

1 **Proteomics of diphtheria toxoid vaccines reveals multiple proteins that are immunogenic and**
2 **may contribute to protection of humans against *Corynebacterium diphtheriae***

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28 **Keywords:** corynebacteria, *Corynebacterium ulcerans*, exoproteome, mass spectrometry,
29 proteomics, secretome, vaccination

30

31 **Abstract**

32 Introduced for mass immunization in the 1920s, vaccines against diphtheria are among the oldest
33 and safest vaccines known. The basic principle of their production is the inactivation of purified
34 diphtheria toxin by formaldehyde cross-linking, which converts the potentially fatal toxin in a
35 completely harmless protein aggregate, which is still immunogenic. Since in addition to diphtheria
36 toxin also other proteins may be secreted by *Corynebacterium diphtheriae* during cultivation, we
37 assumed that diphtheria toxoid might not be the only component present in the vaccine. To address
38 this question, we established a protocol to reverse formaldehyde cross-linking and carried out mass
39 spectrometric analyses. Different secreted, membrane-associated and cytoplasmic proteins of *C.*
40 *diphtheriae* were detected in several vaccine preparations from across the world. Based on these
41 results, bioinformatics and Western blot analyses were applied to characterize if these proteins are
42 immunogenic and may therefore support protection against *C. diphtheriae*. In frame of this study, we
43 could show that the *C. diphtheriae* toxoid vaccines induce antibodies against different *C. diphtheriae*
44 proteins and against diphtheria toxin secreted by *Corynebacterium ulcerans*, an emerging pathogen
45 which is outnumbering *C. diphtheriae* as cause of diphtheria-like illness in Western Europe.

46

47 **1. Introduction**

48 Diphtheria is an infection of the upper respiratory tract of humans and was a major cause of
49 morbidity and mortality especially of children until the beginning of the 20th century (for recent
50 reviews, see [1-3]). In 1884 Löffler showed that *Corynebacterium diphtheriae* is the etiological agent
51 of diphtheria and postulated that a toxin secreted by this bacterium is responsible for the often fatal
52 damages observed to heart and kidneys [4]. This hypothesis was verified by Roux and Yersin
53 (1888). When filter-sterilized supernatants of *C. diphtheriae* cultures were injected to guinea pigs,
54 damages similar to those observed in cases of human diphtheria infections were found [5]. Classical

55 diphtheria of the upper respiratory tract is spread from person to person by respiratory droplets
56 produced by coughing. Additionally, other secretions and contaminated materials may be sources of
57 infection especially in cases of cutaneous diphtheria. After infection and colonization of
58 nasopharyngeal epithelia by the bacteria within two to five days, patients are infectious for two to
59 three weeks. Today, penicillin and erythromycin are drugs of choice to stop the infection [6] and
60 quickly render patients non-infectious [7]. Before introduction of mass vaccination, diphtheria was
61 observed as an infection especially of children, indicating that *C. diphtheriae* was widely
62 disseminated among the population leading to early contact with the pathogen.

63 The development of vaccines starting in the 1920s and the introduction of mass
64 immunization using diphtheria toxoid vaccines led to a dramatic reduction of worldwide diphtheria
65 cases. After the global introduction of the “Expanded Programme on Immunization” (EPI) in 1974,
66 only relatively small and local outbreaks occurred until the 1990s [8]. However, with the breakdown
67 of the former Union of Socialist Soviet Republics, a large scale outbreak was observed, leading to a
68 diphtheria pandemic with more than 157,000 cases and over 5,000 deaths reported between 1990
69 and 1998 [9-12]. The outbreak started in the Russian Federation [13-15], but it quickly spread to the
70 Baltic States, former Asian Soviet Republics and other states such as Finland, Poland and Turkey
71 [16]. In contrast to former epidemics, children were less affected, while diphtheria cases among
72 adolescents and adults reached up to four fifths of total cases in some states [9,17]. The outbreak
73 was finally stopped by mass immunization, especially of adults. Despite this success and the
74 continuing global EPI, diphtheria is not eradicated today and its etiological agent *C. diphtheriae* is
75 still present on the list of the most important global pathogens [18,19]. In fact, reported global cases
76 increased from about seven thousand in 2016 to almost nine thousand in 2017 with a focus on
77 countries with poor access to public health systems, for example India, Indonesia, Nepal, Pakistan,
78 Venezuela and Yemen [20]. Furthermore, recent analyses of respiratory and cutaneous diphtheria
79 cases among Spanish, Belgian, German and British citizens as well as in Asian and African
80 refugees in Finland, Denmark, Germany and Sweden indicated the circulation of toxigenic *C.*
81 *diphtheriae* not only among immigrants, but also among the indigenous population of the European

82 Union [21-25]. Among toxigenic *C. diphtheriae* isolates, the highly virulent 'Sankt-
83 Petersburg/Rossija' epidemic clone that caused the large diphtheria outbreak in Russia and
84 neighboring countries in the 1990s is still in circulation in the European Union [26]. Moreover, in
85 Europe the number of cases of human diphtheria-like disease associated with pet animals, i.e. cats
86 and dogs, has increased, which are caused by *Corynebacterium ulcerans*, a close relative of *C.*
87 *diphtheriae* [24,27].

88 Infections with diphtheria can be successfully treated with antitoxin and antibiotics [6];
89 however, an efficient vaccination regime is most effective to prevent this potentially fatal disease.
90 The basis for vaccination was laid by scientists such as Ehrlich, Fraenkel, Park, Ramon, von
91 Behring and others at the beginning of the 20th century, leading to the development of today's
92 diphtheria toxoid vaccine (for review, see [28]). The vaccines are commonly injected intramuscularly
93 as a 0.5 ml dose and typically combinations with tetanus and pertussis vaccines are administered
94 (DT and DPT vaccines). The immunization schedule recommended by the World Health
95 Organization (WHO) includes a primary immunization series of three doses for infants followed by
96 optional booster immunizations for adults [19]. After the primary immunization series 94 to 100 % of
97 children develop at least minimal protective antibody levels (> 0.01 IU/ml) [19]. For 2018 the WHO
98 reported a global estimated diphtheria, tetanus and pertussis (DTP3) coverage of 86 % [29], while
99 almost 20 million infants worldwide did not receive a routine primary immunization series of DTP3
100 vaccine [30].

101 Although diphtheria vaccine is thought to be directed exclusively against the toxin and
102 immunization is not expected to be prevent carriage of *C. diphtheriae* on epithelia and skin [31], an
103 influence of vaccination on the increasing emergence of non-toxigenic strains was discussed [32]. In
104 this case, diphtheria toxoid vaccines may also prevent bacterial infections due to the presence of
105 trace amounts of other immunogenic proteins in the vaccine.

106 This hypothesis seems plausible, when the production process is analyzed in detail. After
107 cultivation of the most common used toxigenic vaccine strain of *C. diphtheriae* PW8 [33] in beef-
108 derived peptides or casein hydrolysate, the bacteria are removed by centrifugation [28,34,35]. For

109 inactivation of the secreted diphtheria toxin, 0.75 % of formaldehyde is added to the supernatant and
110 the solution incubated for up to six weeks at 37 °C [35-37]. After the supernatant is fully detoxified,
111 the toxoid-containing solution is filtered and as further purification and concentration step ammonium
112 sulfate precipitation is often applied, before the toxoid is tested for potency [28,34,35,38]. To
113 enhance the immune response, aluminum salts are added as adjuvant to the toxoid vaccine [28].
114 This well-established production process makes it very likely, that besides the toxin other secreted
115 proteins, which were described earlier to be part of culture supernatants of *C. diphtheriae* [39], are
116 also present in toxoid preparations. To investigate this hypothesis, we developed a protocol to purify
117 proteins from commercially available vaccines and reverse the formaldehyde cross-linking, which
118 was described to be irreversible previously [40]. A number of proteins was identified by mass
119 spectrometry. Bioinformatics analyses and Western blotting experiments were carried out to
120 elucidate, if these proteins may contribute to immune protection against *C. diphtheriae*.

121

122 **2. Methods**

123 *2.1. Human ethics*

124 Human blood was collected from three adult patients six months after hematopoietic stem cell
125 transplantation before re-vaccinations (first serum) and one year after three vaccinations with
126 Pentavac® (DTaP-Hib containing 30 IU of diphtheria toxoid and 40 IU of tetanus toxoid,
127 SanofiPasteur) (second serum). First booster and second booster vaccinations were given 4 weeks
128 after the primary or first booster vaccinations, respectively. All methods were performed in
129 accordance with the relevant guidelines and were approved by the University Hospital Ethics
130 Committee „Ethik-Kommission der Friedrich-Alexander-Universität Erlangen-Nürnberg“
131 (Krankenhausstraße 12, 91054 Erlangen, Germany; <https://www.ethikkommission.fau.de>) under
132 registration number 147_12B. All patients enrolled gave written informed consent before
133 participation.

134

135 *2.2. Preparation of vaccines for mass spectrometry analysis*

136 Since the protein content of the commercial vaccines analyzed in this study (Table 1) was
137 considered low, multiple vaccine doses (0.5 ml each) were pooled and precipitated by addition of 10
138 % (w/v) trichloroacetic acid (TCA) and incubation at 4 °C for 16 h to get a concentrated sample for
139 mass spectrometry analysis [41]. After incubating for 16 h at 4 °C the samples were centrifuged
140 (8000 x g, 30 min, 4 °C). The precipitated proteins were dried on ice and solved in rehydration buffer
141 (2 % sodium deoxycholate, 10 mM dithiothreitol (DTT), 50 mM Tris, pH 8.0). To reverse the
142 formaldehyde cross-linking of the inactivated toxins, the samples were incubated for 20 min at 95 °C
143 [42]. Subsequently, the protein amount of rehydrated and heat-treated samples was determined
144 using a spectrophotometer (NanoDrop LITE, Thermo Fisher Scientific, Bremen, Germany) at 280
145 nm.

146

147 *2.3. Tryptic digest and C18 clean up*

148 About 10 µg soluble proteins prepared from the vaccine samples (see above) were transferred to 10
149 kDa vivacon 500 membrane filters and the flow-through was discarded after centrifugation for 30
150 min at 12,000 x g to remove all salts from the vaccine (e.g. aluminum adjuvant), TCA precipitation
151 and rehydration step. The tryptic digest of the prepared vaccines samples occur within modified
152 Filter Aided Sample Preparation (FASP) protocol [43]. The proteins were reduced by addition of 200
153 µl of reduction buffer (25 mM DTT, 8 M urea, 50 mM triethylammonium bicarbonate buffer (TEAB))
154 for 30 min at 37 °C. Alkylation of sulfhydryl groups was carried out with 200 µl alkylation buffer (25
155 mM chloroacetamide (CAA), 8 M urea, 50 mM TEAB) for additional 30 min on a shaker at 600 rpm
156 in the dark. The proteins were subsequently washed with 300 µl of 8 M urea in 50 mM TEAB
157 followed by another washing step with 200 µl 6 M urea in 50 mM TEAB. Afterwards 0.5 µg mass
158 spectrometry grade LysC endopeptidase was added onto the filter unit and incubated on a shaker at
159 37 °C and 600 rpm for 3 h, followed by a second digest with 1 µg trypsin and 250 µl dilution buffer
160 (50 mM TEAB) to reach a final concentration of 1 M urea. The sample was incubated over-night at
161 37 °C at 600 rpm on a shaker. Peptides were then collected by centrifugation at 12,000 x g for 20
162 min. For acidification of the peptide solution 20 µl of 10 % trifluoroacetic acid (TFA) was added to

163 reach a final concentration of 0.5 % TFA. To remove all remaining salts a clean-up of the peptides
164 with C18 stage tips were performed. Prior to LC-MS/MS analysis, peptides were vacuum dried and
165 solved in 0.1 % trifluoroacetic acid (TFA) [44].

166

167 *2.4. Mass spectrometry*

168 Mass spectrometric analyses were carried out as described [44,45] and resulting raw data files were
169 analysed using the *C. diphtheriae* ATCC 700971/NCTC 13129/Biotype gravis database (Proteome
170 Id: UP000002198) and the Proteome Discoverer 1.4 program package (Thermo Fisher Scientific,
171 Bremen, Germany). As described by Schäfer and co-workers [46] theoretical masses for peptides
172 were generated by trypsin digestion with a maximum of 2 missed cleavages. Product ions were
173 compared to the measured spectra using the following parameters: carbamidomethyl modification
174 on cysteine was set as fixed and oxidation of methionine as dynamic modification. Mass tolerance
175 was set to 10 ppm for survey scans and 0.6 Da for fragment mass measurements. For protein
176 identification the thresholds were set on 1 % false discovery rate (FDR). For each vaccine, between
177 two and six mass spectrometry runs were carried out (24 in total).

178

179 *2.5. Label-free quantitative protein analysis*

180 For protein quantification of vaccines, three single vaccine doses from the Russian vaccine were
181 prepared and analyzed by mass spectrometry using approximately 250 ng of each sample. The
182 peak area which correlates with the concentration of peptides was used to determine the quantity of
183 proteins present in the vaccines [47-49]. Only peaks ranged from 2×10^7 up to 10^{11} were used for
184 quantification as described previously [50].

185

186 *2.6. Proteome prediction and prevalence of the proteins among other C. diphtheriae strains*

187 Cellular localization and the characteristics of *C. diphtheriae* proteins identified in different samples
188 of vaccines were extracted from the previously available data [51]. The prevalence of these proteins
189 among diverse *C. diphtheriae* strains was inferred by searching the pan-genome of 117 strains [52].

190

191 *2.7. Prediction of putative immunogenic proteins*

192 To analyze the role of proteins present in vaccines as putative antigens the VaxiJen database
193 (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) was used with a threshold of 0.4 [53].

194

195 *2.8. Data availability statement*

196 The mass spectrometry proteomics data of 24 runs carried out have been deposited to the
197 ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner
198 repository [54]. Data are available via ProteomeXchange with identifier PXD009289 (Reviewer
199 account details: username reviewer37203@ebi.ac.uk; password 5Q1HhAzo).

200

201 *2.9. Growth conditions*

202 *C. diphtheriae* and *C. ulcerans* strains used in this study (Table 2) were grown in Heart Infusion (HI,
203 Oxoid, Wesel, Germany) at 37 °C under shaking at 125 rpm in baffled flasks. Growth was monitored
204 by measuring the optical density at 600 nm (OD₆₀₀). For preparation of protein extracts, bacteria
205 were inoculated to an OD₆₀₀ of 0.1 and grown to an OD₆₀₀ between 0.4 and 0.6, which was reached
206 after about 4 h. For toxin production 2-2'-bipyridyl was added at a final concentration of 0.5 mM
207 during exponential phase and bacteria were incubated for further 2 h under iron starvation
208 conditions [55].

209

210 *2.10. SDS-PAGE and Western blotting*

211 For SDS-PAGE of proteins in the Td-pur vaccine, proteins were precipitated, rehydrated (see
212 section 2.2 for buffers) and incubated at 95 °C for different time intervals. 125 µg of proteins were
213 loaded per well and separated according to their apparent molecular mass using Tricine-buffered
214 12.5 % (w/v) polyacrylamide gels [56]. After gel electrophoresis proteins were visualized by silver-
215 staining [57].

216 For SDS-PAGE of cell extracts of the different *C. diphtheriae* and *C. ulcerans* strains studied,
217 bacteria were harvested by centrifugation, resuspended in PBS (0.137 M NaCl, 2.7 mM KCl, 10 mM
218 Na₂HPO₄, 2mM KH₂PO₂, pH 7.4) to identical OD₆₀₀ equivalents (OD₆₀₀ of 0.8) and incubated at 95 °C
219 for 20 min. Protein concentrations of these crude cell extracts were determined using a standard
220 Bradford assay [58]. Equal amounts (approximately 2 µg protein per slot) of protein extracts from the
221 bacteria were loaded onto Tricine-buffered 12.5 % (w/v) polyacrylamide gels [56]. After separation
222 by SDS-PAGE, proteins were transferred onto PVDF membranes using a Bio-Rad semi-dry
223 apparatus for 1 h at 0.8 mA/cm². The membranes were washed for 15 min in TBS-T (19.8 mM Tris,
224 150 mM NaCl, pH 7.6, 0.1 % Tween 20) and subsequently blocked in blocking solution (5 % non-fat
225 dry milk in TBS-T) for 1 h at 4 °C. Human serum from three different donors (K002, K003 and K009)
226 and diphtheria antitoxin (DAT) (Microgen, Russia) was used as primary antibody (1:1,000 dilution)
227 and incubated overnight at 4 °C in blocking solution. After three washing steps in TBS-T, the
228 secondary antibody (anti human IgG (Fc-specific) alkaline phosphatase antibody, Sigma-Aldrich,
229 Germany or anti-horse IgG (Fc-specific) alkaline phosphatase antibody, Sigma-Aldrich, Germany)
230 was incubated in a 1:15,000 dilution in blocking solution for 1 h at 4 °C followed by three washing
231 steps. Detection of immune-reactive bands was performed with 5-bromo-4-chloro-3-indolyl-
232 phosphate/nitro blue tetrazolium (BCIP/NBT) color development substrate according to the
233 manufacturer's protocol.

234

235 **3. Results**

236 *3.1. Reversibility of formaldehyde cross-linking*

237 Diphtheria toxoid vaccines are among the safest vaccines known. During production, the potentially
238 fatal diphtheria toxin is inactivated by extensive cross-linking with formaldehyde, carried out at 37 °C
239 for several days. While short-term formaldehyde cross-linking is reversible, the long treatment in the
240 vaccine production processes was described to be irreversible. Consequently, it was reported that
241 mass spectrometric analyses of such vaccines are highly problematic or even impossible [40,59]. In
242 this study, we developed a protocol to resolve cross-linking of toxoid preparations from different

243 sources. When subjected to SDS-PAGE, untreated samples precipitated almost completely in the
244 wells of the gel and did not enter the polyacrylamide matrix due to extensive cross-linking. Heat
245 treatment of these samples for increasing time intervals resulted in the appearance of protein bands
246 with apparent molecular masses between 10 and 70 kDa besides more faint bands in the higher
247 molecular mass range. The most prominent bands with an apparent molecular mass of
248 approximately 65, 55 and 45 kDa may represent subunits of diphtheria and tetanus toxin (i.e. the 62
249 kDa diphtheria toxin with a 22 kDa A and 40 kDa B subunit and the 150 kDa tetanus toxin with a 50
250 kDa A and 100 kDa B subunit), while the identity of the other bands was unclear (Fig. 1). Therefore,
251 we started mass spectrometry analyses to identify the proteins present in vaccine preparations with
252 emphasis on *C. diphtheriae*.

253

254 3.2. Mass spectrometric analysis of toxoid vaccines

255 For mass spectrometry analyses, heat-treated vaccine preparations were precipitated by TCA,
256 purified and subsequently subjected to mass spectrometry. Samples were analyzed in respect to the
257 total number of proteins present in the all vaccines and producer-specific variations. In the six
258 vaccines analyzed, 665 different proteins from *C. diphtheriae* were identified. While the vaccine from
259 Brazil contained 130 distinct proteins, in the vaccine from Germany 205 different proteins were
260 found, 324 proteins were detected in the Bulgarian vaccine as well as 344 and 363 proteins,
261 respectively, in the two different Russian vaccines analyzed. With 436 different proteins the highest
262 number was detected in the vaccine from India (Fig. 2).

263 Predictions of protein localization showed a cytoplasmic localization for 456 (69 %) proteins,
264 93 (14 %) proteins were predicted to be secreted, 90 (13 %) membrane-localized and 26 (4 %)
265 proteins showed an ambiguous localization. Forty-one percent of the secreted proteins are
266 lipoproteins including 4 % with a twin-arginine domain, 28 % non-classical secreted proteins, 28 %
267 proteins with Spl signal peptide, 1 % Tat-secreted proteins without signal peptide, 1 % Tat-
268 dependent proteins with transmembrane domain and 1 % Esx substrate proteins. Twelve percent of

269 the membrane-associated proteins were also detected with a signal peptide, 7 % with a LPXTG
270 domain and 1 % were YidC-like proteins.

271 Further bioinformatics analyses revealed that out of the 665 distinct proteins detected in the
272 different vaccines, 456 proteins were described before as a set of proteins conserved among 117 *C.*
273 *diphtheriae* isolates, which was defined as core proteome of these strains [60]. These included 336
274 cytoplasmic proteins, 57 secreted proteins, 54 membrane proteins and 9 proteins with ambiguous
275 localization. 65 proteins were present in all six vaccines (Fig. 2a, Table 3). Among these an
276 enrichment of secreted and membrane proteins was observed compared to the overall identified
277 proteins (43 % versus 27.5 %) (Fig. 2 b). From the set of 65 proteins detected in all six vaccines 24
278 proteins were identified in a previously carried out secretome study [39]. Recently, in an *in silico*
279 analysis by Hassan and co-workers [61] ten of the 65 proteins were found as conserved target
280 proteins and four (DIP1902, DIP1303, DIP0470, DIP0281) of them as global drug targets of the
281 conserved proteome form *C. diphtheriae* (Table 3) [61]. In addition, the DIP1680 protein was found
282 in an *in silico* approach for the identification of therapeutic targets and putative virulence factor by
283 Jamal and co-workers [62].

284 Besides their presence, also the relative amounts of *C. diphtheriae* proteins were analyzed
285 for the Russian vaccine. As estimated by label-free protein determination, the peak-area of
286 diphtheria toxin (DIP0222) represents up to 78.7 ± 7.6 % of the total proteins from *C. diphtheriae*.
287 The next prominent single proteins were glutamate dehydrogenase (DIP1547) with $1.1 \% \pm 0.5$ %
288 and catalase (DIP0281) with $0.8 \% \pm 0.1$ %, while all other proteins accumulated to $19.4 \% \pm 7.2$ %,
289 (Fig. 3).

290

291 3.3. Prediction of putative immunogenic proteins

292 Due to the fact, that formaldehyde cross-linking for toxin detoxification is not selective for diphtheria
293 toxin, each of the proteins present in the culture supernatant is able to react with each other to build
294 protein complexes. These proteins identified besides the diphtheria toxin in all vaccine samples (see
295 above) may function as additional antigens during vaccination. Of special interest in this respect

296 were proteins of *C. diphtheriae*, which may have direct contact with the host immune system.
297 Therefore, all secreted or cell surface-exposed proteins, which were identified in this study in all
298 vaccines and were found to be conserved among more than hundred *C. diphtheriae* strains recently
299 [60], were analyzed in respect to their putative immunogenicity. In fact, 15 out of 16 proteins were
300 indicated as possible antigens when the VaxiJen database was used to predict immunogenicity of
301 these proteins.

302

303 3.4. Immuno-reaction of diphtheria antitoxin

304 Although the diphtheria toxin is obviously the main component of diphtheria toxoid preparations, also
305 the additionally observed proteins may be immunogenic as predicted by the bioinformatics analyses
306 presented above. As a first approach to address this idea, commercially available diphtheria
307 antitoxin (DAT) was tested in Western blotting experiments. The diphtheria antitoxin used was
308 produced by immunization of horses with toxoid preparations for application in diphtheria cases to
309 scavenge the diphtheria toxin from the patients' blood stream.

310 The immune reaction of DAT against a selection of three toxigenic and seven non-toxigenic
311 *C. diphtheriae* isolates with different strain background and from different countries was tested
312 (Table 2). Cells were grown in absence and presence of bipyridyl, a chelator of metal ions, which
313 induces iron starvation and leads consequently to the induction of toxin synthesis, since transcription
314 of the *tox* gene is regulated by the iron-dependent transcriptional regulator DtxR.

315 In response to bipyridyl addition, a band with an apparent molecular mass of 62 kDa was
316 observed for toxigenic strains, which was absent without starvation and also not found in cell
317 extracts of non-toxigenic strains. The corresponding protein for the toxigenic strain NCTC 13129
318 was only poorly visible, indicating a low toxin production (Fig. 4). In addition to the bands
319 corresponding to diphtheria toxin, immune reactions of DAT with different proteins in toxigenic and
320 non-toxigenic strains were observed. One protein with an apparent molecular mass marginally
321 higher than the toxin was detected in all strains and under iron surplus and iron starvation.
322 Furthermore, a number of bands corresponding to polypeptides with an apparent molecular mass

323 between 25 and 65 kDa were observed, supporting the idea that toxoid preparations induce
324 antibodies directed other proteins besides the inactivated diphtheria toxin.

325

326 *3.5. Antibody response to corynebacterial proteins of humans vaccinated with diphtheria toxoid*

327 To determine the immune reaction of humans after vaccination with diphtheria toxoid vaccines,
328 Western Blot analyses with human sera were carried out. The adult human donors of these blood
329 samples had undergone a hematopoietic stem cell transplantation. As part of therapy, the patients'
330 immune system was depleted before transplantation and had later to be rebuilt by the transplanted
331 hematopoietic stem cells. Consequently, freshly vaccinated donors six month after stem cell
332 transplantation were expected to show a negligible immune response, while one year after the first
333 vaccination and carried out consecutive booster vaccinations, antibodies should be generated. The
334 use of sera from these patients avoids the problem that healthy adult individuals vaccinated during
335 childhood may have responded against environmental corynebacteria during their lifetime and show
336 cross-reactions due to this contact. Any observed immunoreactivity therefore would not necessarily
337 be directed against the proteins in the vaccine in this case.

338 When sera from three individuals taken six months after transplantation but before booster
339 vaccination were tested, only minor immune reactions were observed (Fig. 5a). In contrast, when
340 sera one year after primary vaccination and consecutive booster vaccinations were applied, a strong
341 immune reaction against a protein with apparent molecular mass of 62 kDa was observed for cell
342 extracts of all toxigenic strains, especially when iron starvation was induced (Fig. 5b, panels 2, 4 and
343 10). Besides this main reaction against the diphtheria toxin, additionally bands corresponding to
344 proteins of an apparent molecular mass between 25 and 65 kDa were observed. In principle, these
345 might be truncated forms of the toxin; however, additional bands were also found for cell extracts of
346 four of the seven non-toxigenic isolates tested (Fig. 5b, panels 1, 3, 5 and 6). Obviously, the sera of
347 the vaccinated adults tested contained antibodies directed against other proteins than the toxin. The
348 number of these immune-reactive proteins correlated with the evolutionary distance of strains. Non-

349 toxigenic *C. diphtheriae* strains, which are more closely related to PW8 production strains, showed
350 more bands in Western blots compared to more distantly related isolates (Fig. S1).

351 To further support the idea that the PW8-derived toxoid vaccines induce antibodies directed
352 against additional proteins only in related *C. diphtheriae* strains, we also tested three toxigenic and
353 two non-toxigenic *C. ulcerans* isolates in Western blot experiments. In fact, when grown without
354 bipyridyl, none of the strains showed an antibody reaction with the three human sera tested. In
355 contrast, in case of the three toxigenic isolates KL756, KL758 and KL785, a clear band attributed to
356 the toxin was observed, in extracts from iron-starved cells. Obviously, the diphtheria toxoid vaccine
357 is also active against *C. ulcerans* diphtheria toxin while immunization does not induce significant
358 levels of antibodies directed against other *C. ulcerans* proteins (Fig. 6).

359

360 **4. Discussion**

361 The development of diphtheria toxoid vaccine is without any doubt a milestone in history of medicine
362 and mankind. Its introduction saved the life of millions of children; however, especially the large
363 scale outbreak starting 1990 in the Russian Federation and Ukraine [14-16] demonstrated that
364 diphtheria is not defeated and eradicated and that surveillance and research are still necessary
365 [2,26].

366 In this study, we identified 665 distinct proteins in addition to the diphtheria toxin in six
367 vaccines from different sources around the world. This astonishingly high number corresponding to
368 about one fifth of the proteins encoded in the *C. diphtheriae* genome is the result of physiological
369 protein secretion, shearing of cell surface proteins and cell lysis as indicated by the predicted
370 localization of proteins. Since between 130 and 436 proteins were observed in the vaccine samples
371 from the different manufacturers, cultivation and production process seem to have a major influence
372 on the protein composition. Bioinformatics analyses of the 65 proteins common in all six vaccines
373 studied here revealed that at least 60 proteins are predicted antigens which may induce antibody
374 formation upon vaccination. In addition, Western blot experiments showed that antibodies of
375 vaccinated persons are directed against different *C. diphtheriae* proteins besides the toxin. Putative

376 target proteins identified by mass spectrometry, which may influence the outcome of infection since
377 antibodies against these proteins may result in a decreased fitness of the pathogen, are
378 components of iron uptake systems, proteases, superoxide dismutase and catalase. The latter
379 enzyme, found in all vaccine sample and one of the most abundant proteins in the Russian vaccine,
380 is crucial for detoxification of reactive oxygen species secreted by host cells upon infection. Effects
381 of vaccination on general pathogen fitness were already suggested for *Clostridium botulinum* since
382 in this case administration of toxoid vaccines to cows reduced the number of *C. botulinum* spores in
383 feces significantly [63].

384 Although it was previously suggested that DT vaccines only induce immune responses
385 against the toxin but are not effective against non-toxigenic strains that are increasing isolated from
386 cases of systemic infections [24,32,52,64], our data indicate that also the distribution of *C.*
387 *diphtheriae* strains may be affected by vaccination. The human antisera tested showed a strain-
388 dependent antibody binding and diphtheria toxoid vaccines may consequently not only put toxigenic
389 strains at disadvantage, but also impair non-toxigenic strains, especially in case that these are
390 taxonomically closely related to the PW8 production strains. The phenomenon that vaccination
391 influence population and genetic drift of strains is discussed for *Bordetella pertussis* [65,66]. In this
392 species, vaccination is assumed to affect the evolution of strains based on the observation that the
393 molecular clock rate of mutations correlated with the vaccination coverage [67]. An effect of
394 vaccination on *B. pertussis* strains was reported for the pertactin gene. The *prn1* allele, which is
395 present in the vaccine strain, was predominant in the pre-vaccine era. After introduction of the
396 whole-cellular vaccine, strains carrying the *prn2* allele increased from 39 % (1993-1996) to 90 %
397 (1998-2004) [67, for review see 69]. Moreover, a study from Sweden showed a shift in the serotype
398 of *B. pertussis* populations from serotype Fim2 in unvaccinated populations to serotype Fim3 and
399 serotype Fim2,3 in vaccinated populations [69].

400 In case of *C. diphtheriae*, studies on allelic replacements over time are not practical due to
401 use of the toxoid vaccine for decades; however, the results obtained here may provide a basis for
402 studies on genomic variations and the clonal distribution of toxigenic and non-toxigenic *C.*

403 *diphtheriae* strains. From the amount of cross-reactions observed, the prediction can be made that
404 strains closely related to PW8 should be at disadvantage compared to more distantly-related strains,
405 a hypothesis, which can be tested in future taxonomical investigations.

406

407 **Conflict of interest**

408 The authors declare that the research was conducted in the absence of any commercial or financial
409 relationships that could be construed as a potential conflict of interest.

410

411 **Authors' contributions**

412 Möller: protein preparation, Western blot experiments, data collection/analysis and manuscript
413 preparation.

414 Kraner: mass spectrometric analyses.

415 Sangal: bioinformatic analyses.

416 Tittlbach, Winkler, Winkler: provided and characterized the human sera.

417 Melnikov: involved in study design.

418 Melnikov, Lang, Mattos-Guaraldi: provided the vaccines.

419 Sing: isolation and characterization of *C. ulcerans* strains used in this study.

420 Burkovski: data analysis/ interpretation and manuscript preparation.

421

422 **Acknowledgments**

423 The study was supported by the *Deutsche Forschungsgemeinschaft* in frame of SFB796 (project B6
424 and Z1) and the German Academic Exchange Service (DAAD fellowship to V.M.).

425

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617

618

619 **Tables**

620

621 **Table 1.** Vaccines used for proteome analyses in this study. Sample size indicates the number of
 622 doses pooled and prepared for mass spectrometry analysis.

623

Manufacturer/designation	Active components	Country	Sample size
Biological E (BE), Vacina contra a difteria e tétano	Tetanus toxoid \geq 20 I.U., diphtheria toxoid \geq 2 I.U.	India	3 x 10 doses (0.5 ml per dose), two different lots
Butanan Institute, Vacina adsorvida difteria e tétano adulto (dT)	Tetanus toxoid \geq 25 Lf/ml, diphtheria toxoid \geq 2 Lf/ml	Brazil	10 doses (0.5 ml per dose)
GlaxoSmithKline (GSK), Td-pur	Tetanus toxoid \geq 20 I.U., diphtheria toxoid \geq 2 I.U.	Germany	10 doses (0.5 ml per dose)
InterVax for BB-NCIPD, Diftet Vacuna DT	Tetanus toxoid \geq 20 Lf/ml, diphtheria toxoid \geq 30 Lf/ml	Bulgaria	10 doses (0.5 ml per dose)
Microgen, Diphtheria toxoid adsorbed (AD-M-toxoid)	Diphtheria toxoid \geq 10 Lf/ml	Russia	2 x 10 doses (0.5 ml per dose)
Microgen, Diphtheria-tetanus toxoid adsorbed (ADT-M-toxoid)	Tetanus toxoid \geq 10 EC/ml, diphtheria toxoid \geq 10 Lf/ml	Russia	2 x 10 doses (0.5 ml per dose)

624

625

626 **Table 2.** Bacterial strains used in this study.

627

<i>C. diphtheriae</i> strain	GenBank accession no.	tox gene	Description/source	Reference
ATCC 27010 (NCTC 11397)	GCA_001457455.1	- ¹⁾		[70,71]
ATCC 27012	CP003210	+ ²⁾	Laboratory strain	[72]
DSM 43988	GCA_000455785.1	-	Throat culture	[73]
DSM 43989	LJXS000000000.1	+		[70]
INCA 402	CP003208	-	Pneumonia (cancer patient)	[72]
ISS 3319	JAQO000000000	-	Severe pharyngitis/tonsillitis	[51,74]
ISS 4060	JAQN000000000	-	Severe pharyngitis/tonsillitis	[51]
ISS 4749	JAQQ000000000	-	Severe pharyngitis/tonsillitis	[51]
HC04	CP003215	-	Fatal case of endocarditis	[72]
NCTC 13129	BX248353	+	Diphtheria	[75]
<i>C. ulcerans</i> strain	GenBank accession no.	tox gene	Description/source	Reference
809	CP002790.1	-	Human	[76]
BR-AD22	CP002791.1	-	Dog	[77]
KL 756	-	+	Dog	This study
KL 758	-	+	Human	This study

KL 785	-	+	Human	This study
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628

629 ¹⁾ -: absent, ²⁾ +: present

630

631 **Table 3.** Proteins identified in all analyzed vaccine samples. Annotation and predicted localization as well as localization signals are given
632 for the identified proteins. The number of strains in which the proteins are conserved among 117 *C. diphtheriae* sequences analyzed are
633 indicated as “prevalence” and a previous identification of these proteins in different proteome analyses is shown.
634

Identifier	Localization [51]	Prevalence	Antigen	Annotation	[39]	[62]	[61]
DIP0025	Cyt	117	+	Peptidyl-prolyl cis-trans isomerase	Yes	No	Yes
DIP0108	Sec-Lipo	116	+	Ferrisiderophore receptor Irp6A	Yes	No	Yes
DIP0154	Cyt	117	-	Putative endopeptidase	No	No	No
DIP0169	Tat-Lipo	117	-	Putative secreted protein	No	No	No
DIP0178	Cyt	117	+	Putative phenylalanine aminotransferase	No	No	No
DIP0222	Sec-Spl	49	+	Diphtheria toxin	No	No	No
DIP0225	Sec-Lipo	111	+	Putative secreted polysaccharide deacetylase	Yes	No	No
DIP0257	Cyt	108	+	Uncharacterized protein	No	No	No
DIP0281	Sec-NC	117	+	Catalase	Yes	No	Yes
DIP0350	Sec-Spl	101	+	Putative secreted protease	Yes	No	No
DIP0365	Sec-Spl	117	+	Surface layer protein A	Yes	No	No
DIP0368	Cyt	117	+	Dihydrolipoyl dehydrogenase	No	No	No
DIP0383	Cyt	117	+	Uncharacterized protein	Yes	No	No

DIP0442	TM-Sec	117	+	Putative membrane protein	No	No	No
DIP0469	Cyt	117	+	Elongation factor G	Yes	No	Yes
DIP0470	Cyt	117	+	Elongation factor Tu	No	No	Yes
DIP0483	Amb	116	+	Putative secreted protein	No	No	No
DIP0491	Sec-Spl	116	+	Putative secreted amino acid hydrolase	No	No	No
DIP0515	Tat-Lipo	117	+	Putative transport system secreted protein	No	No	No
DIP0534	Sec-Lipo	117	+	Putative sugar-binding secreted protein	Yes	No	No
DIP0543	TM-Sec	114	+	Putative sialidase	Yes	No	No
DIP0575	Cyt	117	+	10 kDa chaperonine	Yes	No	No
DIP0582	Sec-Lipo	117	+	Putative iron transport system binding (secreted) protein	Yes	No	No
DIP0611	Sec-Lipo	117	+	Putative ABC transport system secreted protein	No	No	No
DIP0615	Sec-Lipo	117	+	ABC transport system exported protein	No	No	No
DIP0631	Cyt	117	+	Isocitrate dehydrogenase [NADP]	No	No	No
DIP0680	Amb	117	+	Uncharacterized protein	Yes	No	No
DIP0775	Sec-Spl	114	+	Uncharacterized protein	Yes	No	No
DIP0856	TM	117	+	Putative serine protease	No	No	No
DIP0917	Cyt	117	+	Enolase	Yes	No	No

DIP0956	Sec-Lipo	117	+	Putative peptide transport system secreted protein	Yes	No	No
DIP1062	Sec-Lipo	117	+	Putative iron siderophore uptake system exported solute-binding component	Yes	No	No
DIP1086	Sec-Lipo	117	+	Putative iron transport system exported solute-binding component	No	No	No
DIP1100	Cyt	117	+	Ketol-acid reductoisomerase (NADP(+))	No	No	No
DIP1204	Cyt	117	+	Oxoglutarate dehydrogenase inhibitor	No	No	No
DIP1229	Cyt	116	+	Putative cobalamin biosynthesis related protein	No	No	No
DIP1303	Cyt	117	+	Transaldolase	No	No	Yes
DIP1308	Cyt	117	+	Triosephosphate isomerase	No	No	No
DIP1309	Cyt	117	-	Phosphoglycerate kinase	Yes	No	Yes
DIP1310	Cyt	117	+	Glyceraldehyde-3-phosphate dehydrogenase	Yes	No	No
DIP1390	Sec-Lipo	115	+	Putative secreted protein	No	No	No
DIP1419	Cyt	117	+	Alkyl hydroperoxide reductase AhpD	Yes	No	No
DIP1420	Cyt	117	+	Iron repressible polypeptide (putative reductase)	Yes	No	No
DIP1482	Cyt	117	+	Proline-tRNA ligase	No	No	No
DIP1505	Cyt	117	+	Ribosome-recycling factor	Yes	No	No

DIP1586	Sec-Lipo	117	+	Putative secreted protein	No	No	No
DIP1636	Cyt	117	+	Branched-chain-amino-acid aminotransferase	No	No	No
DIP1637	Cyt	117	+	Probable cytosol aminopeptidase	No	No	No
DIP1644	Cyt	117	+	Glutamine synthetase	No	No	No
DIP1667	TM	116	+	Putative membrane protein	No	No	No
DIP1680	Cyt	117	+	GTP cyclohydrolase 1 type 2 homolog	No	Yes	No
DIP1783	Cyt	117	+	Nucleoside diphosphate kinase	No	No	Yes
DIP1786	Cyt	117	+	Valine-tRNA ligase	No	No	No
DIP1787	Cyt	117	+	Malate dehydrogenase	No	No	Yes
DIP1902	Cyt	116	+	Succinyl-CoA:coenzyme A transferase	No	No	Yes
DIP2010	TM-Sec-LPXTG	88	+	Putative surface-anchored membrane protein	No	No	No
DIP2120	Cyt	117	+	Chaperone protein DnaK	Yes	No	No
DIP2128	Sec-Lipo	116	+	Putative substrate-binding transport protein	No	No	No
DIP2169	TM	111	+	Putative membrane protein	No	No	No
DIP2180	Cyt	116	-	Phosphoenolpyruvate carboxykinase	No	No	No
DIP2193	Sec- Spl	117	+	Putative secreted protein	Yes	No	No
DIP2261	Cyt	117	+	Superoxide dismutase [Mn]	No	No	No
DIP2290	Sec-NC	117	+	Single-stranded DNA-binding protein	No	No	No

635

DIP2331	Cyt	117	-	Putative aldehyde dehydrogenase	No	No	No
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636 **Figure legends**

637

638 **Figure 1.** Influence of heat-treatment on patterns of vaccine proteins from Td-pur (GSK). Proteins
639 were separated by a 12.5 % (w/v) Tricine-buffered SDS-PAGE. The proteins were precipitated by
640 TCA extraction and resuspended in 50 µl rehydration buffer. Approximately 125 µg of protein was
641 loaded per lane. The separated proteins were silver-stained. Lane 1: protein marker, lane 2: without
642 boiling, lane 3: 95 °C for 5 min with loading dye, lane 4: 95 °C for 20 min in addition to 95 °C for 5
643 min with loading dye.

644

645 **Figure 2.** Bioinformatics analysis of proteins identified in diphtheria toxoid vaccines. (a) Venn
646 diagram [78] of identified proteins in the different samples. Country of origin is given (Russia 1:
647 Diphtheria Toxoid, Russia 2: Diphtheria-Tetanus Toxoid). (b) Predicted localization of 65 proteins
648 present in all vaccines studied. Proteins located in the cytoplasm are shown in medium grey,
649 secreted proteins in dark grey, membrane proteins in light grey and proteins with ambiguous
650 localization in black.

651

652 **Figure 3.** Quantitative protein analysis. 250 ng of prepared vaccine samples were analyzed by mass
653 spectrometry. The relative protein amount of diphtheria toxin (DT), the two most prominent proteins
654 beside DT and of the remaining proteins was calculated. Only proteins in a range from 2×10^7 to
655 10^{11} were considered for calculation.

656

657 **Figure 4.** Western blots of cell extracts from toxigenic and non-toxigenic *C. diphtheriae* strains.
658 Bacteria were grown without (-) and with (+) bipyridyl, which induces iron starvation. 2 µg of protein
659 extract were added per lane. Diphtheria antitoxin produced in horses for therapy was used as
660 primary antibody. 1: ATCC 27010 (tox⁻), 2: ATCC 27012 (tox⁺), 3: DSM 43988 (tox⁻), 4: DSM 43989
661 (tox⁺), 5: INCA 402 (tox⁻), 6: ISS 3319 (tox⁻), 7: ISS 4060 (tox⁻),

662 8: ISS 4749 (tox⁻), 9: HC04 (tox⁻), 10: NCTC 13129 (tox⁺). The arrow indicates the diphtheria toxin
663 with an apparent molecular mass of 62 kDa, the asterisk a protein with slightly higher apparent
664 molecular mass present in all cell lysates.

665

666 **Figure 5.** Western blots of *C. diphtheriae* cell extracts. Toxigenic and non-toxigenic strains were
667 grown without (-) and with (+) bipyridyl. 2 µg of protein extract were added per lane. Serum from
668 three different donors (K002, K003 and K009), six months after hematopoietic stem cell
669 transplantation (first serum) (Fig. 5a) and 1 year after primary and two booster vaccinations (second
670 serum) (Fig. 5b) was used as primary antibody. 1: ATCC 27010 (tox⁻), 2: ATCC 27012 (tox⁺), 3:
671 DSM 43988 (tox⁻), 4: DSM 43989 (tox⁺), 5: INCA 402 (tox⁻), 6: ISS 3319 (tox⁻), 7: ISS 4060 (tox⁻), 8:
672 ISS 4749 (tox⁻), 9: HC04 (tox⁻), 10: NCTC 13129 (tox⁺). Diphtheria toxin is indicated by an arrow.

673

674 **Figure 6.** Western blots of *C. ulcerans* cell extracts. Proteins from strains grown without (-) and with
675 bipyridyl (+) were separated by SDS-PAGE. Human serum from three individual donors (a: K002, b:
676 K003 and c: K009), collected 1 year after primary and two booster vaccinations (second serum) was
677 used as primary antibody. 1: 809 (tox⁻), 2: BR-AD22 (tox⁻), 3: KL 756 (tox⁺), 4: KL 758 (tox⁺) and 5:
678 KL 785 (tox⁺). Diphtheria toxin is indicated by an arrow.

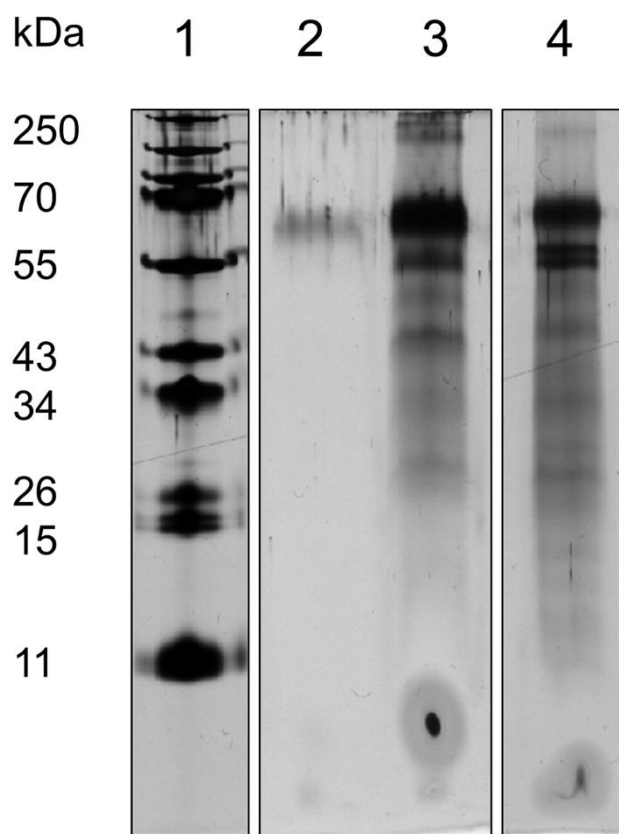
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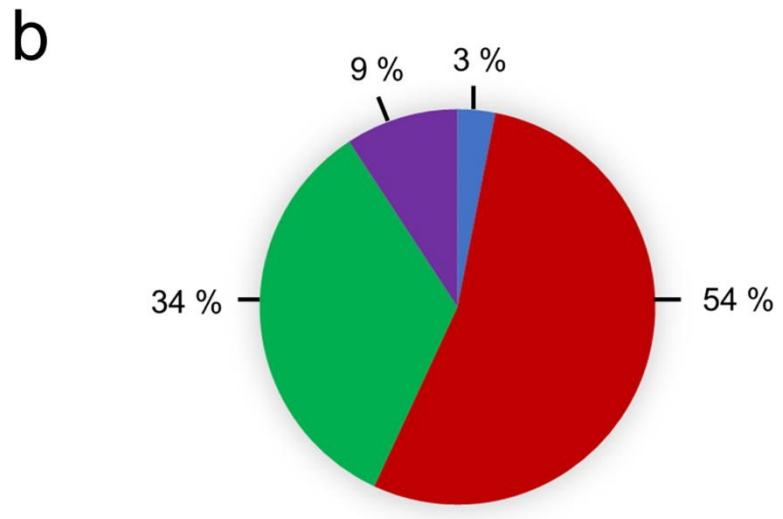
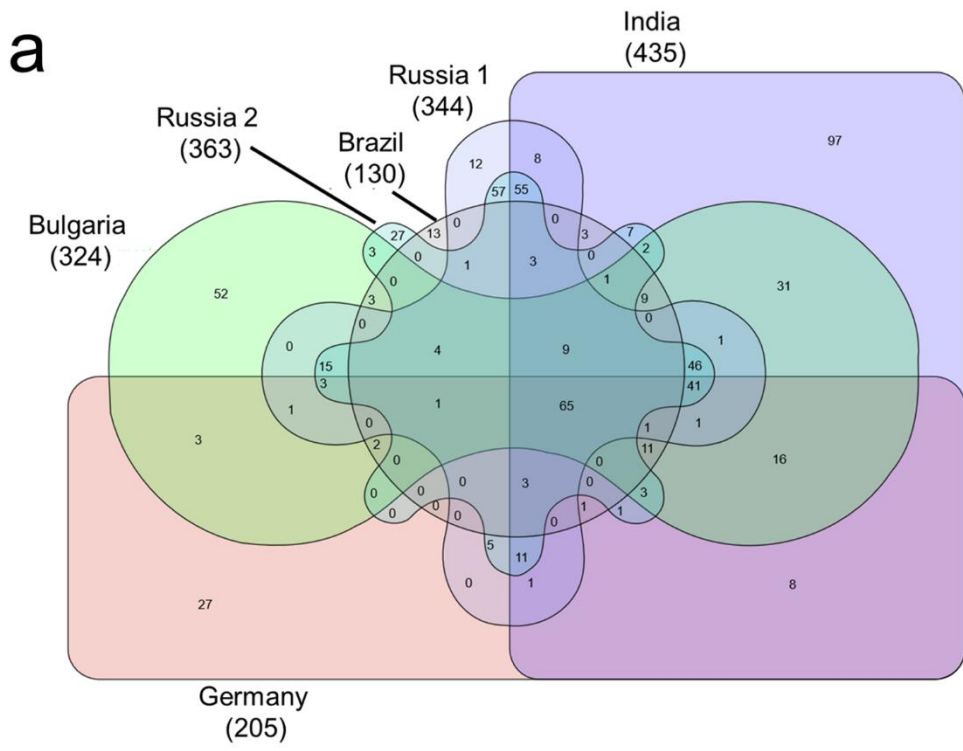
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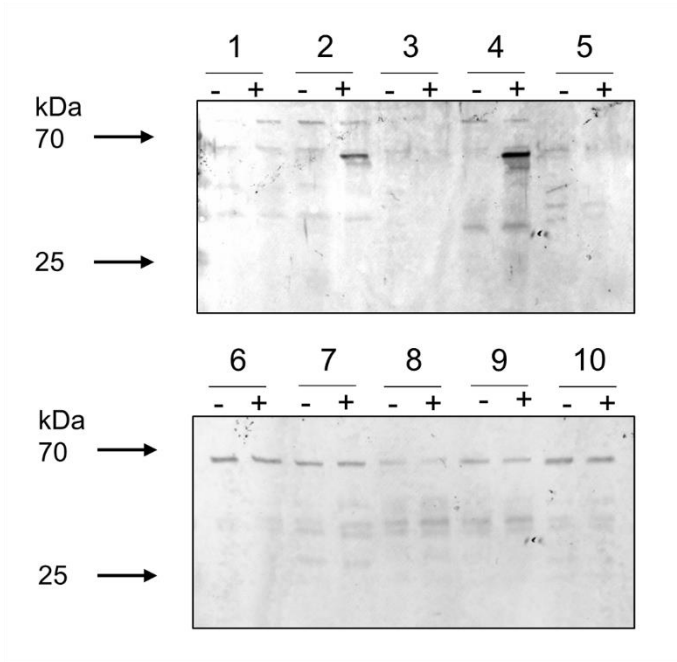
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685 **Figure 1**



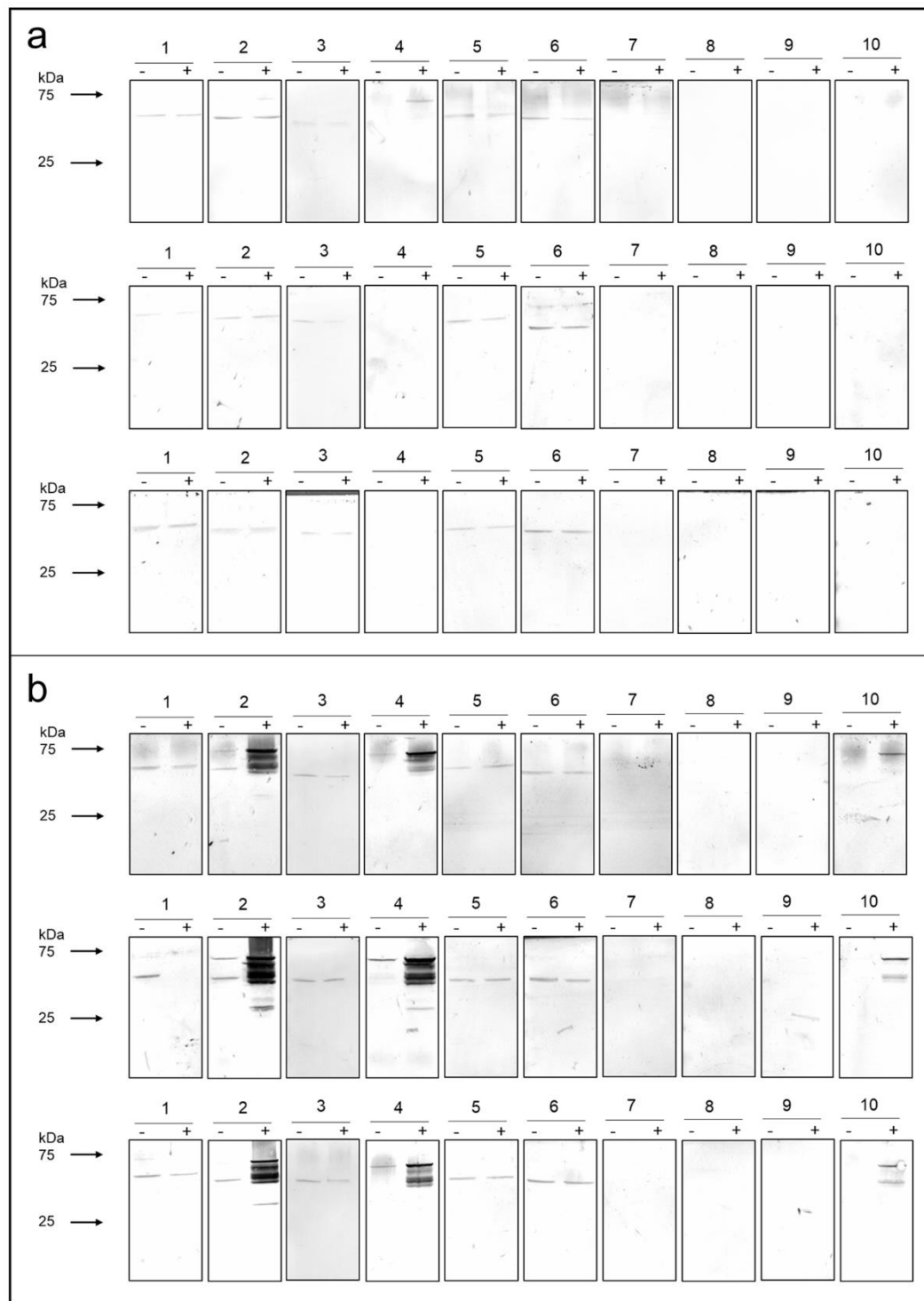
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687 **Figure 2**



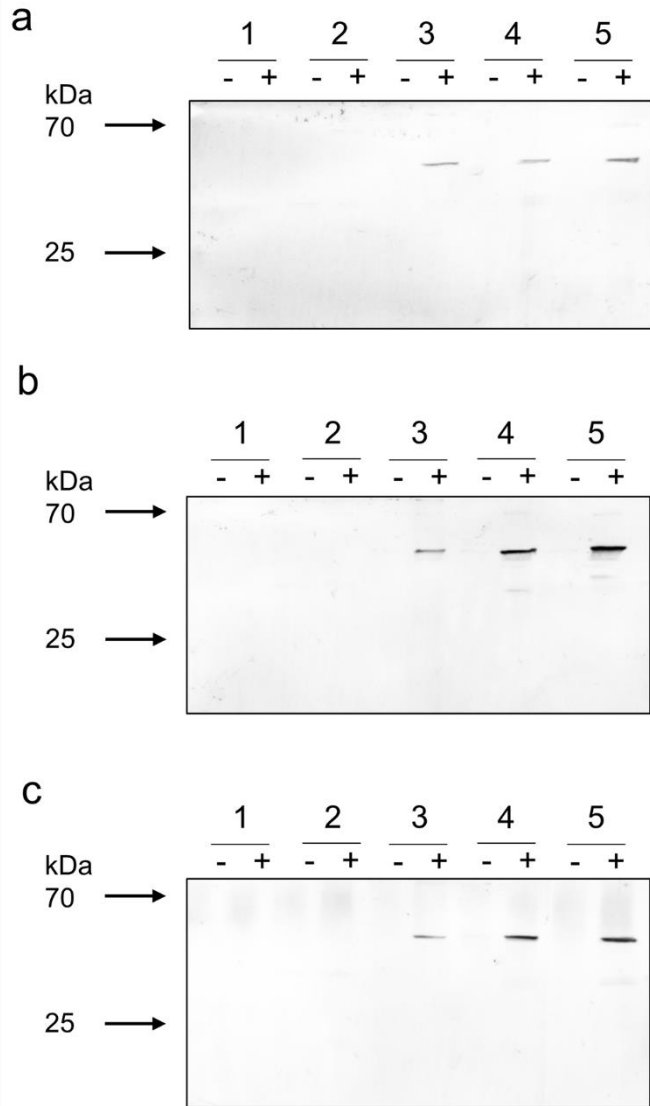
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689 **Figure 3**



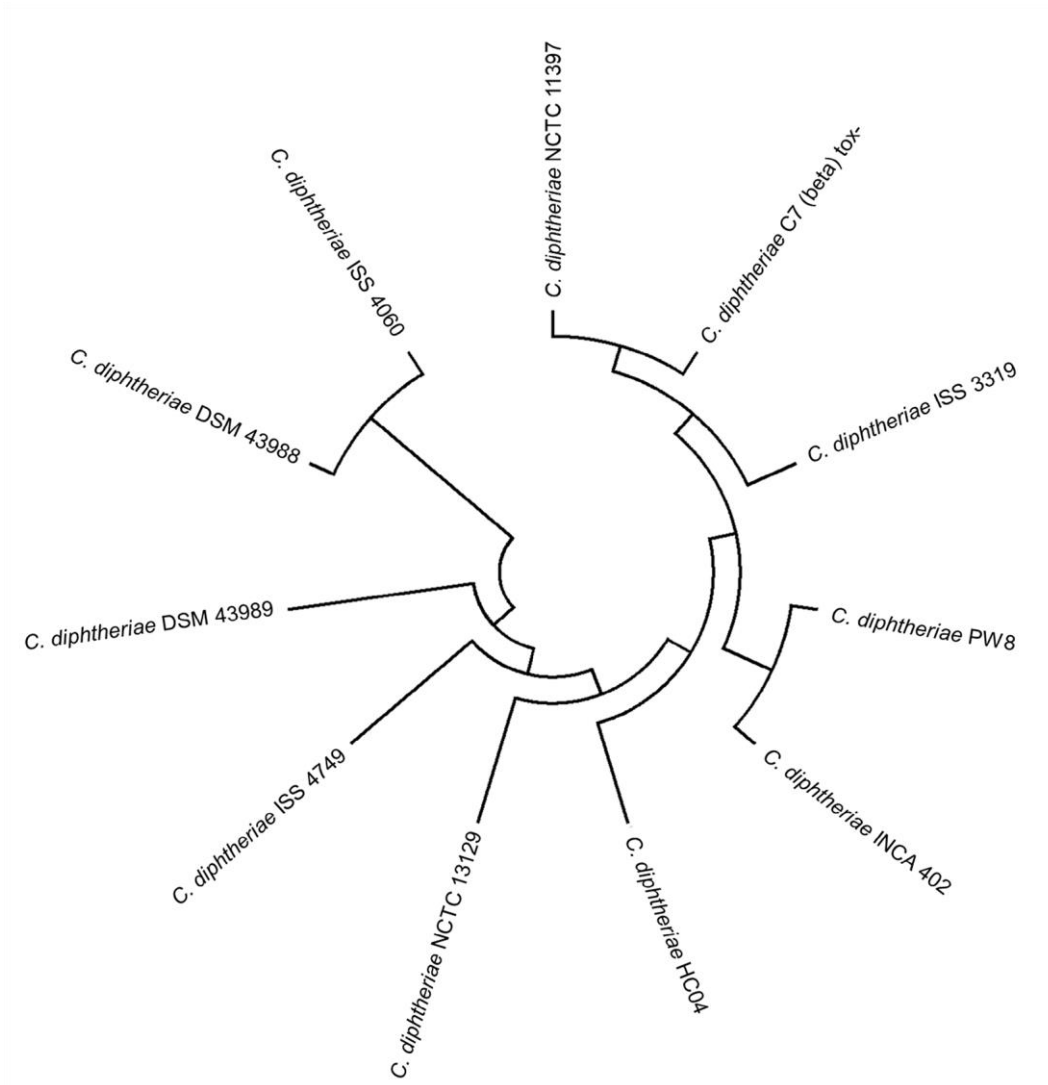
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691 **Figure 4**



692

693 **Figure 5**



694

695 **Figure 6**