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Development of a novel selective agar for the isolation and detection of *Bacillus anthracis*

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Abstract**Aims:** The aim of this study was to develop a novel selective agar for the specific isolation and detection of *Bacillus anthracis*.**Methods and Results:** Based on published data on antibiotic resistance and susceptibility of *B. anthracis* and other closely related species of the *Bacillus cereus sensu lato* group, a new selective agar formulation termed CEFOMA (*Bacillus Cereus sensu lato* group-specific antibiotics, FOscomycin, MACrolides) was developed and evaluated. All tested strains of *B. anthracis* were able to grow on CEFOMA with the same colony number as on non-selective media, whereas CEFOMA inhibited the growth of the other species within the *B. cereus sensu lato* group. In comparison to other selective agars, CEFOMA had a superior performance and considerably reduced the total amount of accompanying flora in soil. Furthermore, *B. anthracis* was successfully isolated from deliberately spiked soil samples.**Conclusions:** CEFOMA is a highly promising selective agar for the efficient isolation of *B. anthracis* from environmental samples with a large bacterial background flora.**Significance and Impact of the Study:** The isolation of *B. anthracis* from environmental samples is severely impaired by the lack of adequate selective agars which suppress the growth of other bacteria. CEFOMA agar represents an important improvement and suitable alternative to currently used selective agars.**Introduction**

Bacillus anthracis is the causative agent of anthrax and occurs naturally in the environment. It is a dangerous pathogen for humans and can affect especially herbivores, due to its high pathogenicity and severe course of disease with high fatality rates depending on the route of infection (World Health Organization 1998; Turnbull 2008). The ability of *B. anthracis* to form highly stable spores, resistant to heat as well as many chemicals and disinfection agents, has resulted in efforts to abuse this feature for the development of biological weapons in the past (Goel 2015). Unfortunately, culture-based detection of *B. anthracis* from matrices with high concentrations of accompanying bacterial flora is a serious challenge. Especially soil samples contain only small concentrations of *B.*

anthracis spores in the presence of large bacterial numbers of closely related species of the *Bacillus cereus sensu lato* group and other environmental strains which render any attempts for isolation complicated, tedious and often unsuccessful (Silvestri *et al.* 2015). These difficulties of culture-based detection result to a great extent from the lack of adequate selective media (World Health Organization 1998). PLET agar (polymyxin, lysozyme, ethylenediaminetetraacetic acid and thallium acetate) is a selective agar which is specifically designed to isolate and select *B. anthracis* (Knisely 1966). However, the incorporation of highly toxic thallium acetate limits its use today due to stricter work safety and environmental protection regulations including a more diligent waste management than in the past (Tomaso *et al.* 2006; Turnbull 2008). In addition, it was shown that many other *Bacillus* strains are

also able to grow on PLET and that the germination of *B. anthracis* spores is significantly reduced on this medium (Dragon and Rennie 2001; Klee et al. 2006; Marston et al. 2008). The majority of agars currently used aiming to isolate *B. anthracis* target the whole *B. cereus sensu lato* group, which includes species like *B. cereus* and *B. thuringiensis*. However, growth on these chromogenic or differential agars such as Cereus Ident™ Agar (CEI), mannitol egg yolk polymyxin B agar or sulfamethoxazole trimethoprim blood agar is not always easy to interpret and, thus, generally prone to misclassification (Juergensmeyer et al. 2006; Tomaso et al. 2006; Marston et al. 2008; Silvestri et al. 2015). Furthermore, many environmental samples, including soil, contain high numbers of members of the *B. cereus sensu lato* group which can easily overgrow *B. anthracis* on agar plates (Fasanella et al. 2013).

In this study, we developed and tested a novel selective agar suitable for environmental samples, termed CEFOMA (*Bacillus Cereus sensu lato* group-specific antibiotics, FOsfomycin, MAcrolides), with superior selectivity for *B. anthracis* compared to currently used selective or differential agar formulas.

Materials and methods

Literature research

A literature research was performed screening the English and German literature in Google Scholar and PubMed. Search terms for gathering data on antimicrobial resistance within the *B. cereus sensu lato* group included 'antimicrobial', 'antibiotic', 'antibacterial', 'susceptibility', 'testing', 'resistance', 'MIC', '*B. anthracis*', '*B. cereus*', '*B. thuringiensis*', '*B. weihenstephanensis*', '*B. mycoides*', and '*B. pseudomycoides*'. After identifying a potentially suitable antimicrobial agent, the literature research was repeated by including the name of the antibiotic in the search term list. Search terms to identify components for the development of a selective agar for *B. anthracis* included '*B. anthracis*', '*B. cereus*', '*B. thuringiensis*', 'selective', 'isolation', 'detection', 'cultural', 'agar', 'medium', 'media', 'chromogenic', 'soil', 'enrichment', 'broth' and 'PLET'.

Strains and cultivation

A detailed list of all *Bacillus* reference strains and environmental isolates (RKI ZBS 2 strain collection) is given in Table 1. All *Bacillus* strains were cultivated on tryptic soy agar as solid non-selective medium or in lysogeny broth as non-selective liquid medium under aerobic conditions at 37°C. For selective plating, spores or freshly grown bacterial cultures were used and agar plates were

incubated for 24–48 h. To avoid CFU losses due to spore clumping, Triton X-100 was added to the working solutions of all spore preparations in a final concentration of 0.01%. Spores were prepared on manganese sulphate agar following the instructions of DIN EN 14347:2005-08.

Preparation and use of selective agars

1 l CEFOMA agar (for approximately 50 agar plates) was prepared as follows: 38 g brain heart infusion broth modified (BD, Franklin Lakes, NJ) and 13 g agar (no. 1; Oxoid, Hampshire, UK) were dissolved in water and

Table 1 List of all *Bacillus* sp. strains used for testing the specificity of CEFOMA agar

Species (no. of strains)	Strains/isolates	Growth on CEFOMA after 24 h
<i>B. anthracis</i> (12)	Sterne (34F2) Wirt (CDC 1014) Pasteur (ATCC 4229) 11/38 527 22/39 Jena UD III-7 Delta Ames Vollum Three environmental isolates (Icelandic horse, heroin user, wool factory)	Growth without CFU loss, attenuated strains with translucent morphologies (3), virulent strains opaque (9)
<i>B. cereus</i> (10)	DSM 31 ATCC 10987 Hohenheim BW-A DSM 4312 BW-B ATCC 14579 301101RA0432 ATCC 11778 DSM 9378	No growth (7 strains), strongly inhibited growth with CFU decrease >2 log ₁₀ levels (3 strains)
<i>B. cereus</i> biovar <i>anthracis</i> (1)	CARR (no virulence plasmids)	Strongly inhibited growth with CFU decrease >3 log ₁₀ levels
<i>B. thuringiensis</i> (6)	DSM 350 DSM 2046 DSM 5724 DSM 5815 DSM 6022 DSM 6087	No growth
<i>B. weihenstephanensis</i> (1)	DSM 11821	No growth
<i>B. mycoides</i> (1)	DSM 2048	No growth
<i>B. subtilis</i> (1)	ATCC 66334	No growth

autoclaved. After cooling to 45°C, trimethoprim (dissolved in DMSO (Carl Roth, Karlsruhe, Germany), final concentration 2 mg l⁻¹; Sigma-Aldrich, St. Louis, MI), sulfamethoxazole (dissolved in ethanol, final concentration of 38 mg l⁻¹; Sigma-Aldrich), polymyxin B and lysozyme (dissolved in water, final concentrations of 30 000 U polymyxin B and 300 000 U lysozyme, Anthracis selective supplement; Merck, Darmstadt, Germany), fosfomycin disodium salt (dissolved in water, final concentration of 20 mg l⁻¹; Sigma-Aldrich), erythromycin (dissolved in water, final concentration of 0.03125 mg l⁻¹; Carl Roth), azithromycin dihydrate (dissolved in water, final concentration of 0.0625 mg l⁻¹; Sigma-Aldrich) and defibrinated sheep blood (final concentration of 1%; Oxoid) were added.

In addition to the newly developed CEFOMA agar, two conventional types of selective and differential agar were used for comparison: The commercially available CEI (Merck) is selective for the *B. cereus sensu lato* group and contains the chromogenic substance 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate which is converted by the enzymatic activity of many strains of *B. cereus* and *B. thuringiensis* resulting in green-blue colonies, whereas colonies of *B. anthracis* appear white (Tomaso *et al.* 2006). Trimethoprim sulfamethoxazole polymyxin blood agar (TSPBA) is also selective for the *B. cereus sensu lato* group and allows for haemolysis assessment with most strains of *B. cereus* and *B. thuringiensis* being typically haemolytic while *B. anthracis* is not haemolytic. 1 l TSPBA was prepared with Columbia agar and 5% sheep blood and was supplemented with 3.2 mg trimethoprim, 16 mg sulfamethoxazole and 20 mg polymyxin B (in-house developed formula, adapted from Turnbull 2008).

To test the productivity (the ability to germinate and form colonies) of *B. anthracis* on CEFOMA, spore preparations were adequately diluted (to reach a theoretical colony number of 10–100 colonies per agar plate) and 100 µl of these dilutions were plated on CEFOMA and sheep blood agar (duplicates for fully virulent strains, at least triplicates for the three attenuated strains Sterne, Pasteur and Wirt). After incubation for 24–48 h, all colonies were enumerated and CFU counts between selective and full medium were compared. Additionally, this experiment was performed in a similar manner for vegetative cells using overnight cultures.

To evaluate the stability of CEFOMA after long-term storage at 4°C for up to 10 weeks, plates were tested every 2 weeks for growth of spores of *B. anthracis* (Sterne and Wirt) and inhibition of spores of *B. cereus*, *B. thuringiensis* and *B. subtilis*. Additionally, the number of colonies on CEFOMA obtained by plating two heat-treated soil samples (tested negative for *B. anthracis*) and

after incubation for 48 h was enumerated every 2 weeks to screen for any increase in colony numbers due to diminished selectiveness caused by aged agar media.

Soil samples and deliberate spiking

Two different soil samples were used: Soil sample I (pH of 7.7 after 1 : 10 dilution in water), characterized by a rather sandy composition with a low content of organic matter, was directly taken from the environment (a lawn in Berlin), soil sample II (pH of 7.0 after 1 : 10 dilution in water) was taken from commercial potting soil (Edeka, Gut & Günstig) and was rich in humus. Analysis of soil composition and nutrient content of the two samples was carried out by Reblu GmbH (Bodenanalyse Zentrum, Filderstadt, Germany). Both samples were confirmed to be free of *B. anthracis* by PCR (after 45 min of incubation in LB medium, heat treatment at 95°C for 60 min and processing by DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the pretreatment instructions for Gram-positive bacteria) and extensive plating on CEFOMA and CEI. 1 g of each soil sample was diluted 1 : 10 in water and heat treated for 30 min at 65°C to kill off the majority of vegetative cells. For deliberate spiking, 10³ spores of *B. anthracis* Sterne or Wirt were added to the diluted soil samples (resulting in a final concentration of 100 spores per ml) prior to heat treatment. All samples were rigorously shaken after spiking and again after heat treatment before taking a sample for plating. 100 µl of this mixture was then plated directly on agar and incubated for 24–48 h at 37°C (theoretically resulting in, on average, 10 colonies of *B. anthracis* per plate). All colonies resembling typical morphologies of *B. anthracis* were picked and transferred to fresh plates. Heat lysates (95°C for at least 60 min) were prepared and directly used for colony PCR analysis. All experiments were performed as triplicates. For each experiment, soil samples were plated on two CEFOMA plates (100 µl each).

PCR

For the confirmation of picked isolates of *B. anthracis*, Sterne from spiked soil samples, real-time PCR was used to screen for the presence of the protective antigen (PA) using CGGATCAAGTATATGGGAATATAGCAA as forward primer, CCGGTTTAGTCGTTTCTAATGGAT as reverse primer and CTCGAAGTGGAGTGAAGTGT-TACCGCAAA as the probe (modified with FAM-TAMRA) (Ellerbrok *et al.* 2002). Likewise, for the confirmation of picked isolates of *B. anthracis* Wirt, real-time PCR was used to screen for the dhp-locus using CGTAAGGACAATAAAAGCCGTTGT as forward primer, CGATACAGACATTTATTGGGAAGTACAC as reverse

primer and TGCAATCGATGAGCTAATGAACAATGACCCT as probe (modified with FAM-TAMRA) (Antwerpen *et al.* 2008). All sequences are given in 5'–3' orientation. Amplification and detection were done with ABI PRISM 7500 (40 cycles: denaturing for 15 s at 95°C, annealing and elongation for 60 s at 60°C).

Results

Agar development and specificity testing

A comprehensive literature research was performed to gather all available knowledge dealing with the selective isolation of *B. anthracis* and with available data on antimicrobial resistance. With the exception of PLET, all other agars used components which are thought to provide specificity for the whole *B. cereus sensu lato* group (Turnbull 2008). Therefore, we combined all these ingredients, that is, polymyxin and lysozyme (PLET) and the combination of trimethoprim and sulfamethoxazole (resulting in the *Bacillus cereus sensu lato* group-specific component, termed 'CE' component). Thallium acetate was omitted due to its high toxicity. EDTA was omitted because its effect is believed to be linked to the use of thallium acetate in PLET and its chelating activity (Bowen 2000). As expected, all members of the *B. cereus sensu lato* group were able to grow on this agar while growth of other *Bacillus* species like *B. subtilis* was reduced (data not shown). Next, the literature was systematically screened for antibiotic susceptibility testing of members of the *B. cereus sensu lato* group, including *B. anthracis*, and five antibiotics were identified by this literature search, which seemed to be suitable for a novel selective agar (Table 2). Not surprisingly, the susceptibility to most antibiotics among the *B. cereus sensu lato* group was, in general, rather similar. However, in case of the two macrolide antibiotics erythromycin and azithromycin, it was noted that strains of *B. anthracis* exhibited higher tolerance towards these substances than many other strains of the *B. cereus sensu lato* group (Yamamoto *et al.* 2001; Bryskier 2002; Cavallo *et al.* 2002; Mohammed *et al.* 2002; Frean *et al.* 2003; Jones *et al.* 2003; Schlegelova *et al.* 2003; Turnbull *et al.* 2004; Citron and Appleman 2006; Luna *et al.* 2007; Ikeda *et al.* 2015; Kreizinger *et al.* 2016). It is important to note that many strains of *B. anthracis* are defined as being sensitive to erythromycin and azithromycin (Turnbull 2008). However, they can tolerate the presence of these antibiotics in small amounts, while many other strains of the *B. cereus sensu lato* group cannot grow in the presence of the same antibiotic concentration (Table 2). When these antibiotics (macrolide component; 'MA') were combined with the CE component of the selective agar, strong synergistic

effects (i.e. minimal inhibitory concentrations were smaller in the presence of other antibiotics than in non-selective growth medium) were observed and, thus, concentrations of azithromycin and erythromycin were reduced by 75% of the initially theoretically assumed tolerance limits based on the literature search (Table 2). Additionally, the natural resistance of *B. anthracis* against fosfomycin (while most other members of the *B. cereus sensu lato* group are susceptible) was exploited and consequently incorporated as the 'FO' component (Schuch and Fischetti 2006). Initially, the same concentration of fosfomycin was used as described in the Ground Anthrax *Bacillus* Refined Isolation (GABRI) method by Fasanella *et al.* (2013); but again synergistic effects (resulting from the presence of other antibiotics in the growth medium) were encountered and the fosfomycin concentration was lowered from initially 50 to 20 mg l⁻¹. Finally, sheep blood was added to CEFOMA as a non-selective component which allows for haemolysis testing and identification of non-target colonies. 5% sheep blood tended to reduce the selective properties of CEFOMA as observed in subsequent experiments (data not shown); thus, only 1% sheep blood was used, which was sufficient to distinguish between haemolytic and non-haemolytic strains.

As a first evaluation of the sensitivity and specificity of CEFOMA, 12 *B. anthracis* strains (including six fully virulent reference strains, three attenuated strains and three environmental isolates) and 19 other reference strains of the *B. cereus sensu lato* group (predominantly belonging to the species *B. cereus* and *B. thuringiensis*) were tested. All *B. anthracis* strains were able to grow on CEFOMA after 24–48 h of incubation (Fig. 1), albeit at a reduced speed, roughly estimated 2–3 times slower, compared to media without antibiotics like sheep blood agar (but apparently still faster than on PLET agar; Knisely 1966). Interestingly, some attenuated strains showed slower growth and more irregular, often translucent colony shapes compared with fully virulent strains (Fig. 1), which complicated enumeration after only 24 h of incubation resulting in slightly lower colony number counts compared to 48 h of incubation. In contrast, colony number counts for fully virulent stains did not differ between 24 and 48 h of incubation.

Next, colony numbers were enumerated to determine whether all plated bacteria (spores or vegetative cells) could form colonies. No colony loss was recorded for *B. anthracis* on CEFOMA agar compared to sheep blood agar without any additives as, on average, colony numbers were equal on the two growth media after plating equal amounts of bacteria (spores or vegetative cells). In contrast, all tested non-target *Bacillus* strains were either completely inhibited on CEFOMA or at least the CFU number was remarkably reduced by several orders of

magnitude and the colony size was considerably smaller (Table 1). Importantly, even unusual strains of *B. cereus* (e.g. *B. cereus* BW-A and BW-B) which behave very similar to *B. anthracis* as they do not show haemolysis or phospholipase C activity (and are thus hardly distinguishable from *B. anthracis* on differential media like

CEI or TSPBA agar) were not able to grow on CEFOMA (Klee *et al.* 2006). Furthermore, long-time storage of CEFOMA plates at 4°C up to 1 month had no obvious effect on the inhibition of growth of non-target strains, nor did it effect growth of *B. anthracis* strains (data not shown).

Table 2 Comparison of antimicrobial resistance data on *Bacillus anthracis* and related species for antibiotics considered as suitable selective components for the development of CEFOMA

Antibiotic	MIC ₅₀ /lowest MIC found for <i>B. anthracis</i> in mg l ⁻¹	MIC ₅₀ for <i>B. cereus</i> in mg l ⁻¹	MIC ₅₀ for <i>B. thuringiensis</i> in mg l ⁻¹	Reference	Final concentration in CEFOMA in mg l ⁻¹ (initially tested concentration)
Erythromycin	0.5/0.25	0.094	0.064	Luna <i>et al.</i> (2007)	0.03125 (0.125)
	1/0.5	Not tested	Not tested	Jones <i>et al.</i> (2003)	
	1/0.5	0.064	0.094	Turnbul <i>et al.</i> (2004)	
	1/0.5	Not tested	Not tested	Bryskier (2002)	
	0.5/0.125	Not tested	Not tested	Kreizinger <i>et al.</i> (2016)	
	1/0.5	Not tested	Not tested	Frean <i>et al.</i> (2003)	
	NA/0.06	0.06	Not tested	Yamamoto <i>et al.</i> (2001)	
	1/0.5	Not tested	Not tested	Cavallo <i>et al.</i> (2002)	
	1/0.5	Not tested	Not tested	Mohammed <i>et al.</i> (2002)	
Azithromycin	3/1	0.38	0.19	Turnbull <i>et al.</i> (2004)	0.0625 (0.25)
	1/0.5	Not tested	Not tested	Bryskier (2002)	
	NA/0.12	0.5	Not tested	Yamamoto <i>et al.</i> (2001)	
	2/1	Not tested	Not tested	Jones <i>et al.</i> (2003)	
Rifampicin	0.19/0.064	0.038	0.25	Luna <i>et al.</i> (2007)	Not included in CEFOMA (0.032)
	0.19/0.004	Not tested	Not tested	Kreizinger <i>et al.</i> (2016)	
	0.125/0.125	Not tested	Not tested	Cavallo <i>et al.</i> (2002)	
Daptomycin	2/1	Not tested	Not tested	Bryskier (2002)	Not included in CEFOMA (0.2)
	1.5/0.38	0.25	0.25	Luna <i>et al.</i> (2007)	
Linezolid	0.5/0.38	0.25	0.19	Luna <i>et al.</i> (2007)	Not included in CEFOMA

Only publications containing complete resistance data on *B. anthracis* are displayed (thus, exact upper and lower limit of MICs were stated in the publications). Data on antimicrobial resistance of other members of the *B. cereus sensu lato* group without data on *B. anthracis* (or with insufficient information) were also collected and analysed. These data are not shown here since those (inter-laboratory) results can vary remarkably due to different experimental procedures for MIC testing making direct comparisons between the studies difficult and unreliable. The MIC₅₀ value is the concentration required to inhibit 50% of the bacterial strains tested.

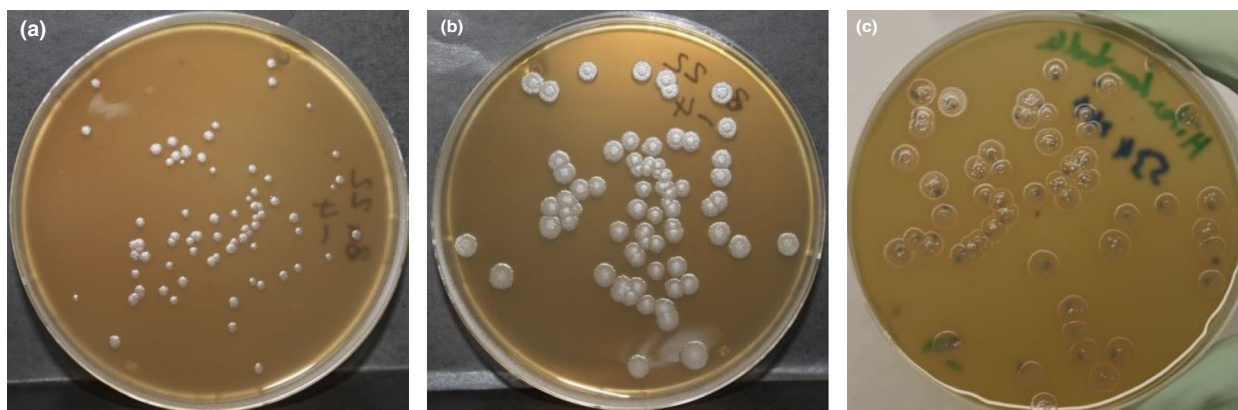


Figure 1 Growth of *Bacillus anthracis* (virulent environmental isolate) on CEFOMA after 24 h (a) and 48 h (b) and of attenuated *B. anthracis* Wirt (CDC 1014) after 48 h (c). [Colour figure can be viewed at wileyonlinelibrary.com]

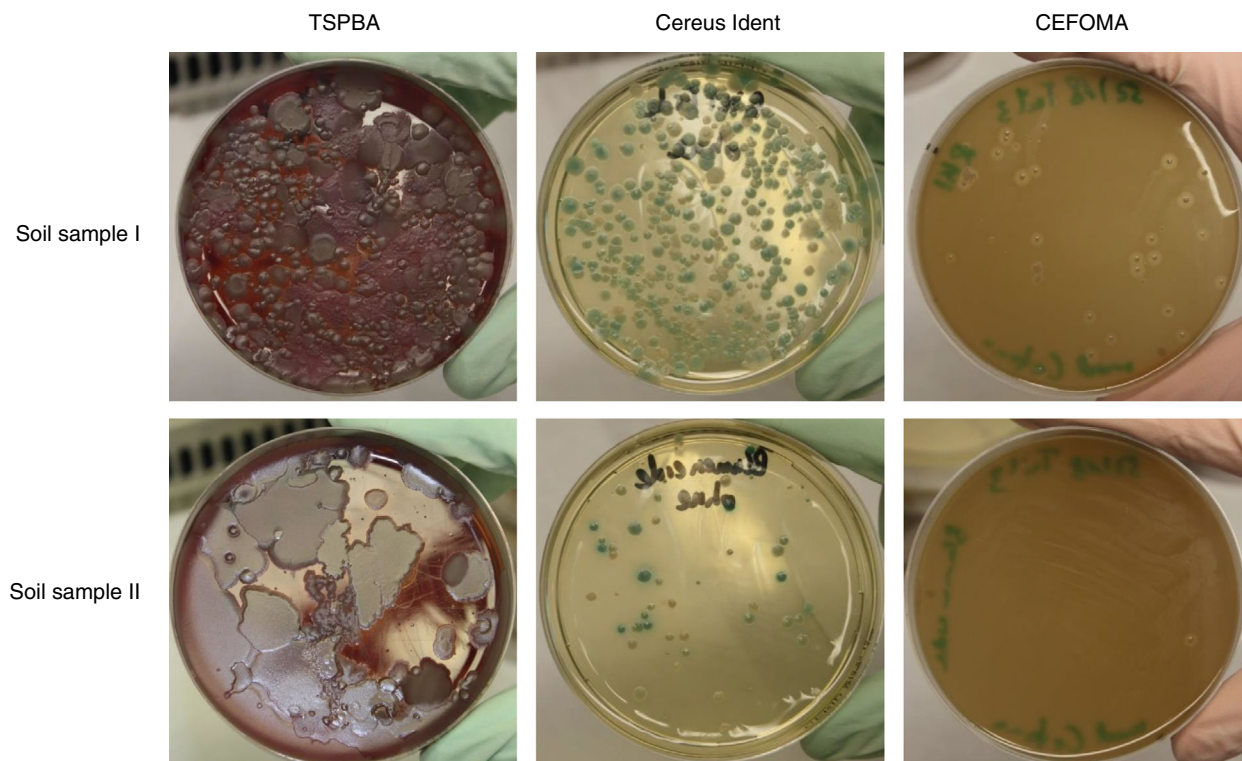


Figure 2 Reduction of accompanying flora in two heat-treated soil samples (soil sample I and II) after 24 h of incubation on TSPBA (left), CEI (middle) and CEFOMA agar (right). [Colour figure can be viewed at wileyonlinelibrary.com]

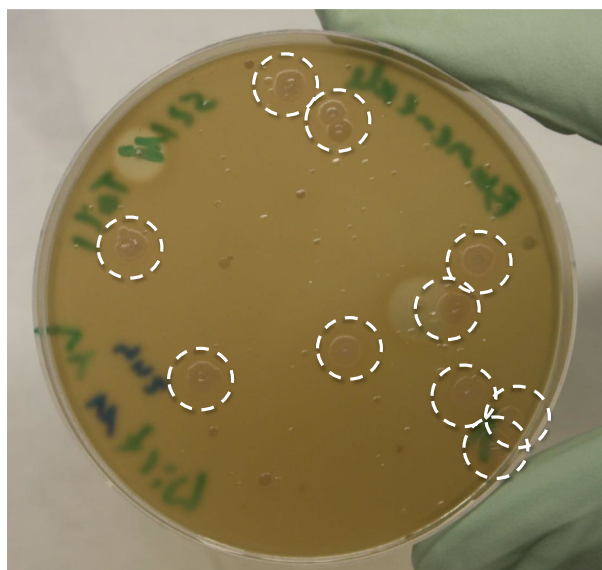


Figure 3 Picking of suspected *Bacillus anthracis* Wirt (CDC 1014) colonies from spiked soil samples after incubation on CEFOMA for 48 h. All picked isolates are circled (white). 57 of 60 picked colonies were later confirmed to be *B. anthracis* by PCR. [Colour figure can be viewed at wileyonlinelibrary.com]

Reduction of accompanying environmental flora and comparison with alternative selective agars

Commercially available reference strains only reflect a small proportion of the true diversity found in nature. To test the selectiveness of CEFOMA under more realistic conditions, two soil samples (tested negative for *B. anthracis*) were used to compare the amount of environmental background flora obtained on CEFOMA with conventional selective and differential media like TSPBA and CEI. Despite the heat treatment at 65°C to kill off most vegetative cells, plates of TSPBA as well as of CEI were overgrown within a day (Fig. 2). Notably, neither haemolysis activity on TSPBA nor the presence of 5-bromo-4-chloro-3-indoxyl myo-inositol-1-phosphate in CEI were adequate to rule out the presence of *B. anthracis*. In contrast, overall colony numbers on CEFOMA were reduced by at least 1–2 log₁₀ levels compared with conventional selective agar (Fig. 2). Moreover, the remaining colonies were significantly smaller than *B. anthracis* and, additionally, haemolysis could be used to distinguish them from *B. anthracis*.

Next, soil samples were spiked beforehand with *B. anthracis* Wirt or *B. anthracis* Sterne (resulting in a

theoretical mean of 10 spores of *B. anthracis* per plate) and treated as described above (Fig. 3). *B. anthracis* could easily be identified on CEFOMA after 24 or 48 h and all potential isolates were, subsequently, confirmed by PCR. Notably, 57 of 60 picked colonies on six CEFOMA plates were confirmed to be *B. anthracis* resulting in a recovery rate (or sensitivity) as well as a specificity of 95%.

Discussion

Despite the fact that the formula of PLET agar was first published more than half a century ago and contains highly toxic components, it remained the only truly selective agar for *B. anthracis* since other selective agars do not suppress the growth of the various *B. cereus sensu lato* group members. The difficulties to develop a selective agar can be attributed to the fact that no single antibiotic resistance or physiological property of *B. anthracis* (apart from the slightly higher tolerance towards thallium acetate) could be identified and exploited efficiently to suppress closely related species like *B. thuringiensis* and *B. cereus* (Bowen 2000; Turnbull 2008). Here, we present CEFOMA as a promising alternative to the highly toxic PLET agar as this novel agar was shown to be highly selective for *B. anthracis* enabling the efficient isolation and reliable detection of this pathogen without CFU loss. This agar is not relying on a single substance but instead combining fosfomycin, azithromycin and erythromycin, each suppressing a fraction of the closely related non-target strains. Due to the numerous antibiotics employed, it is conceivable that not all spores are able to germinate or, additionally, that the presence of fosfomycin might induce prophages in the genome causing reduced colony numbers (Schuch and Fischetti 2006). However, no such effects were observed in our experiments.

During our literature search, three other antibiotics, rifampicin, daptomycin and linezolid, were also identified as potentially interesting substances (Table 2) which, apparently, can be tolerated by *B. anthracis* in higher concentrations than its related species (Dabbs *et al.* 1995; Bryskier 2002; Cavallo *et al.* 2002; Mohammed *et al.* 2002; Citron and Appleman 2006; Luna *et al.* 2007; Horii *et al.* 2011; Ikeda *et al.* 2015; Kreizinger *et al.* 2016). During our experiments, these substances were not able to improve CEFOMA selectivity with the tested panel of non-target reference strains. However, it cannot be ruled out that the inclusion of these antibiotics might further optimize the reduction of accompanying flora in complex soil samples. Furthermore, the incorporation of cycloheximide in CEFOMA as a fungicide might be useful, especially if the crucial heat pretreatment at 65°C to reduce the vegetative accompanying bacterial flora is not desired (e.g. in case vegetative forms of *B. anthracis* should be detected). 1% blood was added to CEFOMA to assess haemolytic activity. Alternatively, blood

(which might influence selectivity) may be replaced by 5-bromo-4-chloro-3-indoxyl myo-inositol-1-phosphate, the chromogenic component from CEI, to obtain similar information. However, this proprietary substance is considerably more expensive. Interestingly, the translucent appearance of strains of *B. anthracis* with only one or no virulence plasmid (e.g. Pasteur or Sterne) was already described for PLET agar as well (Marston *et al.* 2008; Luna *et al.* 2009).

Efficient plating media are an integral part to obtain bacterial isolates. In addition, liquid enrichment media are often used beforehand to lower the limit of detection. This would be highly useful for *B. anthracis* as well because the amount of spores in environmental samples is typically low. More research is thus needed to check whether the composition of CEFOMA can be a suitable starting point for an improved enrichment media.

Conflict of Interest

The authors declare no conflicts of interest.

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