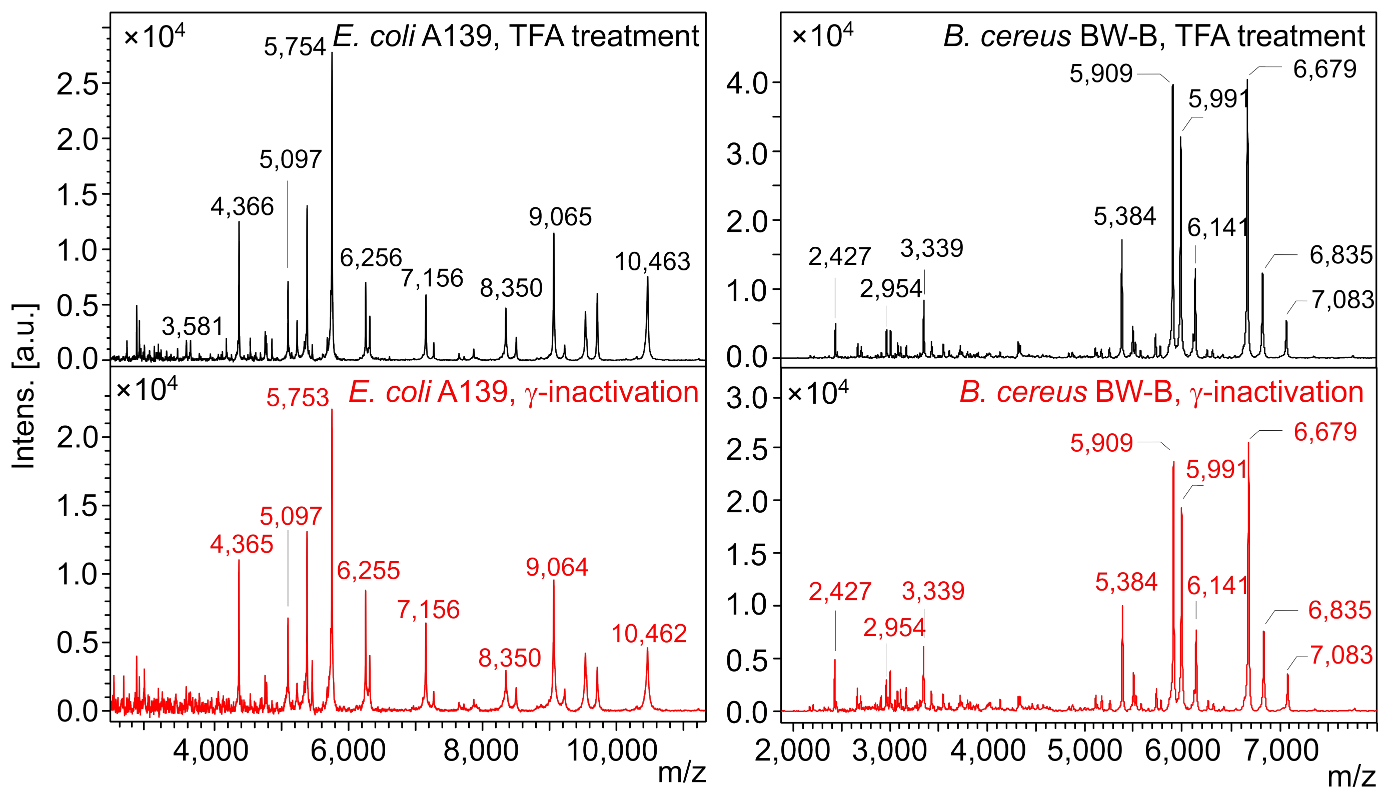

Figure 1. MALDI-TOF mass spectra of control samples (black traces) and microorganisms inactivated by means of high-dose γ-irradiation (red traces). Irradiated samples of *E. coli* A 139 and *B. cereus* BW-B (spores) were prepared for MALDI-TOF MS in the same way as the retained control samples by means of the TFA inactivation method (24). The spectra (smoothed, baseline corrected) demonstrate only insignificant differences between the irradiated and control samples suggesting that γ-irradiation is compatible with the routine sample preparation protocols used by the partner institutions (see also text for details).

Lasch et al., Figure 1, ver. Jan 15, 2015

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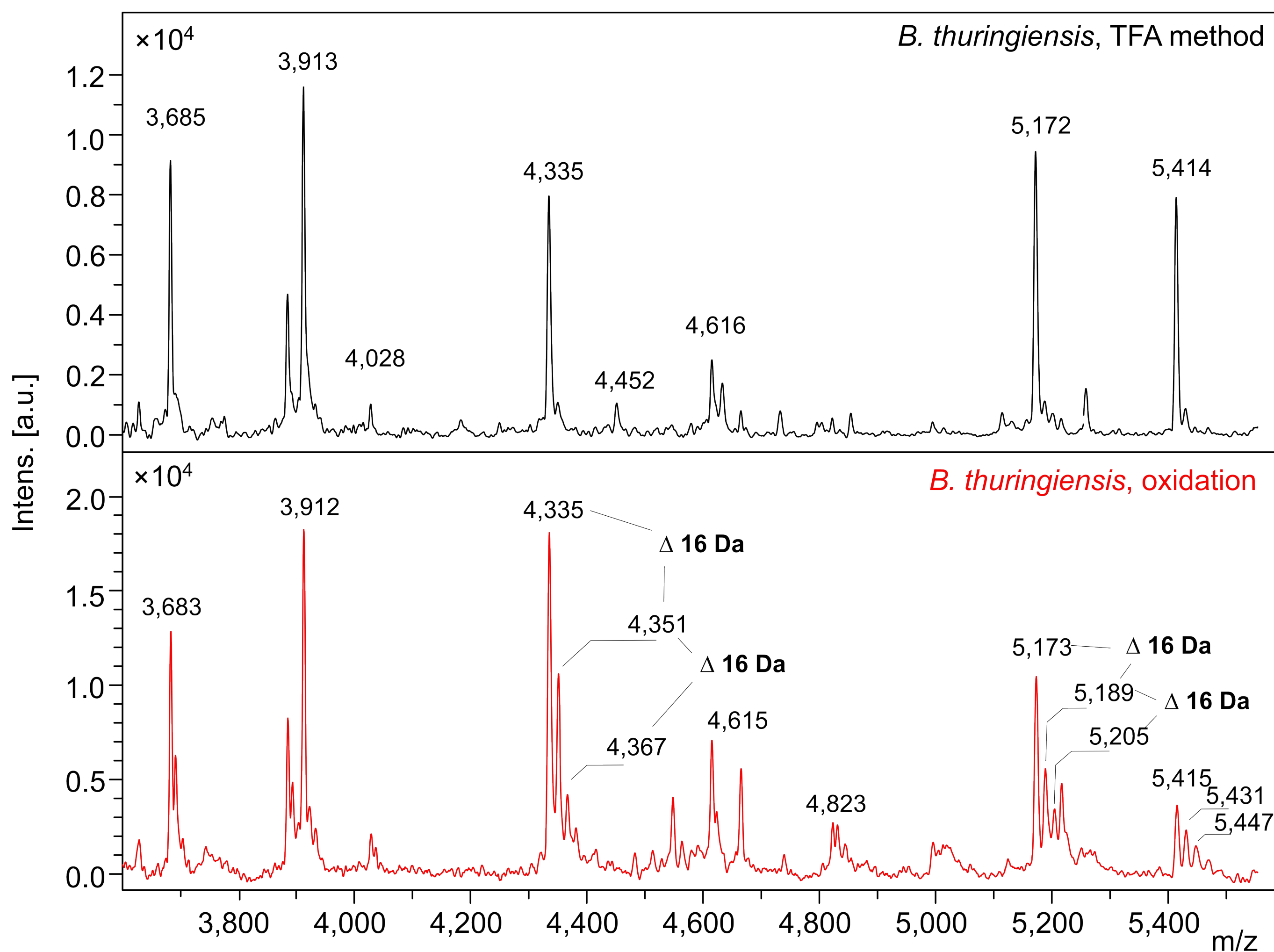

Figure 2. Oxidation of microbial extracts of *Bacillus thuringiensis* by sodium hypochlorite (NaClO). Top panel: Reference mass spectrum of a *B. thuringiensis* sample prepared on the basis of the trifluoroacetic acid (TFA) inactivation technique (34). Lower panel: TFA-treated sample of the same *Bacillus* strain with a likely contamination by sodium hypochlorite. The spectral differences - satellite peaks at +16 Da-intervals – are attributed to a contamination by the oxidant NaClO which was employed for external sterilization of sample vials during outward transfer from a BSL-3 laboratory (spectra were smoothed and baseline corrected, see text for further details).

Lasch et al., Figure 2, ver. Jan 15, 2015

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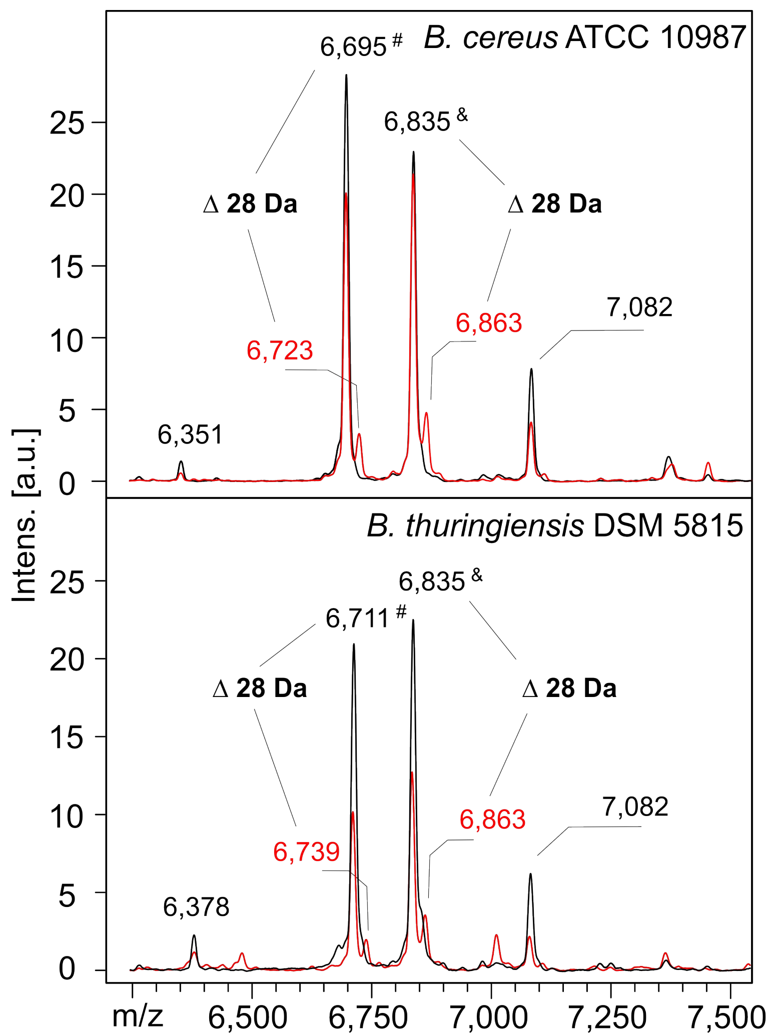

Figure 3. Formylation of spore marker proteins, small acid-soluble proteins (SASP) in test samples of *Bacillus cereus* and *Bacillus thuringiensis* as a possible result of prolonged treatment by highly concentrated (70%) formic acid (FA) (24). # peaks at m/z 6,695 or 6,711 corresponding to two possible variants of β–SASP in *B. cereus* and *B. thuringiensis.* & peaks at 6,835 (α–SASP, UniProt ID Q73CW6 in *B. cereus* ATCC 10987). All mass spectra were smoothed, baseline corrected and intensity normalized).

Black curves: reference MALDI-TOF mass spectra of *Bacillus* samples prepared by the trifluoroacetic acid (TFA) inactivation method (34).

Red curves: Spectra from identical strains processed on the basis of the ethanol/FA method (33).

Peaks marked by red number denote additional mass peaks at a distance of +28 Da with reference to the α–SASP (m/z 6,835), or the β–SASP (m/z 6,695/6,711) peaks, respectively.

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