

ORIGINAL ARTICLE

A DNA microarray facilitates the diagnosis of *Bacillus anthracis* in environmental samplesK.M. Felder¹, K. Hoelzle¹, M.M. Wittenbrink¹, M. Zeder², R. Ehricht³ and L.E. Hoelzle¹¹ Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland² Department of Limnology, Institute of Plant Biology, University of Zurich, Zurich, Switzerland³ Clondiag Chip Technologies GmbH, Jena, Germany**Keywords**ArrayTube, *Bacillus anthracis*, biotinylation, DNA microarray, environmental samples.**Correspondence**

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Abstract**Aims:** In order to improve the diagnosis of *Bacillus anthracis* in environmental samples, we established a DNA microarray based on the ArrayTube technology of Clondiag.**Methods and Results:** Total DNA of a bacterial colony is randomly biotinylated and hybridized to the array. The probes on the array target the virulence genes, the genomic marker gene *rpoB*, as well as the selective 16S rDNA sequence regions of *B. anthracis*, of the *Bacillus cereus* group and of *Bacillus subtilis*. Eight *B. anthracis* reference strains were tested and correctly identified. Among the analysed environmental *Bacillus* isolates, no virulent *B. anthracis* strain was detected.**Conclusions:** This array clearly differentiates *B. anthracis* from members of the *B. cereus* group and other *Bacillus* species in environmental samples by chromosomal (*rpoB*) and plasmid markers. Additionally, recognition of *B. cereus* strains harbouring the toxin genes or atypical *B. anthracis* strains that have lost the virulence plasmids is feasible.**Significance and Impact of the Study:** The array is applicable to the complex diagnostics for *B. anthracis* detection in environmental samples. Because of low costs, high security and easy handling, the microarray is applicable to routine diagnostics.**Introduction**

The rapid identification of *Bacillus anthracis* in complex substrates such as dust and powder samples is a challenging diagnostic task and important for routine laboratories. *Bacillus anthracis* is difficult to differentiate phenotypically from members of the *Bacillus cereus* group by culture methods (Turnbull 1999). Further, *B. anthracis* has a nearly identical 16S rDNA sequence to *B. cereus*. Therefore, a molecular identification of *B. anthracis* can be achieved by PCRs targeting the plasmid-encoded virulence genes and specific chromosomal DNA sequences such as Ba813, *virA* and *rpoB* (Andersen *et al.* 1996; Jackson *et al.* 1997; Ramišse *et al.* 1999; Qi *et al.* 2001; Patra *et al.* 2002). This requires time-consuming tests susceptible to cross-contamination. Recently, a variety of

molecular assays, e.g. real-time PCR, multiplex PCR and a combination of multiplex PCR with a DNA microarray, were developed to improve the diagnostics (Ellerbrok *et al.* 2002; Wang *et al.* 2004; Bavykin *et al.* 2008).

The objective of the study was to develop a DNA microarray to differentiate *B. anthracis* from environmental *Bacillus* isolates obtained from samples released as 'white powders' with regard to potential bioterrorism threats. Aforementioned microarrays are based on fluorescence-labelled PCR products. As the labelling reagents are expensive and the development of the techniques is time consuming, such systems are not ideal for routine use in diagnostic laboratories. In this study, the ArrayTube™ (AT) platform (Clondiag, Jena, Germany) and biotinylated whole bacterial DNA were used to diagnose *B. anthracis*. This platform is rapid and uses inexpensive reagents.

Table 1 Strains used in this study. Characterization was performed by PCRs of the virulence genes, 16S gene sequencing and hybridizations to the array

Reference strains or isolates respectively	Accession number	<i>rpoB</i>	<i>pag</i>	<i>lef</i>	<i>cya</i>	<i>capAC</i>	<i>capB</i>	Highest homology with the 16S rRNA gene of	Sequence identity in percent	Result of ArrayTube hybridization	Phenotypic group
<i>Bacillus anthracis</i> A15*	-	+	+	+	+	-	-	<i>B. anthracis</i>	99.9	<i>B. anthracis</i> ; toxin genes	ND
								<i>Bacillus cereus</i>	99.9		
<i>B. anthracis</i> A73*	-	+	-	-	+	+	+	<i>B. anthracis</i>	99.3	<i>B. anthracis</i>; capsule genes	ND
								<i>B. cereus</i>	99.3		
<i>B. anthracis</i> 12/07/60	-	+	+	+	+	+	+	<i>B. anthracis</i>	99.7	<i>B. anthracis</i> ; all virulence genes	ND
								<i>B. cereus</i>	99.7		
<i>B. anthracis</i> 07/11/62	-	+	+	+	+	+	+	<i>B. anthracis</i>	99.9	<i>B. anthracis</i>; all virulence genes	ND
								<i>B. cereus</i>	99.9		
<i>B. anthracis</i> 09/11/61	-	+	+	+	+	+	+	<i>B. anthracis</i>	99.7	<i>B. anthracis</i> ; all virulence genes	ND
								<i>B. cereus</i>	99.7		
<i>B. anthracis</i> TV 06/81	-	+	+	+	+	+	+	<i>B. anthracis</i>	99.8	<i>B. anthracis</i> ; all virulence genes	ND
								<i>B. cereus</i>	99.8		
<i>B. anthracis</i> Sterne†	GenBank:NC005945	+	+	+	+	-	-	<i>B. cereus</i>	99.2	<i>B. anthracis</i>; toxin genes	ND
<i>B. anthracis</i> A58†	-	+	-	-	-	-	-	<i>B. cereus</i>	99.3	<i>B. anthracis</i>; no virulence genes	ND
<i>B. cereus</i> ATCC 14579	GenBank:NC004722	+	-	-	-	-	-	<i>B. cereus</i> ATCC 14579	99.7	<i>B. cereus</i> group	ND
<i>Bacillus mycoides</i> ATCC 6462	GenBank:AF155956	-	-	-	-	-	-	<i>B. anthracis</i> Ames	99.0	<i>B. cereus</i> group	ND
<i>Bacillus thuringiensis</i> ATCC 10792	GenBank:AF290545	-	-	-	-	-	-	<i>B. cereus</i>	99.3	<i>B. cereus</i> group	ND
								<i>B. thuringiensis</i>	99.1		
<i>Bacillus subtilis</i> ATCC 6051	GenBank:AB042061	-	-	-	-	-	-	<i>B. subtilis</i>	99.4	<i>B. subtilis</i>	ND
M63/3	-	-	-	-	-	-	-	<i>B. subtilis</i>	97.2	<i>B. subtilis</i>	2
M63/4_2	-	-	-	-	-	-	-	<i>B. subtilis</i>	99.6	<i>B. subtilis</i>	3
M65/1_1	-	-	-	-	-	-	-	<i>Bacillus licheniformis</i>	88.9	NP	5
M71/1_4	-	-	-	-	-	-	-	<i>B. subtilis</i>	96.9	<i>B. subtilis</i>	4
M96/2	-	-	-	-	-	-	-	<i>B. licheniformis</i>	97.7	NP	7
M100	-	+	-	-	-	-	-	<i>B. anthracis</i> Ames	99.1	<i>B. cereus</i> group	5
M101/1	-	+	-	-	-	-	-	<i>B. cereus</i>	98.1	<i>B. anthracis</i> ; no virulence genes	6
M125/1_1	-	-	-	-	-	-	-	<i>Bacillus clausii</i>	61.9	genus <i>Bacillus</i>	4
M125/2	-	-	-	-	-	-	-	<i>B. subtilis</i>	100.0	<i>B. subtilis</i>	7
M131/1_2	-	-	-	-	-	-	-	<i>B. clausii</i>	99.3	NP	2
M134	-	-	-	-	-	-	-	<i>Bacillus pumilus</i>	98.8	<i>B. subtilis</i>	1
M135/1_4	-	-	-	-	-	-	-	<i>B. clausii</i>	95.4	NP	1
M141/11	-	+	-	-	-	-	-	<i>B. cereus</i>, <i>B. thuringiensis</i>, <i>B. anthracis</i>	96.0	<i>B. anthracis</i>; no virulence genes	6

rpoB, gene encoding the subunit β of the RNA polymerase; *pag*, *lef* and *cya*, genes encoding the toxin components protective antigen, lethal factor and oedema factor respectively; *capAC* and *capB*, genes encoding the capsule proteins; +, a PCR product could be detected; -, no PCR product could be detected; NP, strain assignment by hybridization signals not predictable; ND, not done; Bold, hybridizations of these strains are shown in Fig. 2b-o.

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Table 2 Oligonucleotides and annealing temperatures

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	T, °C	References
16S rDNA	CAGAGTTTGATCCTGGCTCAG	TACGG(CT)TACCTTGTTACGACTT	58	Lane (1991)
<i>rpoB</i>	TTGAAATTTATGAGCGTCTA	TAAGATTGTTCTTCTGTC	45	GenBank:NC_003997 forw: 109091–109111, rev: 109303–109320
<i>pag</i>	TCCTAACACTAACGAAGTCG	GAGGTAGAAGGATATACTGT	55	Beyer <i>et al.</i> (1995)
<i>cya</i>	AGTATTATATCCTTTTCAGTATT	ATTTCAATTCATTATAGGC	50	GenBank:NC_007322 forw: 122641–122663, rev: 124889–124909
<i>lef</i>	ATGTAATTAAGCTCCG	CCTTTATTACTAATCAGCTT	45	GenBank:NC_007322 forw: 150957–150975, rev: 151106–151126
<i>capB</i>	GGCTCAGTGTAACCTCTA	TACTGACGAGGAGCAACC	55	Beyer <i>et al.</i> (1995)
<i>capAC</i>	AATTTGATTTCCAATTTATCATC	ATCAGCCCGTATTATG	55	GenBank:NC_007323 forw: 53991–54013, rev: 55444–55460

Because of the hybridization with whole bacterial DNA instead of PCR products, the cross-contamination risk is minimized. All important genomic and plasmid markers can be analysed in parallel.

Materials and methods

Bacterial strains and growth conditions

Strains used are listed in Table 1. Environmental samples (1 g) were homogenized in 10 ml phosphate buffered saline and pasteurized (65°C, 10 min) to inactivate vegetative cells. Blood-TSA (blood-trypticase-soy-agar; Oxoid, Basel, Switzerland) was used for cultivation. *Bacillus anthracis* strains were handled under biosafety level 3 conditions.

Phenotypic characterization of 'environmental' bacillus strains

All environmental isolates were checked for the absence of *B. anthracis* virulence genes by PCR. Gram-staining characteristics (Romeis 1989), haemolysis, motility and penicillin susceptibility were evaluated.

DNA preparation

An inoculation loop was tipped into a bacterial colony, and the attaching cells were lysed at 56°C for 2 h in 400 µl lysis buffer (0.1 mol l⁻¹ Tris-HCl, pH 8.0, 0.05% Tween 20, 0.24 mg ml⁻¹ proteinase K), denatured at 95°C for 10 min and filtrated [0.2 µm (Acrodisc[®], Syringe filters; Pall Corporation, Ann Arbor, MI, USA); (Perreten *et al.* 2005)]. DNA concentrations were determined spectrophotometrically.

PCR and sequencing

16S rDNA, *rpoB* and *B. anthracis* virulence genes were amplified by PCR. Oligonucleotides and annealing

temperatures are listed in Table 2. PCRs were performed using 100 ng template DNA, the HotStarTaq Master mix (Qiagen, Hombrechtikon, Switzerland) and 0.8 µmol l⁻¹ primers.

Sequences of the 16S rDNA (MWG-Biotech, Ebersberg, Germany) were aligned with databank entries using FASTA and the GCG package software (<http://www.bio.uzh.ch/bioc/>).

DNA array

Twenty-two probes target the plasmid-encoded virulence genes and five probes cover the *rpoB* gene of *B. anthracis*. Fifteen 16S rDNA targeting probes were designed on sites with higher variability between the members of the *B. cereus* group as well as of *Bacillus subtilis* compared with the corresponding *B. anthracis* sequence. Target genes of the microarray were selected from the GenBank database or from published primer/probe sequences (Table 3; Fig. 1). Common features are length between 22 and 30 nt, melting temperature between 67 and 69°C and GC content of 35–45%. Probes were custom-spotted by Clontech onto a glass surface integrated into the AT system. Genetic markers and gene groups are represented by at least two different probes. All probes were spotted twice.

DNA biotinylation and microarray hybridization

Genomic DNA was biotinylated and hybridized to the array as described by Perreten *et al.* (2005). Instead of QMT hybridization buffer, 3× DNA Buffer (Clontech) was used. Prehybridization (100 µl 1× BSA/1× SSPE; 10 mmol l⁻¹ NaHPO₄, 0.18 mol l⁻¹ NaCl, 1 mmol l⁻¹ EDTA, pH 7.4) was performed at 50°C for 15 min.

Results were obtained by reading the microarray in an AT Reader (ATR01; Clontech) at 25°C taking a picture after 40 min. Analysis was performed by the IONCLUST software provided by Clontech. Hybridiza-

Table 3 Oligonucleotide probes spotted on the array

ID	Probe-name	Probe-sequence (5'-3')	Probe-target	Reference	Probe position
1	capA_1	CCAAAACCCAGTTGCCAGTGCTGG	Capsule-biosynthesis protein A	Dang et al. (2001)	—
2	capA_2	CATTTACGTGATAATGGTACTGCAATTC	Capsule-biosynthesis protein A	GenBank:NC_007323	54 166–54 139
3	capA_3	ACGTGATAATGGTACTGCAATCTTG	Capsule-biosynthesis protein A	GenBank:NC_007323	54 136–54 161
4	capA_4	GGTACTGCAATCTTGATGTTGTACCT	Capsule-biosynthesis protein A	GenBank:NC_007323	54 125–54 151
5	capB_1	GAAGCAGAAGCACTTATTTGTGAATGTA	Capsule-biosynthesis protein B	GenBank:NC_007323	56 453–56 480
6	capB_2	ATATGGATGTTATGGACCTACACTTGA	Capsule-biosynthesis protein B	GenBank:NC_007323	56 338–56 365
7	capB_3	AAGAGGTTGCAGAAGAGAAATACAAA	Capsule-biosynthesis protein B	GenBank:NC_007323	56 227–55 254
8	capB_4	TAGAAGGCTGTCAACAAGTGAAATTTAT	Capsule-biosynthesis protein B	GenBank:NC_007323	55 729–55 756
9	capC_1	CCACGGAATCAAAAATCTCAAAATGGCAT	Capsule-biosynthesis protein C	Ellerbrok et al. (2002)	—
10	capC_2	GGCAACGCTAAATTACAGGTTATTGTT	Capsule-biosynthesis protein C	GenBank:NC_007323	55 326–55 351
11	capC_3	ATCCGTGGTATTGGAGTTATTGTTTC	Capsule-biosynthesis protein C	GenBank:NC_007323	55 248–55 273
12	capC_4	GCAATACAAATTCAAAAGCAAGGGTTAC	Capsule-biosynthesis protein C	GenBank:NC_007323	55 209–55 236
13	cya_1	TAAATGAAATGTAGCTGTGCGCAAG	Adenylate-cyclase (oedema factor)	GenBank:NC_007322	122 376–122 403
14	cya_2	TACTATTTGCTATATCCTCTCACAGGC	Adenylate-cyclase (oedema factor)	GenBank:NC_007322	122 663–122 690
15	cya_3	CACCTGACCATAGAACGGTTATAGAGTT	Adenylate-cyclase (oedema factor)	GenBank:NC_007322	123 356–123 383
16	lef_1	TTGCATATTATTCGAGCCACAGCATCGTG	Zinc-endopeptidase (lethal factor)	Dang et al. (2001)	—
17	lef_2	AATGAGGTACAAGAATTTTGCAAAAG	zinc-endopeptidase (lethal factor)	GenBank:NC_007322	151 030–151 057
18	lef_3	GGTACAAGAATTTTGCAAAAGCTT	Zinc-endopeptidase (lethal factor)	GenBank:NC_007322	151 027–151 052
19	pag_1	AAAGGTTACAGGACGGATTGATAAGAAAT	Protective antigen	GenBank:NC_007322	144 576–144 603
20	pag_2	CTAGTGAAGTACATGGAAATGCAGAAAT	Protective antigen	GenBank:NC_007322	144 764–144 791
21	pag_3	GTCTTTGATATGTTGGGAGTGTATCT	Protective antigen	GenBank:NC_007322	144 801–144 828
22	pag_4	AATTGATCATTCATCTCTAGCAGGG	Protective antigen	GenBank:NC_007322	144 864–144 891
23	rpoB_T	TCCAAAAGCGTATGATTTAGCAAATGT	RNA-polymerase β	Qi et al. (2001)	—
24	rpoB_C	GGTCGCTACAAGATCAACAAGAAAGTTACAC	RNA-polymerase β	Qi et al. (2001)	—
25	rpoB_C1	ACTTGTCTCGTTTCTTGATCCAAAAGCG	RNA-polymerase β	Ellerbrok et al. (2002)	—
26	rpoB_C2	TAGGTCGTACAAGATCAACAAGAAAT	RNA-polymerase β	GenBank:AE_017334	109 190–109 225
27	rpoB_T1	TAGGTCGTATAAAGATCAACAAGAAAT	RNA-polymerase β , mismatch	GenBank:AE_017334	109 190–109 225
28	rrs-low-var	TCGTCAGCTCGTCTGTGAGATGTT	16S rDNA low variability region	GenBank:NC_007530	1045–1069
29	rrs-Banth-high1_2xC	GACATCTCTGCAACCCTAGAGATA	16S rDNA high variability region	GenBank:NC_007530	976–1001
30	rrs-Bthur-high1_AC	GACATCTCTGAAAACCCCTAGAGATA	16S rDNA high variability region	GenBank:AF_290545	986–1011
31	rrs-Bsub-high1_CT	GACATCTCTGACAACTCTAGAGATA	16S rDNA high variability region	GenBank:AB_042061	1002–1027
32	rrs-Banth-high2	AGGGCTTCTCTTCGGGAGCAGAGTG	16S rDNA high variability region	GenBank:NC_007530	1001–1026
33	rrs-Bmyc-high2	AGGGCTTCCCTTCGGGGCAGAGTG	16S rDNA high variability region	GenBank:AF_155956	1018–1043
34	rrs-Bsub-high2	AGGACTCCCTTCGGGGCAGAGTG	16S rDNA high variability region	GenBank:AB_042061	1027–1052
35	BsuR1_22	AGGTTTCGGATCGTAAAGCTC	16S rDNA	GenBank:AB_042061	422–443
36	BsuR2_22	GATCGTAAAGCTCTGTTGTTAG	16S rDNA	GenBank:AB_042061	431–452
37	BsuR3a_22	GAACAAGTACCGTTCGAATAGG	16S rDNA	GenBank:AB_042061	457–478
38	BsuR3b_22	GAACAAGTCCGTTCGAATAGG	16S rDNA	GenBank:AB_042061	457–478
39	BsuR4_22	TTCGAATAGGGCGGTACCTTGA	16S rDNA	GenBank:AB_042061	469–490
40	GneR1_22	AGGCCCTCGGTTGTAAGATAC	16S rDNA	GenBank:NC_000913	416 596–416 626
41	GneR2_22	GGTTGTAAGTACTTTCAGCGGG	16S rDNA	GenBank:NC_000913	224 194–224 215

Table 3 (Continued)

ID	Probe-name	Probe-sequence (5'–3')	Probe-target	Reference	Probe position
42	EcoR3_22	GGAAGGGAGTAAAGTTAATACC	23S rDNA	GenBank: NC_000913	4 034 002–4 034 023
43	EcoR4_22	TAAATACCTTTGCTCATITGACG	23S rDNA	GenBank: NC_000913	4 034 016–4 034 037
44	BanR1_22	AGGCTTTCGGGTCGTAAACTC	16S rDNA	GenBank: NC_007530	29 524–29 545
45	BanR2_22	GGTCGTAAACTCTGTTGTTAG	16S rDNA	GenBank: NC_007530	29 533–29 554
46	BanR3_22	GAACAAGTGCTAGTTGAATAAG	16S rDNA	GenBank: NC_007530	9765–9786
47	SalR3a_22	GGAAGGTGTTGGTTAATAAC	16S rDNA	GenBank: NC_006905	4 328 702–4 328 723
48	SalR3b_22	GGAAGGTGTTGGTTAATAAC	16S rDNA, mismatch	GenBank: NC_006905	4 328 702–4 328 723
49	P13'NH2	Unknown	Internal hybridization control of Clondiag		
50	htag4-Bio wisp2_129_158r Bio	Biotin			

Bold: nucleotide differences from the sequence of *B. anthracis* Ames Ancestor NC_007530, as indicated in Fig. 1

tion signals were considered to be specific if they appeared in duplicates and if the mean intensity was 0.05 or above.

Results

Phenotypic and genotypic characterization of the *Bacillus* isolates

A total of 158 environmental samples were submitted to our laboratory for *B. anthracis* diagnostic. Out of these samples, we obtained 43 *Bacillus* isolates that were characterized phenotypically. Based on the phenotype, the strains were assigned to seven groups. From each group, two isolates were chosen for array hybridization (group three consisted of only one member).

The 16S rDNA of the 13 environmental *Bacillus* isolates that were used for hybridization were sequenced (Table 1). Additionally, all isolates were examined using a *B. anthracis*-specific *rpoB* PCR (Qi et al. 2001). PCR products were obtained for three of the isolates (M100, M101/1 and M141/11). The PCRs targeting *B. anthracis* virulence genes were negative for all isolates.

Specificity testing

To evaluate the specificity of the array probes, biotinylated PCR amplicons of the *capAC*, *capB*, *pag*, *cya*, *lef*, *rpoB* and the 16S rRNA gene region of *B. anthracis* A73 (capsule) and *B. anthracis* A15 (toxin genes) were hybridized to the AT. Specific hybridization signals were observed in all experiments (Fig. 2a). Hybridization with reference strains (Table 1) revealed the expected probe signals confirming their specificity (Fig. 2b–h). Four *B. anthracis* field isolates were also hybridized to the array (Fig. 2g). All virulent *B. anthracis* strains showed positive hybridization signals with spots 1–22. Only probe 18, detecting a region on the *lef* gene, was negative. *Bacillus anthracis* strains harbouring only one virulence plasmid could be recognized and differentiated by their hybridization patterns (positive for spots 1–12 and for 13–22 indicating the presence of pXO2 and pXO1 respectively). Strain A58 (cured of both plasmids) differed from *B. cereus* by its hybridization pattern of the genomic *rpoB* gene region (spots 23–27).

Hybridization with strains obtained from environmental samples

The discriminatory power of the array for *B. anthracis* from other members of the genus *Bacillus* was tested with DNA of strains isolated from environmental samples. Thirteen *Bacillus* isolates representing seven different groups were tested (Table 1).

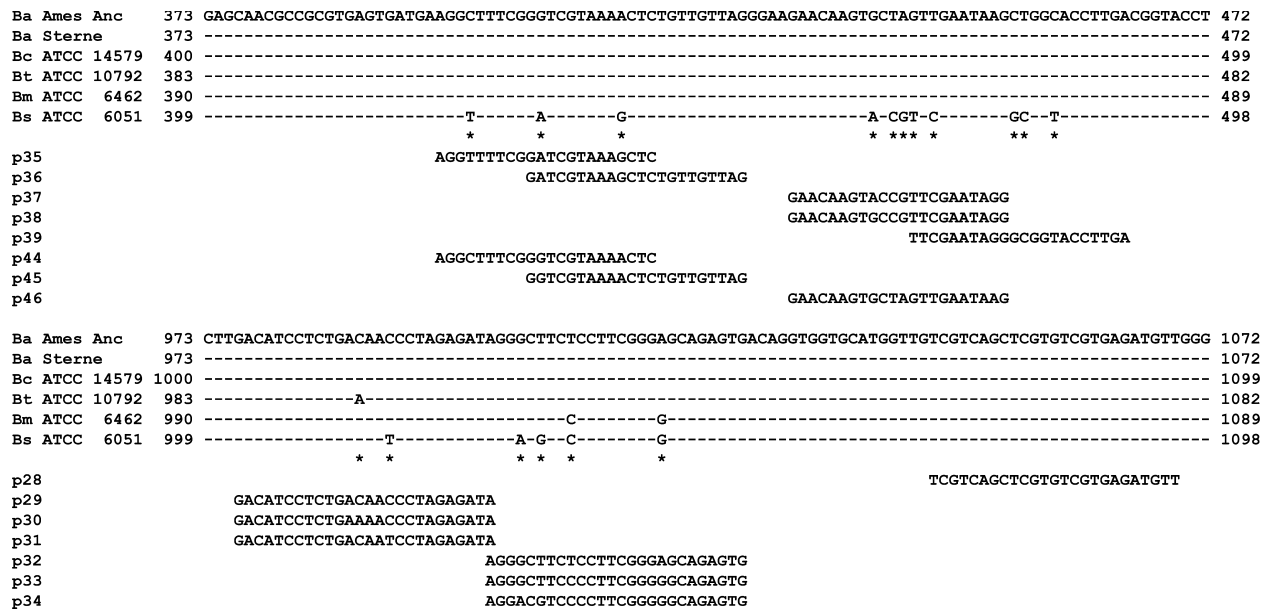


Figure 1 Probe design of the 16S rDNA region. The 16S rRNA gene sequences of the *Bacillus cereus* group members are nearly homologous. Here, an alignment of these sequences as well as of the corresponding *Bacillus subtilis* sequence of higher variable regions is shown. *nt positions showing differences between the sequences; Ba Ames Anc: *Bacillus anthracis* Ames Ancestor NC_007530. Other strains and accession numbers are given in Table 1. The probes are described in Table 3.

All isolates could be differentiated from virulent *B. anthracis* because no positive hybridization results for any *B. anthracis* virulence gene probes were detected. Considering the *rpoB* and 16S rDNA probes, M101/1 and M141/11 could be typed as *B. anthracis* without plasmid-encoded genes. M63/3, M63/4_2, M71/1_4 and M125/2 were identified as *B. subtilis* and M100 as *B. cereus*. This result was confirmed by 16S rDNA sequencing. For the other isolates, no species identification was possible. Figure 2i–o illustrates arrays representing each phenotypic group.

Discussion

State-of-the-art diagnosis of *B. anthracis* in environmental samples requires simultaneous detection of chromosomal markers as well as of plasmid-encoded virulence genes. An unambiguous routine diagnosis is time-consuming and sometimes uncertain because *Bacillus* strains with atypical characteristics have been described recently, i.e. penicillin-resistant *B. anthracis* strains (Bradaric and Punda-Polic 1992; Lalitha and Thomas 1997) or *B. cereus* isolates harbouring the virulence plasmids (Hoffmaster *et al.* 2004). In this study, we developed a microarray for the parallel detection of chromosomal markers (*rpoB*) and the virulence genes of *B. anthracis*. Furthermore, 16S rDNA-specific probes were included in the array to distinguish the *B. cereus* group from other *Bacillus* species.

Instead of fluorescence labelling of multiple PCR fragments, we biotinylated the entire bacterial DNA. The advantage is that it is not necessary to establish several PCRs or a multiplex PCR and multiple and expensive labelling reactions. This minimizes the required working steps, the total costs as well as the cross-contamination risk in routine application. Using our array, a definite diagnosis is achievable within 12 h.

Specificity of our microarray was proven by hybridization of biotinylated control PCR fragments and whole DNA of well-defined *Bacillus* strains. Thereby, all reference strains and field isolates of *B. anthracis* could be identified and unambiguously differentiated from other *Bacillus* strains. Only probes 18, 45 and 46 did not reveal the expected hybridization signals. However, the application of the whole bacterial DNA instead of specific PCR products as a template allows customizing the probe composition on newly produced microarray batches.

Interestingly, our results indicate that the reference strain *B. cereus* ATCC 14579 could be distinguished from the *B. anthracis* strains at the 16S rDNA level. Probe 30, originally designed to differ between *Bacillus thuringiensis* and *B. anthracis*, revealed a specific hybridization signal with *B. cereus* ATCC 14579. No signal was obtained with *B. anthracis*. It remains to be elucidated whether this is true for other *B. cereus* strains.

The hybridization results of the environmental *Bacillus* isolates confirmed the discriminatory power of our array,

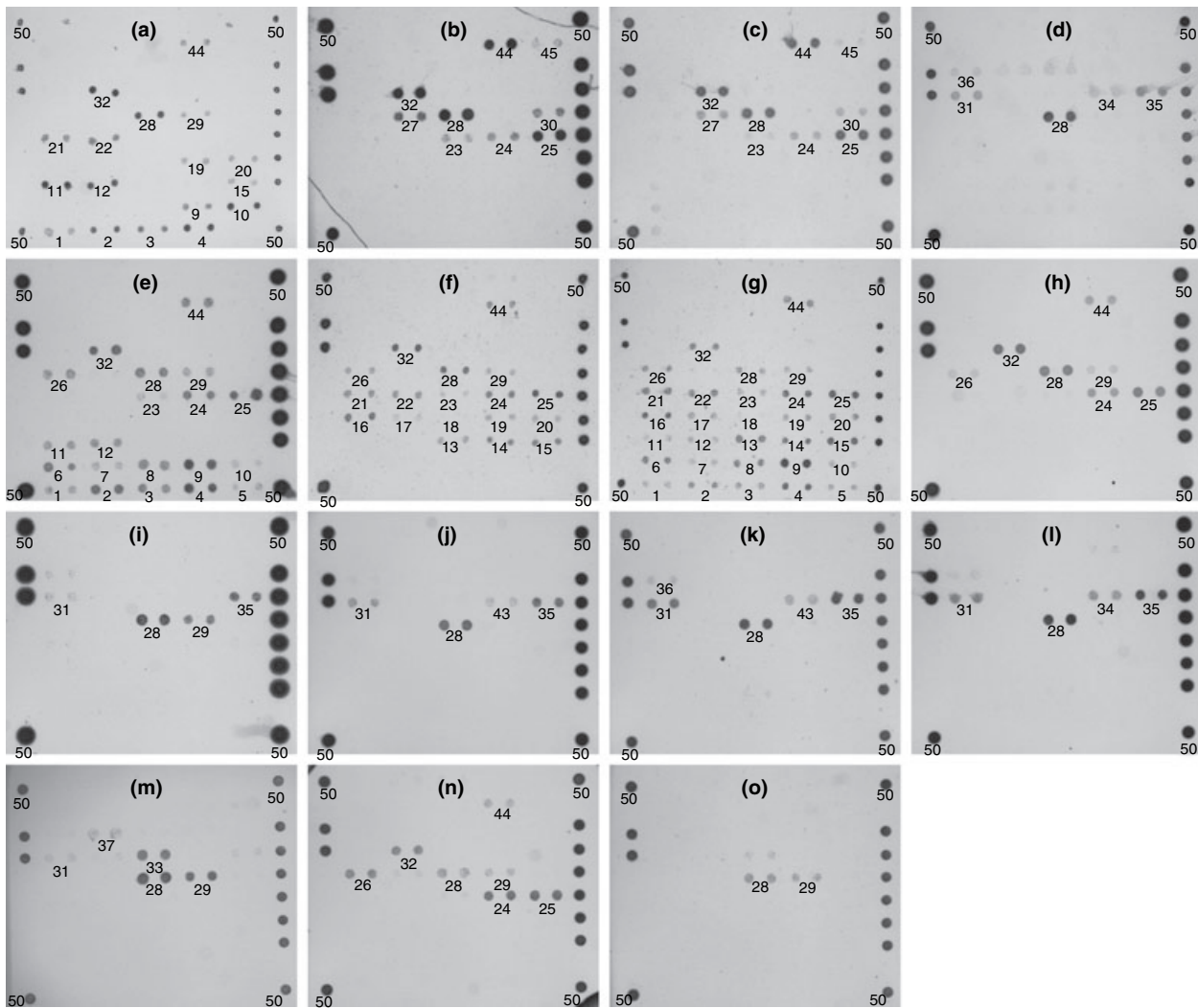


Figure 2 Hybridizations of several DNA samples to the ArrayTube. (a) Biotinylated PCR products of *capAC* amplified from strain *Bacillus anthracis* A73, *cya*, *lef* and the 16S rRNA gene region amplified from strain *B. anthracis* A15; (b–n) whole biotinylated DNA content of tested strains: (b) *Bacillus cereus*; (c) *Bacillus thuringiensis*; (d) *Bacillus subtilis*; (e) non-virulent *B. anthracis* A73 (pX01–; pX02+); (f) non-virulent *B. anthracis* Sterne (pX01+; pX02–); (g) virulent diagnostic *B. anthracis* isolate 07/11/62; (h) non-virulent *B. anthracis* A58; (i) M134; (j) M63/3; (k) M63/4_2; (l) M71/1_4; (m) M65/1_1; (n) M141/11 and (o) M96/2.

i.e. all isolates were clearly diagnosed as non-*B. anthracis* or avirulent *B. anthracis*. The 16S rDNA sequence of the strain M100 showed the highest identity with the *B. anthracis* strain Ames. The DNA did not hybridize with probes 24 and 26 on the array. Thus, it was identified as *B. cereus*; not as *B. anthracis*. The isolates M101/1 and M141/11 were assigned to *B. cereus* and the clusters *B. cereus*, *B. thuringiensis* and *B. anthracis* by 16S rDNA sequencing. By hybridization, both strains could be identified as *B. anthracis* without virulence genes because of high similarity to the *rpoB* hybridization pattern of *B. anthracis* A58. Therefore, the array has the potential to recognize *B. anthracis* strains cured of the virulence

plasmids that would be overseen by 16S rDNA sequencing. Such strains are of epidemiological significance (Bode *et al.* 2004) and important to be recognized for bio-warfare prevention.

We conclude that our array is a reliable tool for the diagnosis of *B. anthracis* in environmental samples. It is possible to clearly distinguish all constitutions of *B. anthracis* (fully virulent, harbouring only one plasmid or without plasmids). Further, *B. cereus* harbouring the virulence plasmids would be detected. It is applicable in routine diagnostics because of its safety and simple handling. The cross-contamination risk and therefore false-positive results are minimized. Furthermore, the

application of the entire bacterial DNA as a template enables the adaptation of the array layout to novel findings in *B. anthracis* research.

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