Extremophiles (2007) 11:133–143
DOI 10.1007/s00792-006-0024-3

ORIGINAL PAPER

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A catalytic carbohydrate contributes to bacterial antibiotic resistance

Received: 31 May 2006 / Accepted: 10 July 2006 / Published online: 18 October 2006

Abstract Penicillins are widespread in nature and lethal to growing bacteria. Because of the severe threat posed by these antibiotics, bacteria have evolved a wide variety of strategies for combating them. Here, we describe one unusual strategy that involves the activity of a catalytic carbohydrate. We show that the cyclic oligosaccharide, β-cyclodextrin (βCD), can hydrolyze, and thereby inactivate, penicillin in vivo. Moreover, we demonstrate that this catalytic activity contributes to the antibiotic resistance of a bacterium that synthesizes this oligosaccharide in the laboratory. Taken together, these data not only expand our understanding of the biochemistry of penicillin resistance, but also provide the first demonstration of natural carbohydrate-mediated catalysis in a living system.

Keywords Catalysis · β-lactamase · Alkaliphile · Cyclodextrin · Resistance

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Communicated by K. Horikoshi.

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Introduction

Cyclodextrins are cyclic oligosaccharides containing six to eight α 1-4 linked glucose residues that certain bacteria synthesize from long-chain starch molecules present in the surrounding environment (Szetjli 1998). These oligossaccharides are synthesized outside the bacterial cell by a secreted enzyme, cyclodextrin glycosyltransferase (CGTase) (Fiedler et al. 1996). This enzyme cleaves and cyclizes linear starch molecules to form cyclodextrins which accumulate in millimolar quantities in the extracellular environment. The cyclodextrins are then transported into the cell by a specific uptake system, where they are utilized for carbon and energy.

Cyclodextrins possess well-documented biophysical properties that appear unrelated to their role as carbon sources (Szetjli 1998). These include the ability to bind small molecules and catalyze assorted chemical reactions at pH 10 (Bender and Komiyama 1978; Breslow and Huang 1991; Breslow and Dong 1998). These striking properties result from the three-dimensional structure of cyclodextrins, which are shaped like lamp shades and contain a hydrophobic cavity that can form inclusion complexes with guest molecules. β-cyclodextrin (βCD), which contains seven glucose residues, can hydrolyze the β-lactam ring of penicillins and thereby inactivate the antibiotic under alkaline conditions (Tutt and Schwartz 1970, 1971; Hada et al. 1997). The mechanism of this reaction mimics the well-studied β-lactamase enzymes (Chen et al. 1993), and proceeds in a step-wise fashion (Tutt and Schwartz 1970, 1971). First, a non-covalent

complex between βCD and substrate forms. Next, appropriately positioned βCD alkoxide ions attack the labile β-lactam ring of the substrate, forming an acyl-intermediate. Finally, the intermediate is hydrolyzed and released to produce the inactivated antibiotic and regenerate the βCD catalyst. Importantly, stereospecificity and Michaelis–Menten kinetic parameters have been demonstrated for βCD-mediated penicillin hydrolysis, thus establishing it as a bonafide catalytic phenomenon (Tutt and Schwartz 1970, 1971).

Despite more than 30 years of research into the chemistry of cyclodextrins, and their use in the chemical and pharmaceutical industries, no in vivo biological significance has been attributed to the catalytic activities of these molecules. Moreover, there has been no suggestion that βCD-mediated β-lactamase activity has any relevance for the organisms that produce the carbohydrate. Because optimal βCD catalysis occurs only under alkaline conditions (pH > 10), it has been viewed solely as a laboratory phenomenon. An emerging interest in microbes that grow optimally at high pH (Horikoshi 1991, 1999), however, serves to challenge these assumptions. This group of microbes, the alkaliphiles, constitutes an important component of the terrestrial microbial environment, and includes many cyclodextrin-producing bacteria (Nakamura and Horikoshi 1976a, b, c). Horikoshi and colleagues have reported, for example, that cultivatable alkaliphiles can be recovered (10^2–10^5 CFU/g) from both alkaline and non-alkaline soil samples (Horikoshi 1999). In addition, some of these bacterial isolates possess protein β-lactamases, thus providing compelling evidence that β-lactam antibiotic challenge is a real phenomenon in the growth environment of these microbes (Kato et al. 1989).

We describe here a model biological system where a βCD-producing alkaliphile (ATCC 21594) shows penicillin resistance and βCD-mediated β-lactamase activity in the laboratory. These data expand our understanding of biological catalysis and raise the possibility that catalytic carbohydrates may contribute to bacterial antibiotic resistance in the environment.

Materials and methods

Media and reagents

Growth media

All recipes are per 1 l and the medium pH is 10.2 unless otherwise specified. All starch-containing media were stirred on a hot plate and heated overnight to fully dissolve the starch after autoclaving.

Complex media

Complex media were made using a 4× concentrated stock of alkaline medium (ALKM) solution containing peptone (20 g), yeast extract (20 g), K_2HPO_4 (4 g), $MgSO_4$ (0.8 g). The 4× concentrated ALKM stock was employed to make glucose-, starch-, and glucose/βCD-containing complex media (ALKM/G, ALKM/S, and ALKM/G/βCD, respectively).

Alkaline medium/glucose contained one quarter volume of 4× ALKM (250 ml), 1% Na_2C0_3 (final), and 40% filtered glucose (25 ml). ALKM/S was prepared in a similar fashion, except that soluble starch (15 g) was substituted for glucose. ALKM/G/βCD contained 1/4 volume of 4× ALKM (250 ml), 1% Na_2C0_3 (final), 40% glucose (25 ml), and βCD powder (5.67 g). All solutions were either autoclaved or filter sterilized before use.

Minimal media

Some experiments employed defined minimal media. These minimal media were derived from a basic minimal medium (Min) that contained the following (per liter): 20× M9 Salts (50 ml), 1 M $MgSO_4$ (2 ml), 1 mg/ml thiamine (2 ml), 1 M $CaCl_2$ (100 µl), 10 µg/ml Biotin (200 µl), 10% Na_2CO_3 (100 ml). Twenty times M9 salts contained [Na_2HPO_4 (120 g), KH_2PO_4 (60 g), NH_4Cl (20 g), pH 7.4] per liter. All solutions were filter sterilized (0.22 µm filter) before use. Minimal-glucose (Min/G) and Minimal starch (Min/S) media were prepared by adding 40% glucose (25 ml) or 15 g soluble starch to 1 l Min media. Minimal glucose medium containing βCD (Min/G/βCD) was prepared by adding 40% glucose (25 ml) and βCD (5.67 g) to Min media. Some media contained phenolphthalein (0.0004%), methyl orange (0.005%) or starch azure (0.75%). Solid growth media were prepared the same as liquid media, except that bactoagar was added to a final concentration of 2%.

Conditioned media

Conditioned media were made in a number of steps. First, from a frozen stock culture, bacteria were streaked onto ALKM/G plates. Single colonies were then picked to fresh ALKM/G medium (5 ml) and incubated overnight at 37°C with rotary shaking. An amount of 0.5 ml of this overnight culture was used to seed 50 ml of the Min to be conditioned. This culture was incubated overnight at 37°C with shaking, and then centrifuged (Sorvall RC5B, SS34 Rotor, 10,000*g*, 4°C) to pellet the bacteria. The supernatant was collected and then boiled for 30 min to kill bacteria and denature proteins. After boiling, the pH of the medium was adjusted to 10.2 (by titration with 1 M NaOH), and the medium was boiled again (30 min). Finally, the boiled solution was cooled on ice, centrifuged as before to remove insoluble material, and employed as described. This medium was routinely plated on solid ALKM and incubated at 37°C for 48 h to confirm that it was sterile.

Reagents

Cyclodextrins were obtained from Sigma Chemical Company (St. Louis, MO, USA), and were stored as per the manufacturers recommendations. All antibiotics (Sigma Inc.) were prepared as 100 mg/ml stock solutions, filter sterilized, and stored at −80°C. Sterile antibiotic disks were obtained from Becton, Dickinson & Co. (Sparks, MD, USA) and used and stored as per the manufacturers recommendations. Nitrocefin was purchased from EMD Biosciences (San Diego, CA, USA), and stored as a 100 mM stock solution in accordance with the manufacturer's instructions. Water and organic solvents used in analytical experiments were high performance liquid chromatography (HPLC) grade and obtained from Fisher Inc. (Houston, TX, USA). All common laboratory reagents were obtained from Sigma Chemical Company.

Strains

Bacillus ATCC 21594 (JCM 9147) was obtained from the American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* TOP10 cells were obtained from Invitrogen Inc. (Carlsbad, CA, USA). *Bacillus subtilis* strain ALKO 2013 was a generous gift from Drs. Marja Paloheimo and Eino Vaisanen (Roal Oy, Rajamaki, Finland) (Paloheimo et al. 1992).

Isolation of mutant *Bacillus* strain (ATCC 21594)

Methane sulfonic acid ethyl ester (EMS) (Sigma Inc.) was used to mutagenize *Bacillus* ATCC 21594, as described by Burkholder et al. (2001). Briefly, 50 µl of a *Bacillus* culture grown overnight in ALKM/G was used to seed 5 ml of freshly prepared ALKM/G media. These cultures were incubated in a rotary shaker at 37°C until the optical absorbance at 600 nm of visible light (Ab_{600}) reached 1.0, and then centrifuged in a clinical centrifuge to pellet the cells. The bacterial pellet was washed twice with ice-cold dH_2O, and then resuspended in 5 ml ice-cold phosphate-buffered saline (PBS, pH 7.4). Various amounts of EMS were added (0–1.5% final concentration) to the resuspended cells, which were then incubated at room temperature (RT) in an end-over-end mixer for either 30 or 60 min. Cells were placed on ice, washed three times with ice-cold ALKM/G, and finally incubated for 60 min at 37°C on a rotary shaker (to allow recovery from the EMS treatment), diluted 1:1 with glycerol, and stored at −80°C. The kill frequency of frozen stocks was assessed as previously described (Burkholder et al. 2001). Stocks with kill frequencies between 80 and 90% were used for the screen. *Bacillus* strain BT13 (see below) was isolated following screening cells that had been subjected to treatment with 1.5% EMS for 30 min (kill frequency = 84%).

Screening for β-cyclodextrin mutants

β-cyclodextrin indicator plates (Park et al. 1989) were employed to screen for *Bacillus* ATCC 21594 mutants with defects in βCD accumulation. Pure βCD (but not pure glucose or αCD) spotted onto these red plates turns the plates yellow. Similarly, colonies that accumulate βCD generate yellow halos, and colonies with defects in βCD production generate small or no halos at all. To perform the screen, EMS-mutagenized cells were thawed and incubated in ALKM/G for 60 min at 37°C. The cells were then diluted $1{:}10^6$, and 100 µl of the diluted sample were plated onto βCD indicator plates, resulting in about 100 colonies per indicator plate. The plates were inverted, incubated for 72 h at 37°C, and then screened for colonies with decreased halo formation. Of 80,000 colonies screened, 32 colonies possessed diminished halos and were re-screened. One strain (BT13) was used in all subsequent experiments.

Growth curves

Single colonies picked from complex solid media plates were used to seed media that were then incubated overnight. Samples (50 ml) from these cultures were then diluted into Min (5 ml) and grown to Ab_{600} = 0.3. Samples of these cultures were then diluted into the same medium and the Ab_{600} of the cultures were determined at approximately 2-h intervals for 24 h using a Hitachi U-2000 spectrophotometer.

Antibiotic resistance assays

Liquid assays

Overnight cultures of *Bacillus* ATCC 21594 strains were used to seed fresh or conditioned minimal media (5 ml). The cultures were then incubated at 37°C with rotary shaking until mid-log phase (Ab_{600} = 0.6). Five milliliter of the same media were seeded with 50 µl of these mid-log phase cultures. When the cultures reached early log phase (Ab_{600} = 0.3), various amounts of antibiotics were added, and the optical density was measured every 2 h, over 16 h. A 40-h measurement was also taken. Finally, the absorbance at 600 nm was plotted as a function of antibiotic concentration, and the concentration of antibiotic that inhibited growth by 50% (IC_{50}) was estimated. All experiments were repeated at least three times. The results were averaged, and the standard deviations calculated.

Plate assays

Antibiotic resistance was determined using the Kirby–Bauer disk agar diffusion procedure (Bauer et al. 1966) using the following antibiotic discs: no antibiotic

(control); ampicillin (10 mg); cefotaxime (10 mg); erythromycin (15 mg); gentamicin (10 mg); novobiocin (5 mg); rifampin (5 mg); nalidixic acid (30 mg); streptomycin (10 mg). Cell pellets from overnight cultures grown in minimal media were washed three times with sterile dH_2O, resuspended in the same, and then plated on solid minimal media. A single antibiotic disk (Becton, Dickinson & Co.) of standard concentration (i.e., 10 mg/ml) was placed on each plate. Plates were inverted, incubated at 37°C for 48 h, and the diameter of the zones of clearance around disks were measured in millimeter. At least six replicates were performed for each condition tested, and the results reported represent the average.

PCR detection of β-lactamase genes

To investigate whether *Bacillus* ATCC 21594 cultures were contaminated with laboratory strains harboring plasmid-encoded β-lactamase genes, we periodically performed PCR (35 cycles of 95°C 1 min, 52°C 1 min, and 72°C 2 min) using an Eppendorf Mastercycler (Eppendorf Inc., New York, NY, USA) on boiled whole cell extracts of *Bacillus* cells using primers complementary to these gene sequences (AMP forward, ATG AGT ATC CAA CAT TTC CGT GTC; AMP reverse, TTA CCA ATG CTA ATC AGT GAG GC). The β-lactamase positive pBAD TOPO 2.1 plasmid (Invitrogen) alone was used as a positive control in these experiments. No *Bacillus* ATCC 21594 cultures harboring these gene sequences were ever found. In addition, we employed previously described methods and primers to perform PCR on boiled whole cell extracts of *Bacillus* cells grown in starch (Bert et al. 2002). These experiments failed to detect any β-lactamase genes coding for the PSE, CARB, OXA, LCR-1, TEM and SHV groups of protein β-lactamases (Bert et al. 2002), or the lipo-penicillinase from *Bacillus sp*. strain 170 (Kato et al. 1989).

Biological assay for cell-associated β-lactamase activity

A biological assay was employed to examine β-lactamase activity in *Bacillus* ATCC 21594 cells grown in the absence of starch. Briefly, $\sim 10^6$ TOP10 *E. coli* cells harboring the β-lactamase-expressing plasmid (pBAD-TOPO 2.1) (Invitrogen), or $\sim 10^9$ *Bacillus* cells, were lysed by sonication (3 × 15 s) on ice using a Cole Parmer Ultrasonic Homogenizer (Model 4710) with MicroTip. The duty cycle and output control were set at 50% and 4, respectively. The lysates were clarified by centrifugation in a Spectrafuge 16 M Microfuge (Labnet Inc., Spanish Fort, AL, USA) at 13.3*g* for 5 min in the cold, and then the supernatant was passed through a 22 μM filter. The filtrate was spotted to the center of Luria-Bertani (LB) agar plates containing 10 μg/ml aminobenzylpenicillin. Plates were inverted and incubated at 37°C for 24 h. Finally, the plates were spread with a lawn of penicillin-sensitive *B. subtilis* cells (strain ALKO 2013), and bacterial growth assessed by visual inspection after incubation at 37°C for 24 h.

Nitrocefin assays

Single colonies, picked from freshly streaked ALKM/S plates, were inoculated into 5 ml overnight cultures in ALKM/S medium. These cultures were then used to seed 5 ml cultures in Min/S media. *B. subtilis* (ALKO 2013) and *E. coli* (TOP10, pBAD-TOPO 2.1) cells were also picked from LB plates to LB liquid medium. These strains served as positive and negative controls, respectively. Cultures were incubated with shaking at 30°C for 48 h. Because *Bacillus* (ATCC 21594) might harbor β-lactamase genes that are induced by β-lactam antibiotics, a sub-lethal dose (1 μg/ml) of aminobenzylpenicillin was added to cultures to induce this expression. The bacteria were then incubated for 1 h at 30°C, pelleted, washed with 5 ml ice-cold PBS, pH 7.4, and resuspended in 1 ml of the same. The cells were sonicated, and the lysates clarified as described above. Next, nitrocefin (10 μl/ml) was added to the clarified material. In some experiments, the lysates were boiled for 30 min and clarified by micro-centrifugation before nitrocefin addition. Finally the nitrocefin reaction mixtures were incubated at RT for 20 min, clarified by centrifugation (2 min at RT), and the optical absorbance at 486 nm (Ab_{486}) recorded using a Hitachi U-2000 spectrophotometer. Experiments were performed in triplicate, and the averages and standard deviations were calculated and reported.

In vitro β-cyclodextrin-mediated aminobenzylpenicillin hydrolysis reactions

Typically, an 800 μl reaction was prepared by mixing aminobenzylpenicillin (67 μM final) with (a) pure βCD between 0.7 μM and 5 mM final concentration with ice-cold complex or minimal media (pH 10.2), or (b) various amounts of conditioned complex or minimal media. The reaction mixtures were incubated in a water bath at 37°C for various lengths of time, and then stopped by rapidly placing them on ice. Finally, 100 μl of each sample were removed and analyzed by HPLC as described below.

Reverse phase high performance liquid chromatography analysis of aminobenzylpenicillin hydrolysis

Aminobenzylpenicillin hydrolysis was analyzed using a modification of a previously described method (Ishida et al. 1999). Briefly, for hydrolysis reactions, 12.5 mg of aminobenzylpenicillin was dissolved in 1 ml of the appropriate buffer and incubated for various lengths of time (see Figure legends). The reaction mixture was then passed through a C-18 solid phase extraction disk.

The HPLC system consisted of a Varian Prostar 230 HPLC, column oven, and a Waters 484 UV detector. The column was a Waters μ bondapak™ C-18 (250×4.6 mm^2). The mobile phase consisted of acetonitrile and 10 mM NaH_2PO_4 (6.5:93.5). The column temperature was maintained at 30°C with the UV detector set at 220 nm. The flow-rate was maintained at 1.0 ml/min throughout the analysis. Aminobenzylpenicillin peak heights of duplicate experiments varied by less than 10%.

In some experiments, a previously described reverse phase high-performance liquid chromatography-mass spectrometry (LC-MS) method (Holstege et al. 2002) was modified and employed to analyze the products of reaction mixtures. Separation was performed on an Agilent 1100 liquid chromatograph using the same C-18 reverse phase column as for LC-UV analysis described above (Waters μ Bondapak 250×4.6 mm^2). Analytes were eluted (with a gradient of water/methanol containing 0.05% formic acid at a flow rate of 0.75 ml/min) directly into the electrospray ion source of an ion trap mass spectrometer operating in positive mode (EsquireLC, Bruker Daltonics, Coventry, UK). The retention times for aminobenzylpenicillenic acid (PA) ($C_{16}H_{21}N_3O_5S$ MW 367) and aminobenzylpenicillin (PEN) ($C_{16}H_{19}N_3O_4S$, MW 349) were 11.8 and 13.6 min, respectively. Quantitation was performed as previously described (Holstege et al. 2002). All experiments were performed in triplicate and the results of representative experiments are shown.

High performance thin layer chromatography analysis

Mono- and oligosaccharides were separated on silica gel 60 high performance thin layer chromatography (HPTLC) plates (Merck, Whitehouse Station, NJ, USA) in (*n*-butanol-pyridine-water (70:15:15) or chloroform/methanol/water (35:60:15) and visualized by orcinol staining (Stahl 1969). The amounts of βCD on plates were determined by scanning densitometry. Supernatants from starch-cultures grown in complex or minimal media for 24 h were found to contain 8.8 and 7.7 mM βCD, respectively.

Positive- and negative-ion electrospray ionization-mass spectrometry of β-cyclodextrin-penicillin solution

A diluted solution of βCD and penicillin in water/methanol (10 μM) was infused directly into the ion source of the LCQ Duo ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) at a flow rate of 5 ml/min. The spray voltage was set to 5 kV and capillary temperature to 220°C. Low energy collision induced dissociation was carried out at a relative collision energy of 35–45%.

Results

To assess the possibility that a cyclodextrin can confer resistance to penicillin on organisms that synthesize this oligosaccharide, we analyzed an alkaliphilic strain of *Bacillus*. The *Bacillus* strain ATCC 21594 was isolated and characterized more than 30 years ago (Yamamoto et al. 1972), thrives above pH 10, and produces large amounts of extracellular βCD (7.7 mM) when grown with starch. Conversely, no βCD was detected in cultures grown without starch (Fig. 1a). Therefore, starch provides a convenient and natural switch for modulating βCD synthesis and bacterial antibiotic resistance. In addition, when *Bacillus* (ATCC 21594) is cultured in weakly alkaline (pH 9.0) medium containing glucose or starch, the pH of the medium does not increase over time (data not shown). Thus, the pH of the bacterial culture medium in this system can be tightly controlled.

We employed aminobenzylpenicillin (PEN) in all experiments, and assayed resistance by two methods. First, we added various amounts of the antibiotic to early log phase cultures and assessed bacterial growth by measuring the optical density of cultures following antibiotic addition. This assay determined the concentration of antibiotic that inhibited growth by 50% (IC_{50}). Second, we measured zones of clearing around antibiotic disks placed on solid media spread with a lawn of *Bacillus* cells. Measurement of the clearance radius provides a quantitative method for assessing antibiotic resistance (Bauer et al. 1966).

Penicillin resistance increases in β-cyclodextrin

To modulate βCD synthesis, we grew bacterial cultures in both liquid and on solid media, with and without starch. As predicted, cultures grown in the absence of starch exhibited negligible penicillin resistance (IC_{50} = 7 μg/ml). Upon addition of starch, however, penicillin resistance was observed at an IC_{50} of up to 450 μg/ml, a 64-fold increase (Fig. 2a). Further, the zone of clearing was reduced (Table 1). Importantly, no carbon sources other than starch induced penicillin resistance (data not shown), and starch did not induce resistance to non-penicillin antibiotics (Fig. 2b). Moreover, the degree of penicillin resistance correlated directly with the amount of starch present in the culture medium (data not shown). These observations demonstrate that only starch stimulated the production of a specific and βCD dose-dependent resistance to penicillin.

We next assayed β-lactamase activity in medium conditioned by growth of the alkaliphile (Fig. 3a). This medium, which harbored protein, carbohydrate, lipid, and other organic components derived from the bacterium, was prepared by growing bacteria in liquid media followed by removal of bacteria. Penicillin was incubated at 37°C for various lengths of time with conditioned medium from either starch-supplemented

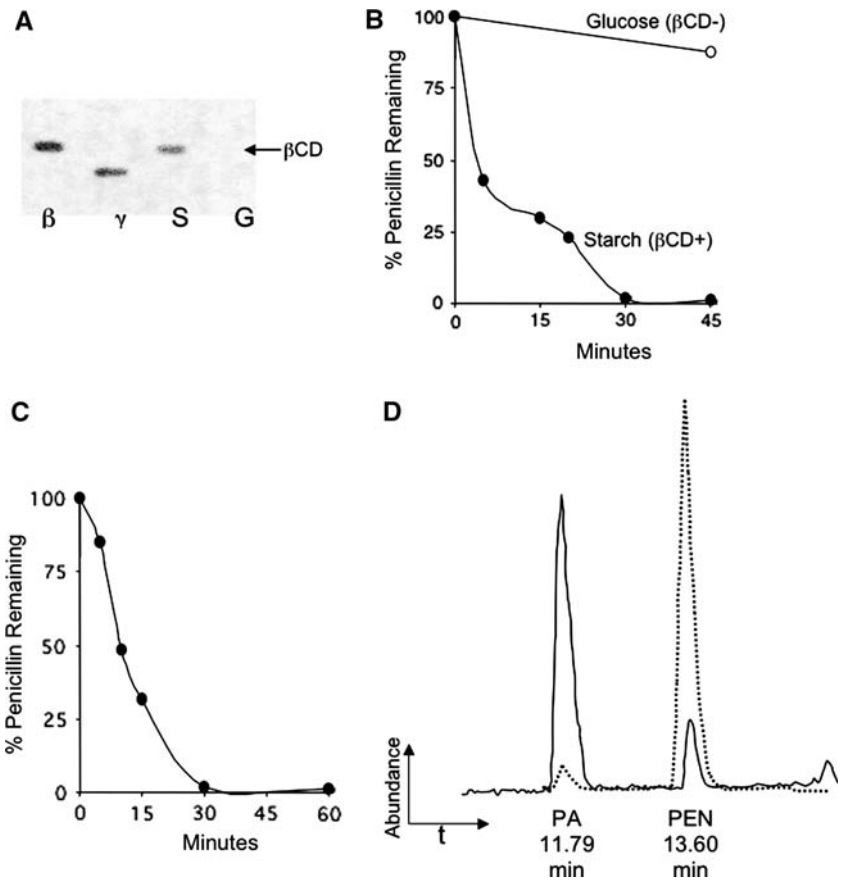

Fig. 1 Analysis of *Bacillus* growth medium and penicillin hydrolysis. **a** HPTLC of conditioned medium following the growth of *Bacillus* with starch (*S*) or glucose (*G*). Pure *β*CD (*β*) and *γ*CD (*γ*) standards are also shown. **b** Conditioned medium or **c** pure *β*CD was incubated with aminobenzylpenicillin (*PEN*) for various lengths of time. The unhydrolyzed penicillin substrate was then analyzed and quantified by HPLC. **d** Aminobenzylpenicillenic acid (*PA*) accumulates in reaction mixtures containing PEN and conditioned medium from cultures grown with starch. Reaction mixtures were analyzed by LC-MS as described, and total ion chromatograms displaying the abundance of penicillin and penicillenic acid after 0 min (*dotted line*) and 60 min (*solid line*) incubations are shown

cultures, or from control cultures grown without starch. After 30 min incubation with conditioned medium from starch-supplemented cultures, the amount of penicillin remaining in the reaction was less than 2% of controls (Fig. 1b). This striking loss of penicillin was accompanied by a corresponding increase in aminobenzylpenicillenic acid, a product of penicillin hydrolysis (Fig. 1d). Importantly, both glucose-grown (control) and starch-grown culture supernatants were examined for *β*-lactamase activity under similar alkaline conditions (see Materials and methods). Therefore, the differences in penicillin hydrolysis observed in these samples could not be attributed to the alkaline pH of the medium. Instead, these data indicate that starch-conditioned media in which the *Bacillus* had grown harbored a penicillin-hydrolyzing activity.

Protein *β*-lactamase activity

Several experiments were performed to examine the possibility that proteins with *β*-lactamase activity were present in media conditioned by this bacterium. First, conditioned media from starch-grown cultures were boiled and then assayed for *β*-lactamase activity on penicillin. These media, predicted to contain denatured proteins and *β*CD, retained *β*-lactamase activity comparable to that of non-boiled control media (data not shown). Second, a biological *β*-lactamase assay failed to detect *β*-lactamase protein enzymes in glucose-grown (*β*CD-free) conditioned media (Fig. 4b). Third, experiments with nitrocefin, a *β*-lactam containing chromogenic substrate recognized by all known protein *β*-lactamase enzymes (Livermore and Brown 2001), were performed to examine the role that protein *β*-lactamases play in this system. Specifically, *Bacillus* cells were grown in starch or glucose-containing ALKM, and then washed, lysed, and incubated with nitrocefin in PBS at neutral pH. In this neutral buffered system, *β*CD alone fails to hydrolyze the chromogenic indicator (data not shown). However, robust *β*-lactamase activity was detected in the positive control sample derived from *E. coli* cells harboring a plasmid containing a penicillin resistance cassette (Fig. 5). Taken together, the data indicated that the differences in *β*-lactamase activity observed in glucose and starch-grown cultures could not be attributed to protein activity alone. Finally, degenerate PCR experiments using DNA from the *Bacillus* strain failed to detect *β*-lactamase genes known to be present in other penicillin resistant bacterial strains, including the PSE, CARB, OXA, LCR-1, TEM, and SHV group *β*-lactamases commonly found in gram-negative bacteria (Bert et al. 2002), and the lipo-penicillinase from the alkaliphilic *Bacillus sp*. strain 170 (data not shown) (Kato et al. 1989). Taken together, these data indicate that non-protein components contributed

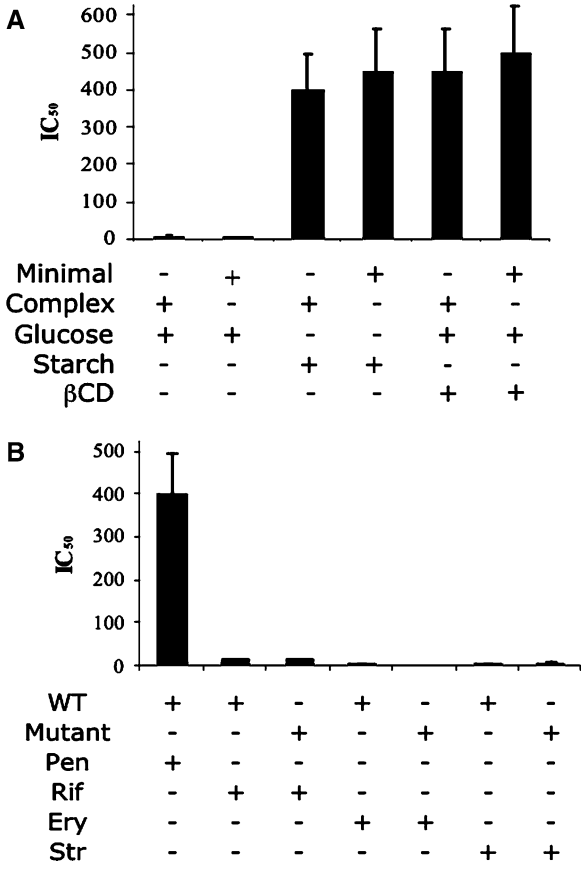

Fig. 2 *β*-cyclodextrin effects on antibiotic resistance. **a** *Bacillus* was grown in complex or minimal media in the presence or absence of starch, glucose, and *β*CD. The IC_{50}s for aminobenzylpenicillin (*PEN*) were then determined. **b** Wild-type (*WT*) and mutant *Bacillus* resistance to penicillin PEN, rifampin (*RIF*), erythromycin (*ERY*), and streptomycin (*STR*) in starch

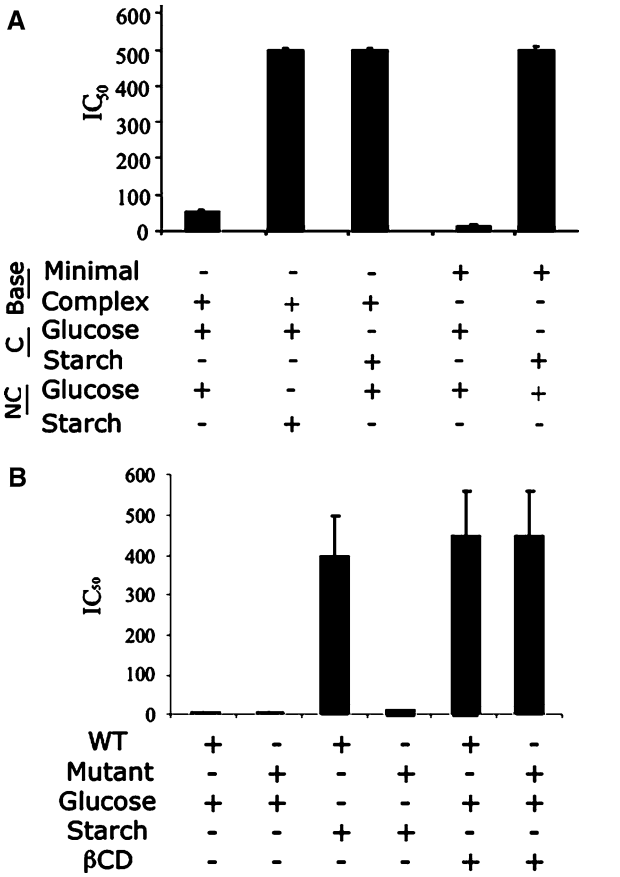

Fig. 3 Penicillin resistance in wild-type and mutant *Bacillus* when assayed in liquid media. **a** Resistance to PEN in cultures containing mixtures of conditioned (*C*) and non-conditioned (*NC*) media in the presence of a base medium (*base*). **b** Complementation of PEN resistance in mutant and wild-type (*WT*) strains by the addition of glucose, starch, or *β*CD

Table 1 *β*-cyclodextrin contributes to bacterial penicillin resistance on solid minimal media containing combinations of *β*CD (5 mM), glucose, and starch

Carbon source			Clearance (mm)
Glucose	Starch	*β*CD	
+	–	–	8.1
–	+	–	3.3
+	–	+	1.7
–	+	+	0.0

Antibiotic discs containing penicillin (10 mg) were added to a fresh lawn of *Bacillus* cells. After ~48 h, zones of antibiotic clearance were measured from the outside of the disc to the closest colony. All data represent the average of at least six replicates

to the *Bacillus* *β*-lactamase activity observed in starch-grown cultures, and suggest that *β*CD played a role in hydrolyzing the antibiotic.

β-Cyclodextrin mediates the hydrolysis and inactivation of penicillin

We examined whether *β*CD *β*-lactamase activity directly contributed to antibiotic resistance in several ways. First, pure *β*CD was added to *Bacillus* cultures grown without starch. Under these conditions, the bacteria demonstrated enhanced resistance to penicillin (IC_{50} = 450 μg/ml) (Fig. 2; Table 1). The corresponding control culture lacking the added oligosaccharide, however, retained its sensitivity to the antibiotic (IC_{50} = 7 μg/ml). Second, we compared the antibiotic resistance of the wild-type and *Bacillus* mutant we isolated that did not accumulate *β*CD (Figs. 2b, 3b, 4). The mutant was almost 50-fold more sensitive to penicillin than the wild-type strain (Figs. 2b, 3b). Importantly, the addition of pure *β*CD to a culture of the mutant bacteria restored the level of penicillin-resistance shown by the wild-type strain (Fig. 3b). However, soluble starch alone, or a methylated form of *β*CD (*β*CD-Met) with impaired ability to form catalytic alkoxide ions, failed to display similar levels of penicillin hydrolysis (data not shown). Taken together, these data indicate that pure *β*CD possesses an alkaline-dependent penicillin hydrolyzing activity and plays a direct role in mediating penicillin resistance.

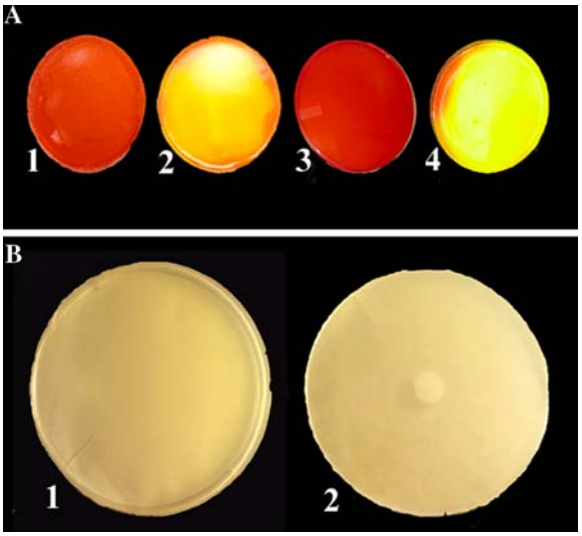

Fig. 4 Phenotypes of wild-type (*WT*) and mutant *Bacillus* cells. **a** βCD accumulation phenotypes resolved on βCD indicator plates. Untreated plates are *red*, but are *yellow* when incubated with βCD. WT *Bacillus* were streaked onto indicator plates containing glucose (and lacking starch) (1) or containing starch alone (2). Mutant cells were streaked onto an indicator plate containing starch (3). Pure βCD powder was sprinkled onto an indicator plate containing glucose and lacking starch (4). All plates were photographed after incubation for 48 h at 37°C. **b** Biological protein β-lactamase assay. Solid medium with penicillin was spotted with (2) a cell-free extract from an *Escherichia coli* strain expressing β-lactamase proteins, or (1) a cell-free extract from *Bacillus* (ATCC 21594). A lawn of penicillin sensitive *B. subtilis* was then plated on this solid medium. Growth of the *B. subtilis* indicator strain is observed on the positive control plate containing the *Escherichia coli* extract (2). However, no growth is observed on the plate spotted with the *Bacillus* (ATCC 21594) extract (1)

Fig. 5 Protein β-lactamase assays. TOP10 *Escherichia coli* carrying pBAD2.1 (Amp^R) (+ control), *B. subtilis* (− control), whole cell lysates from wild-type (*WT*) and BT13 mutant (*mut*) *Bacillus* (ATCC 21594) cells were examined for β-lactamase activity using nitrocefin. Some lysates were boiled to inactivate heat-labile bacterial protein enzymes before the nitrocefin assay was performed

β-Cyclodextrin acts as a catalyst

If βCD is acting as a catalyst, it should not be used up in the reaction. Therefore, we investigated whether βCD was destroyed in our in vitro system. When diluted conditioned Min harboring βCD was incubated with a 100-fold molar excess of penicillin, 98% of the oligosaccharide remained at the end of the reaction (Table 2). Similar results were obtained when conditioned complex media harboring βCD, or pure βCD alone, was employed (99 and 94% of the starting material remained, respectively). Finally, using electrospray ionization mass spectrometry (ESI-MS), we showed that pure βCD physically interacted with penicillin (Fig. 6), and then demonstrated that this interaction led to the hydrolysis and inactivation of the antibiotic at alkaline pH (Fig. 1c, d). No such hydrolysis was seen if glucose was substituted for βCD, if soluble starch alone was employed, or if the reaction was performed at neutral pH. Taken together, these data demonstrate that the oligosaccharide was not consumed in the reaction, and indicate that the molecule is a true catalyst.

Discussion

More than 30 years ago Bender et al. first reported that cyclodextrins have the remarkable ability to catalyze the hydrolysis of small molecules at high pH (Bender and Komiyama 1978). This observation set the stage for the pioneering work of Breslow and colleagues who demonstrated that cyclodextrins and their derivatives can catalyze a wide variety of chemical reactions under non-physiological conditions (Breslow and Dong 1998). We have taken another step in this project by demonstrating cyclodextrin mediated catalysis in a biological system in the laboratory. βCD hydrolyzes the β-lactam ring of penicillin in vivo, and thereby contributes to the penicillin resistance of a model alkaliphilic microbe (*Bacillus* ATCC 21594) that synthesizes this cyclic oligosaccharide.

Bacillus (ATCC 21594) grows under highly alkaline conditions (pH > 10) in both complex and minimal media and produces large amounts of cyclodextrin when starch is available. βCD accumulates as the predominant cyclodextrin component. However, minor amounts of γCD (< 10%) were also observed. Because αCD and γCD do not possess significant β-lactamase activity at alkaline pH this distribution of cyclodextrin species favored our analysis of βCD-mediated catalysis in vivo.

Several lines of evidence indicate that βCD plays a direct role in penicillin hydrolysis. Catalysis was not observed in reactions where βCD was absent, and penicillin was not hydrolyzed in control reactions containing glucose or βCD-Met. However, when penicillin was added to sterile medium containing βCD or to βCD-containing conditioned medium, antibiotic hydrolysis was dramatically accelerated. These data are consistent with previous reports demonstrating Michaelis–Menten kinetic parameters for the βCD-mediated hydrolysis of penicillin in water (Tutt and Schwartz 1970, 1971). Although we failed to detect β-lactamase gene sequences in

Table 2 β-Cyclodextrin is not consumed by co-incubation with penicillin

Medium	Conditioned medium?	Carbon source	Pure βCD added?	βCD (ng) 0 min	βCD (ng) 60 min	βCD remaining (%)
Minimal	+	Glucose	–	0	0	NA
Minimal	+	Starch	–	342	336	98
Complex	–	Glucose	+	221	209	95
Complex	–	Starch	–	387	382	99

Pure βCD was incubated for 60 min in sterile minimal or complex media, or conditioned media derived from starch or glucose grown cultures, containing a 100-fold molar excess of penicillin. βCD remaining at the end of the reaction was resolved by high performance thin layer chromatography (*HPTLC*) and quantified using scanning densitometry
NA not applicable

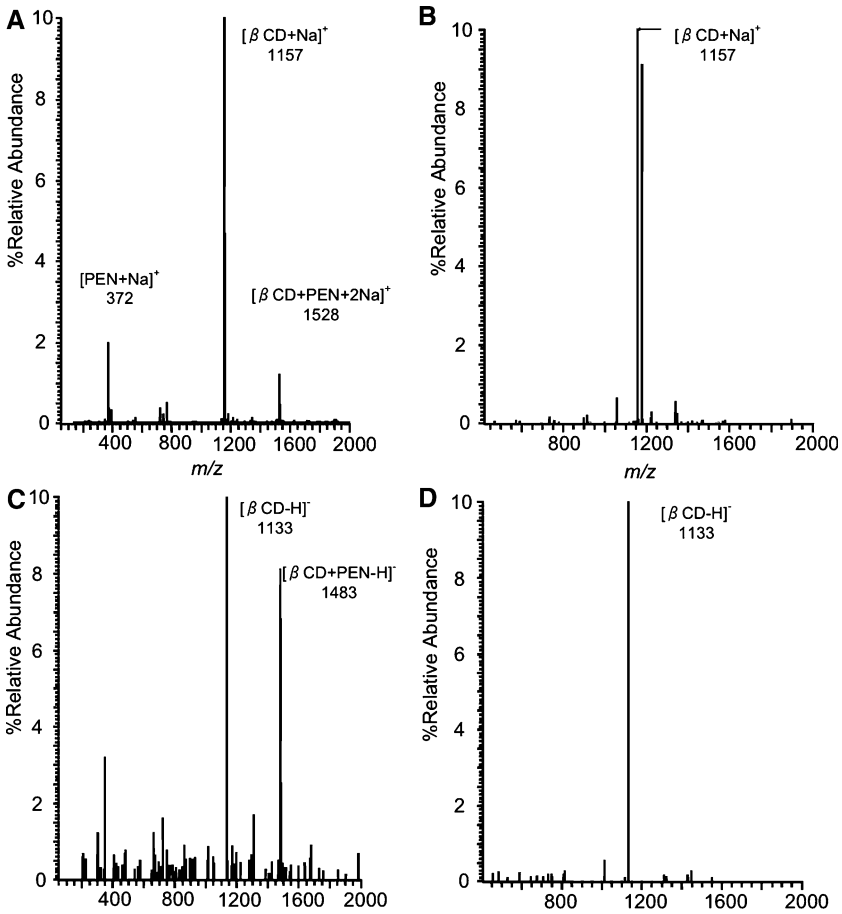

Fig. 6 Electrospray ionization mass spectrometry (*ESI-MS*) analysis of βCD and aminobenzylpenicillin (*PEN*). **a** Positive ion mass spectrum reveals PEN, βCD and complex βCD + PEN detected as $[\beta CD + PEN + 2Na]^+$ at m/z 1528. **b** Low energy collision induced dissociation (*CID*) spectrum of the ion at m/z 1528. Similarly, **c** depicts a negative ion mass spectrum of the mixture showing PEN, βCD and the complex detected as $[\beta CD + PEN - H]^-$ at m/z 1483. **d** Low energy CID of the ion at m/z 1483 confirms the complex structure

the *Bacillus* ATCC 21594 genome using established degenerate PCR methods, we cannot rule out the possibility that this microbe contains protein β-lactamase genes. DNA sequence divergence within the protein β-lactamase gene family is large (Livermore and Brown 2001) and includes members outside the gene families tested (Kato et al. 1989; Bert et al. 2002) Therefore, the possibility that this *Bacillus* species harbors a novel β-lactamse gene cannot be excluded. Our nitrocefin and biological protein β-lactamase assays, however, support the conclusion that non-protein components contribute to the *Bacillus* β-lactamase activity observed in starch-grown cultures, and indicate that βCD plays a direct role in hydrolyzing the antibiotic under alkaline conditions in vivo and in vitro.

In contrast to protein catalysts, cyclodextrins are small (MW = ~1 kDa), chemically simple molecules that do not require folding for catalytic activity. Nevertheless, the rate of βCD-mediated penicillin hydrolysis approaches that seen for some protein-catalyzed hydrolysis reactions. For example, the rate of chymotrypsin-catalyzed proteolysis (k_{cat} = 0.14/s) is only approximately ten-fold faster than βCD mediated penicillin hydrolysis (Tutt and Schwartz 1970; Mathews and van Holde 1990). Although the βCD rate is significantly slower than typical protein β-lactamase

catalyzed reactions ($k_{cat} = \sim 1 \times 10^3$/s) (Chen et al. 1993), the oligosaccharide accumulates in millimolar concentrations in bacterial culture supernatants. This high concentration compensates for βCD's catalytic inefficiency, and greatly facilitates bacterial antibiotic clearance.

Since the catalytic activity of βCD requires alkaline conditions, it can only be biologically relevant in alkaline environments, which are surprisingly common in nature. Alkaline soils, lakes, and streams, for example, are typically found in arid climates (Horikoshi 1991, 1999), and alkaline marine environments have been described (Takaki et al. 2004; Takai et al. 2005). In addition, facultative alkaliphilic microbes are found in neutral pH soil samples at high frequencies (Horikoshi 1991). Horikoshi and colleagues have reported, for example, that cultivatable alkaliphiles can be recovered (10^2–10^5 CFU/g) from both alkaline and non-alkaline soil samples (Horikoshi 1991, 1999). Importantly, alkaliphiles that produce cyclodextrin are routinely isolated and cultivated from these soils (Horikoshi 1991, 1999; Salva 1997). Given the prevalence of alkaliphiles and alkaline environments, it is intriguing to speculate that the catalytic potential of these molecules may be widespread.

Our data highlight the multi-functionality of βCD. Cyclodextrin is a carbon source for some bacteria, and it also contributes to the penicillin resistance of cyclodextrin-producing microbes in an alkaline environment. This multi-functionality is reminiscent of that seen for both protein and RNA enzymes. Protein catalysts can play structural roles, and RNA carries both genetic information and catalytic potential (Sumner 1926; Kruger et al. 1982). Multi-functionality may therefore be a hallmark of biological catalysts.

It is noteworthy that in the laboratory the catalytic activities of a variety of natural and substituted cyclodextrins are not limited to penicillin substrates. In fact, these molecules have been shown to catalyze assorted reactions using DNA (Spies and Schowen 2002), ribonucleotides (Breslow and Huang 1991), and organophosphate substrates (Ishiwata and Kamiya 1999). Although the physiological relevance of these catalytic events remains unexplored, these observations provide the tantalizing possibility that βCD may catalyze other physiologically relevant biochemical reactions in nature.

Acknowledgments We thank Drs. Milton Gordon, Jim Staley, Marion Brodhagen, Robert Edmonds, and members of the Nester, Hakomori, and Gordon laboratories for their insightful comments and criticisms, and Drs. Carlos Semino and William J. Brown for stimulating discussions during the early stages of this work. Ms. Emily Lee and Mr. Adonis Acuario provided expert technical assistance. This work was supported by a National Science Foundation Small Grant for Exploratory Research (MCB-0135592) and a National Institutes of Health Research grant (GM 32618) to E. W. N., a National Institutes of Health/National Cancer Institute grant (R01-CA80054) to S. Hakomori that also provided support to T. H., and an American Cancer Society postdoctoral fellowship to P. dF.

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