Microbial-mediated release of bisphenol A from polycarbonate vessels

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Abstract:

Aim: To identify the source of bisphenol A (BPA) [2,2'-bis(4-hydroxyphenyl) propane] in cultures of an antibiotic-producing Bacillus sp. strain grown in polycarbonate flasks.

Methods and Results: Although a culture of an antibiotic-producing Bacillus sp. strain grown in a new, rinsed polycarbonate flask yielded BPA, duplicate cultures grown in thoroughly washed polycarbonate flasks did not. Cells of Escherichia coli strain C were grown in new polycarbonate flasks rinsed three-times with 100 ml distilled H2O. BPA was only recovered from cultures grown in new polycarbonate flasks, but not from the autoclaved medium incubated in parallel.

Conclusions: BPA was present in either Bacillus or E. coli cultures, probably due to its release from inadequately washed polycarbonate flasks. Standard autoclaving did not result in BPA appearance; microbial growth was required. Polycarbonate vessels for microbial cultures should be thoroughly washed to avoid the appearance of BPA in culture medium.

Significance and Impact of the Study: This study rigorously demonstrates that the presence of BPA in culture medium was a consequence of microbial growth or metabolism in inadequately washed polycarbonate flasks. As BPA exhibits antimicrobial and oestrogenic activity, searches for novel drugs or production of recombinant chemotherapeutic agents could be derailed by the artefactual appearance of BPA.

Article:

Introduction

As part of our ongoing research to identify novel antimicrobial agents produced by non-obligate predator bacteria (Cain *et al.* 2000, 2003), an organic extract of a culture of a strain of *Bacillus* sp. recovered from a soil sample collected on the Jordanian shore of the Dead Sea was found to produce high levels of antimicrobial activity against *Staphylococcus aureus* (MIC = 4 μg ml^{-1}). Upon bioactivity-directed fractionation of that organic extract, one of the constituents was found to be bisphenol A [2,2'-bis(4-hydroxyphenyl) propane; BPA]. The isolated BPA had modest antimicrobial activity (i.e. MIC against *Staph. aureus* 288 μg ml^{-1}) in confirmation of data by others (Fabig 1988). In repeated experiments, we sought to confirm the production of BPA by the *Bacillus* sp. isolate. The hypothesis, that the *Bacillus* sp. strain synthesized BPA, was based on evidence of BPA degradation by a gram-negative aerobic micro-organism (Lobos *et al.* 1992; Spivack *et al.* 1994) and other micro-organisms in the environment (Stone and Watkinson 1983; Staples *et al.* 1998). Possibly, the *Bacillus* sp. strain might be capable of BPA biosynthesis by reversing the degradation pathway. As BPA is a major industrial chemical, the discovery of an organism capable of BPA biosynthesis would be desirable. However, in cultures of the same *Bacillus* sp. strain grown under identical conditions in a second experiment,

BPA was not detected, nor was it detected in the sterile medium. This lack of reproducibility of BPA production, coupled with the possibility that the BPA could be a metabolite of that *Bacillus* sp. strain, stimulated us to devise experiments to determine the source of BPA in microbial cultures. One clue that served as the starting point of the experiments was that new polycarbonate flasks had been used for the growth of the *Bacillus* sp. strain in the first experiment, but only repeatedly washed flasks used in the second.

BPA is used widely in the production of polycarbonate, epoxy resins, and flame retardants, and in 1993, 640 000 metric tonnes of BPA were produced (Staples *et al.* 1998). It is used in the manufacture of polycarbonate flasks and bottles used in research laboratories. BPA was detected in water contained in polycarbonate flasks and subjected to 30 min autoclaving at 121°C (Krishnan *et al.* 1993), twice the length of autoclaving used typically for media preparation in microbiology laboratories. A great deal of attention has been focused upon measurement of BPA in foods packaged or stored in polycarbonate vessels (Biles *et al.* 1997a,b; Kang and Kondo 2003), because BPA has antibiotic (Staples *et al.* 1998) and oestrogenic activities (Krishnan *et al.* 1993; Staples *et al.* 1998; Anonymous 2007). Recently, concern over the presence of BPA in plastic bottles has prompted a shift to glass bottles in the San Francisco area (DeFao 2007).

Our investigations were triggered by the absence of BPA in microbiological medium autoclaved 15 min at 121°C. If microbial growth results in release of BPA from polycarbonate, BPA could be a contaminant in cultures of recombinant micro-organisms producing proteins of human or animal origin or, with respect to our own vein of research, in micro-organisms probed for the discovery of new antibiotics. Herein we report the microbial-mediated release of BPA from polycarbonate.

Materials and methods

Bacterial strains

Escherichia coli strain C (ATCC 13706) was used in the study. The *Bacillus* sp. strain A460-4-2-7 was isolated from a soil sample collected in February 2004, approx. 100 m from the shore of the Dead Sea in the Hashemite Kingdom of Jordan (31°41·973'N, 35°34·974'E; elevation 374 m) as a colony producing a zone-of-inhibition on a lawn of *Micrococcus luteus* spread on one-tenth-strength BHIB agar medium (Becton Dickinson, Sparks, MD, USA). *Bacillus* strain A460-4-2-7 is a non-obligate predator strain based upon its ability to grow on *M. luteus*, *Staph. aureus*, and other micro-organisms in the absence of other nutrient and on normal laboratory medium in the absence of prey micro-organisms.

Growth medium and growth of bacteria

Bacillus strain A460-4-2-7 and *E. coli* strain C were grown in ¼-strength Tryptic Soy Broth (TSB) (Becton Dickinson & Co.) containing 0·2% (w/v) glucose (TSB + G). Single isolated colonies on ¼-strength TSB + G agar were picked and used to inoculate 10 ml of TSB + G medium in 125 ml glass Erlenmeyer flasks and incubated for 2 days without shaking at 30°C. Those 10 ml cultures were used to inoculate 90 ml of TSB + G medium in new, thoroughly washed, baffled 250 ml polycarbonate flasks and incubated for 2 days with aeration (60 rev min^{-1}) at 30°C. The thorough washing was adapted from (Biles *et al.* 1997b) and consisted of: (i) wash with detergent (Bio-Clean Detergent, Stanbio Laboratory, Boerne, TX, USA) and water (ii) rinse three times with 20% flask volume of tap and then three times with 20% flask volume of distilled H_2O (iii) fill with distilled H_2O and autoclave (15 min at 15 psi) and (iv) rinse three times with 20% flask volume of distilled H_2O. About 50 ml of the resulting 100 ml cultures were used to inoculate 450 ml of TSB + G medium contained in new polycarbonate flasks (Nalgene, Rochester, NY, USA) rinsed three times with 100 ml distilled H_2O. The inoculated cultures were incubated for 5 days with aeration (60 rev min^{-1}) at 30°C. After incubation, the

contents of the flasks were transferred to either new, rinsed or new, thoroughly washed 500 ml centrifuge bottles, frozen at −70°C, and shipped to Research Triangle Institute for fractionation and identification of compounds with antimicrobial activity. At each transfer, the purity of the culture was assessed by streaking on TSB + G agar medium; for all results reported herein, the cultures were pure and not contaminated.

General chemistry procedures

Preparatory HPLC was carried out on a Varian Prostar HPLC system (Walnut Creek, CA, USA), equipped with Prostar 210 pumps operating at 10 ml min^{-1} and a 330 photodiode array detector, with data collected and analysed using a Star Chromatography Workstation; the column was a YMC ODS-A (5 μm; 250 × 25 mm; Waters, Milford, MA, USA). Analytical HPLC utilized the same HPLC system at 1 ml min^{-1} with a Inertsil ODS-3 (5 μm; 250 × 4·6 mm; Metachem Technologies, Torrance, CA, USA), with chromatograms analysed at 270 nm. All NMR experiments were performed in $CDCl_3$ with TMS as an internal standard using a Varian Unity Inova-500 (Varian Inc., Palo Alto, CA). Low-resolution ESIMS and APCIMS were determined on an Applied Biosystems/MDS Sciex API 150 EX single quadrupole LC/MS system (Applied Biosystems, Foster City, CA, USA).

Results

Isolation and identification of bisphenol A from *Bacillus* strain A460-4-2-7

A culture of predator *Bacillus* sp. strain A460-4-2-7 was processed for the identification of antimicrobial compounds using a modification of procedures described previously (Cain *et al.* 2003). Briefly, the entire 500 ml culture was subjected to one freeze-thaw cycle before being freeze dried. The resultant powder was stirred with 1 : 1 chloroform: methanol overnight, the solution was filtered to remove insoluble materials, and the volume of the filtrate was reduced *in vacuo*. The resulting extract was then partitioned between 4 : 1 : 5 chloroform : methanol : water in a separatory funnel. Upon settling, the bottom layer (organic) was collected, and the solvent was removed under reduced pressure to generate the organic extract (35·0 mg). An aliquot of that organic extract was purified via reverse phase HPLC using an acetonitrile : water gradient (20 : 80 to 40 : 60 over 30 min, then up to 100 : 0 over 20 min) to yield 1·8 mg of BPA. The structure of BPA was confirmed by analysis of both spectroscopic (^{1}H, ^{13}C, DEPT, HSQC and HMBC NMR data) and spectrometric (low resolution ESI and APCI MS) data and by comparison with an authentic sample of BPA (Alfa Aesar, Pelham, NH, USA; Lot GBFA028690), which co-eluted on HPLC (isocratic acetonitrile : water, 40 : 60, R_t 18·5 min).

Isolation and identification of bisphenol A from *E. coli* strain C

Repeated and independent cultures of *Bacillus* strain A460-4-2-7 grown under the same conditions failed to yield BPA. A clue to its source came from observation that new polycarbonate flasks were used in the first culture of *Bacillus* strain A460-4-2-7, whereas old, repeatedly washed polycarbonate flasks had been used in the subsequent cultures where no BPA had been detected. That observation led us to test the hypothesis that BPA was being released from new flasks as a consequence of microbial growth. *E. coli* strain C was used for these experiments because there has been no report of BPA production by this species, in spite of its widespread use. Further, *E. coli* is often the choice for production of recombinant chemotherapeutic proteins, whose detection might be hindered by the presence of BPA. For all the experiments, a single large volume of TSB + G medium was prepared in a thoroughly washed glass flask and dispensed into different flasks as described in Table 1. To

analyse for the presence of BPA in the *E. coli* extracts, the method described above for *Bacillus* strain A460-4-2-7 was used and a seven-point standard curve was generated over the range of 5 μg to 62·5 ng using triplicate injections of 50 μl for each point ($r^2 > 0{\cdot}99$ for the equation $y = (3{\cdot}76 \times 10^3)x + 2{\cdot}77 \times 10^5$). The limits of quantification and detection were 170 and 51 μg of BPA per litre of culture broth, respectively.

Table 1 Bisphenol A detection experiments with *E coli* strain C

Flask No.	Growth flask	Centrifuge bottle	Medium, inoculation and incubation conditions	Concentration of BPA§
1	Rinsed*	Washed†	TSB + G medium, never in flask, not autoclaved, transferred to centrifuge bottle and frozen	ND**
2	Rinsed	Washed	TSB + G medium, autoclaved in flask, cooled and immediately transferred to centrifuge bottle and frozen	ND
3	Rinsed	Washed	TSB + G medium, autoclaved in flask, incubated at 30°C for 5 days, transferred to centrifuge bottle, frozen	ND
4	Rinsed	Washed	TSB + G medium, autoclaved in flask, cooled, inoculated with *E. coli*, incubated at 30°C for 5 days, transferred to centrifuge bottle, frozen	2·8
5	Glass‡	Rinsed	TSB + G medium, never in flask, not autoclaved, transferred to centrifuge bottle and frozen	ND
6	Glass	Rinsed	TSB + G medium, autoclaved in flask, cooled, immediately transferred to centrifuge bottle and frozen	ND
7	Glass	Rinsed	TSB + G medium, autoclaved in flask, incubated at room temperature for 5 days, transferred to centrifuge bottle, and frozen	ND

*New flasks and centrifuge bottles rinsed three-times with 100 ml distilled H_2O.
†Centrifuge bottles washed thoroughly as described in Materials and Methods.
‡Glass flasks washed thoroughly as described in Materials and Methods.
§mg of BPA per litre of culture.
**ND, Below the limit of detection (0·05 mg BPA l^{-1}).

The only *E. coli* culture combination yielding BPA was Flask 4 (2·8 mg l^{-1} of culture); namely *E. coli* strain C grown in 500 ml of TSB + G medium for 5 days with aeration (60 rev min^{-1}) at 30°C in a new, rinsed polycarbonate flask (Table 1). BPA was not detected in either the *E. coli* or *Bacillus* cultures grown in thoroughly washed, new polycarbonate flasks (data not shown). A repeat of that experiment with *E. coli* strain C gave the same result; only Flask 4 yielded BPA (data not shown). BPA was not detected in the medium in an uninoculated flask incubated in parallel for 5 days with aeration (60 rev min^{-1}) at 30°C (i.e. Flask 3, Table 1). Further, cultures of *E. coli* grown in glass flasks and transferred to new, rinsed centrifuge bottles for storage and transfer (Flasks 5–7, Table 1), did not yield any BPA, demonstrating that microbial growth or metabolism, not simply autoclaving and storage, led to the appearance of BPA. In contrast to the work of Krishnan *et al.* (1993), BPA was not detected in the autoclaved medium (Flask 2). That is likely due to the fact that autoclaving time used here was 15 min, half the time used by Krishnan *et al.* (1993).

Discussion

The results of the experiments described herein identify the source of BPA in cultures of the nonobligate predator *Bacillus* sp. strain A460-4-2-7. Although the results do not rule out the possibility that a micro-organism might exist that can synthesize BPA, particularly in light of the fact that microbial-mediated BPA degradation has been reported (Stone and Watkinson 1983; Lobos *et al.* 1992; Spivack *et al.* 1994; Staples *et al.* 1998), the experiments make it likely that the appearance of BPA in the *Bacillus* cultures was due to microbial-mediated release from inadequately washed polycarbonate flasks. This is the first report demonstrating that growth or metabolism of either *E. coli* or *Bacillus* sp. was required for BPA release. Other factors such as alkalinity could also promote BPA release (Biles *et al.* 1997b). Autoclaving and incubation of uninoculated medium for 5 days with aeration at 30°C was insufficient for release of BPA. However, autoclaving for periods of 30 min at 121°C does result in BPA release in the absence of microbial growth (Krishnan *et al.* 1993). Fortunately, that length of autoclaving is usually avoided by microbiologists, since this could cause hydrolysis of proteins and polysaccharides and carmelization of sugars.

Our objective in writing this report is to alert microbiologists to the possible appearance of BPA in cultures of micro-organisms in inadequately washed polycarbonate containers. This amount of a nuisance material has the potential to derail a natural products chemistry investigation of such a culture, where amounts of promising, biologically active secondary metabolites could be less than 3 mg l^{-1}, especially, before the culture conditions are optimized (Cain *et al.* 2000, 2003). BPA has both antimicrobial (Fabig 1988; Staples *et al.* 1998) and oestrogenic activities (Krishnan *et al.* 1993; Staples *et al.* 1998), and thus its appearance in cultures could lead bioassay-based drug discovery in the wrong direction. Because the polycarbonate flasks ordered at two different times released BPA upon cultivation of either *Bacillus* sp. or *E. coli*, this phenomenon evidently reflects a general characteristic of the polycarbonate flasks. However, it is possible that not all polycarbonate flasks share this characteristic, and we did not perform a survey of different sources to explore this question. Further, growth or metabolism of some micro-organisms may not provide the conditions for extraction of BPA from polycarbonate flasks. Rather, our suggestion is to thoroughly wash all polycarbonate containers as described in the materials and methods and avoid high temperature and caustic conditions, because BPA extraction is higher at alkaline pH (Krishnan *et al.* 1993). The microbial-mediated release described here was not due to alkalinity; although the final pH of the *Bacillus* sp. culture was 8·3, that for *E. coli* strain C was 7·08. Finally, one thorough washing is sufficient to reduce BPA release to undetectable levels (Biles *et al.* 1997b).

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