[21] *Caenorhabditis elegans* Carbohydrates in Bacterial Toxin Resistance

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Abstract

The major virulence factor produced by the bacterium *Bacillus thuringiensis* (Bt) is a pore-forming toxin called crystal (Cry) toxin, which targets and kills insects and nematodes. To understand how this bacterial toxin interacts with its invertebrate hosts, a genetic screen in *C. elegans* for nematodes resistant to Bt toxin was carried out. Four of the five genes that mutated to toxin resistance encode glycosyltransferases. These genes were found to participate in the biosynthesis of *C. elegans* glycosphingolipids. These glycolipids in turn were shown to directly bind Bt toxin. Thus, resistance to Bt toxin in *C. elegans* can develop as a result of loss of glycolipid receptors for the toxin. Here we describe the isolation of Bt toxin resistance mutants in *C. elegans*, isolation of *C. elegans* glycolipids, and their separation by thin-layer chromatography, overlay assays to demonstrate direct binding of Bt toxin to glycolipids, and the purification of specific *C. elegans* glycolipid species.

Overview

The free-living, soil-dwelling, nematode *Caenorhabditis elegans* has shed important insights into many different areas of research. *C. elegans* has a short generation time, a mapped, sequenced genome, well-known forward and reverse genetics, and can be grown into large, genetically homogeneous populations for biochemical purifications. *C. elegans* has been increasingly used in the study of glycobiology (Altmann *et al.*, 2001; Cipollo *et al.*, 2005; Griffitts *et al.*, 2005; Haslam *et al.*, 2002; Schachter, 2004). One recent example involves the interaction of *C. elegans* with a bacterial toxin, the crystal protein Cry5B made by the bacterium, *Bacillus thuringiensis* (*Bt*). Cry5B is a pore-forming toxin (PFT) that intoxicates and kills *C. elegans* and other nematodes on ingestion and binding to receptors on the intestine (Griffitts *et al.*, 2001; Marroquin *et al.*, 2000; Wei *et al.*, 2003). Mutations in five genes were identified that result in *C. elegans* resistant to intoxication by Cry5B—that is, loss of any one of these genes results in animals that are much healthier than wild-type animals when ingesting the

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PFT (Marroquin *et al.*, 2000). It was subsequently demonstrated that four of these genes (*bre-2*, *bre-3*, *bre-4*, and *bre-5*) have significant homology to glycosyltransferases and are part of a single biosynthetic pathway involved in biosynthesis of carbohydrate chains on glycosphingolipids (Griffitts *et al.*, 2001, 2003, 2005). The glycolipid carbohydrates were shown to be intestinal receptors for the PFT. Thus, the resistance phenotype associated with loss of any one of these genes can be attributed to the loss of toxin-binding sites. Research into understanding the molecular mechanisms of resistance to Bt crystal toxins is important for protecting this invaluable natural resource. Bt crystal proteins are increasingly used around the world as topical sprays and in transgenic plants to control insects that cause damage to crops or act as vectors for spreading disease (Crickmore, 2005; Griffitts and Aroian, 2005). For example, in the year 2005, 8.5 million hectares of transgenic cotton expressing a Bt crystal protein were grown, accounting for ~24% of all the cotton in the world (James, 2005).

In this chapter, we will discuss the key techniques used in studying the *bre* mutants, namely isolation of *C. elegans* Cry5B resistance mutants, isolation of *C. elegans* glycolipids, analysis by thin layer chromatography (TLC), overlay binding experiments with labeled Cry5B toxin on TLC-resolved *C. elegans* glycolipids, purification of specific glycolipid species, and enzymatic separation of the oligosaccharide from glycolipids.

Maintenance of C. elegans

All worm strains were maintained on 60-mm NG agar plates, which were seeded with approximately 50 μl of saturated *Escherichia coli* OP50 (Brenner, 1974). Strains were maintained by picking individual L4 stage worms to seeded plates every 2–3 days. Worm plates are incubated at a constant temperature of 20° (Hope, 1999). The *C. elegans* strain N2 Bristol is the standard wild-type laboratory strain (Brenner, 1974).

C. elegans *Chemical Mutagenesis*

Ethyl methanesulfonate (EMS) mutagenesis was the method used to generate mutations in *C. elegans* N2 animals. The use of EMS to isolate mutants in *C. elegans* was used in Brenner's initial genetics studies of *C. elegans* mutants (Brenner, 1974). EMS genetic screens have been shown to be capable of recovering one null mutation for every 2000 copies of a specific gene analyzed in any given screen (Jorgensen and Mango, 2002). EMS is a very efficient mutagen, which typically results in the generation of G/C → A/T point mutations (Anderson, 1995).

Procedure for EMS Mutagenesis

Section 1: Preparing a Synchronized Population of Worms

A P_0 generation of N2 worms synchronized at the L4 stage is typically used for mutagenesis (Sulston, 1988). A large population of worms is obtained first by using a sterile spatula to cut and transfer (like a piece of pie) 1/4 of a starved 60 mm plate of N2 worms onto a 100-mm ENG plate (see recipe in Section 3), which have been seeded the day before with 100 μl of a saturated culture of OP50. A total of three such plates are generated. These three plates are then allowed to grow 2–3 days until the worms have nearly consumed all the bacteria and become gravid adults. The adults are then washed from each ENG plate twice with 4 ml of H_2O and transferred to a 15-ml conical tube (Corning #430052). They are then centrifuged at 500*g* for 45 sec in a swinging bucket clinical centrifuge and the supernatant aspirated; 5 ml of water is then added and spun again to wash the pellet of worms and remove excess OP50. After aspirating the supernatant, 4 ml of bleach solution is added (for 5 ml bleach solution, 3.5 ml ddH_20, 1.0 ml 5% NaOCl, 0.5 ml 5 *N* KOH). The worms are then continually mixed by inversion in the bleach solution while monitoring their lysis on a dissecting microscope. After the worms have lysed and eggs are released, the eggs are centrifuged, the bleach solution aspirated, and 8 ml of sterile double-distilled water is added. The eggs are centrifuged and aspirated as before and washed with water one more time and then one time with 8 ml of M9 buffer (22 m*M* KH_2Po_4, 42 m*M* Na_2HPO_4, 85.5 m*M* NaCl, 1 m*M* $MgSO_4$). Because the eggs do not pellet as well in M9 buffer, increase the spin to 750*g* for 75 sec. Carefully pipette off the supernatant and repeat the M9 wash. After the washes are completed, the eggs are suspended in 2 ml M9 and placed on a rotary platform to hatch overnight at room temperature (Huffman *et al.*, 2004).

After hatching the embryos, synchronized L1 larvae are plated onto 1–10 100-mm ENG plates previously seeded with OP50. The concentration of L1s in the tube can be obtained by counting the number of worms in several 5 μl amounts on an NG plate using a dissecting microscope. Each plate is inoculated with 10,000–20,000 worms. The worms are allowed to grow to the L4 stage (36–42 h at 25°).

Section 2: EMS Mutagenesis

After the P_0 generation has reached the L4 stage, the worms are harvested from the plates with 4 ml of M9 and transferred to a 15-ml conical tube (Corning #430052). The worms are then centrifuged for 30 sec at 200*g*. The supernatant is then aspirated, and the worm pellet is washed with an

additional 8 ml of M9 to remove any residual bacteria. After spinning and aspirating, the worms are resuspended in 2 ml of M9. At this point a 2× solution (0.06 *M*) of EMS (Sigma # M-0880) can be prepared in a fume hood. Because of EMS being volatile and a recognized carcinogen, all steps involving the use of EMS are performed with great care in the fume hood while using skin and eye protection (e.g., double-gloved hands that are frequently changed). In addition to this, all items that come in contact with the EMS (pipettes, pipette tips, tubes) must be decontaminated with 1 *M* NaOH before they can be safely discarded. The 2× EMS solution is made by adding 12 μl EMS to 2 ml of M9 solution and mixing well to completely suspend the EMS (which is oily). This will produce a 60-m*M* EMS solution. The 2× EMS solution is then added to the 2 ml worm suspension. The tube with worms in EMS is capped, sealed with Parafilm, and then placed on a gentle rocker in the fume hood for 4 h. After the 4 h of incubation, the worms are pelleted by centrifuging for 30 sec at 200*g*. The supernatant is removed and discarded into a waste bucket containing 1 *M* NaOH. The worms are then washed two times with 4 ml of M9, and the supernatant is discarded into a waste bucket containing 1 *M* NaOH. At this point the worms are resuspended in 0.5 ml M9 and plated onto two 100-mm ENG plates seeded with OP50. The lids are left slightly open in the fume hood to allow evaporation of any remaining EMS. Once the plates are dry, the mutagenized P_0 worms are grown to gravid adults by incubating overnight at 20°.

Section 3: Resistant Mutant Genetic Screen

After growing the P_0 worms to gravid adults, they are collected and bleached as described in Section 1. After hatching the F1 embryos, the synchronized F1 larvae are plated on 100-mm OP50 seeded ENG plates at 5000–10,000 worms per plate. These worms are allowed to grow to gravid adults at 20°, at which point they are bleached one final time to obtain F2 embryos. The synchronized F2 L1 animals are challenged by *Bt* crystal toxin in an effort to identify possible resistant mutants. The screening for resistance can be done on plates (below and Marroquin *et al.* (2000) or in wells (Marroquin *et al.*, 2000).

ENGIC Plate Method for Cry5B-resistant Mutants. In this method, a toxin-expressing *E. coli* strain functions as both a toxin source and a food source for the animals being screened. The JM103 strain of *E. coli* is transformed with a plasmid containing (QIAGEN) crystal toxin gene under the control of an isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible *T5* promoter (vector pQE9 from QIAGEN). Overnight cultures of two bacteria (a strain with Cry5B cloned into pQE9 vector and a strain with empty pQE9 vector) are spread separately onto 100-mm ENGIC plates (ENGIC media

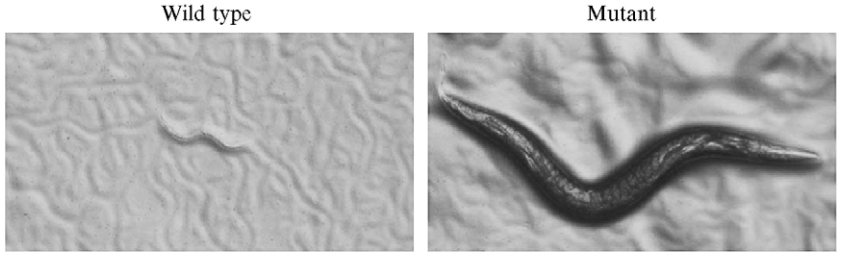

FIG. 1. A wild-type animal (left) and a *bre-3*(*ye28*) (right)–resistant animal grown from the L1 stage on Cry5B-expressing *E. coli* shown at the same magnification. This figure illustrates the ease with which a resistant mutant can be visualized. See Marroquin *et al.* (2000) for more on EMS screening for resistance mutants.

recipe can be found below). After spreading 100 μl of culture on each plate, they are dried overnight at 25°. It is good to set up many Cry5B toxin plates and a few empty vector plates.

F2 populations of mutagenized worms are then plated at the L1 stage (see preceding) at approximately 3500 worms per plate. As a control, 3500 animals are also plated on a plate spread with only empty vector. In addition, 20–30 non-mutagenized L1 worms are plated on one toxin plate and one empty vector plate (controls). After approximately 3 days, the toxin plates with mutagenized worms are scanned for rare (~1/1000–1/5000) healthy well-developed, resistant worms that seem unaffected by the toxin (Fig. 1). In contrast, the nonresistant worms will be severely underdeveloped. The putative resistant mutants are collected onto OP50 (non-toxin plates) and allowed to multiply before their progeny are retested to confirm heritable resistance.

Plate Media

ENG media

5 g Bacto-Peptone
1 g Yeast extract
3 g NaCl
20 g Granulated agar
1000 ml H_2O
Autoclave for 45 min
When cool enough to touch, add:
1 ml 5 mg/mL cholesterol in 100% EtOH
1 ml of 1 *M* $CaCl_2$
1 ml of 1 *M* $MgSO_4$
25 ml of 1 *M* KPO_4

After mixing, distribute the media to 100-mm plates at a volume of 30 ml per plate. Allow the media to dry for 2 days at room temperature before inoculating with a fresh bacterial culture.

For ENGIC plates, in addition add 1 ml of 50 mg/ml carbenicillin and 200 μl of 0.5 *M* IPTG after cooling, for final concentrations of 0.1 m*M* IPTG and 50 μg/ml carbenicillin.

C. elegans *Glycosphingolipids*

C. elegans possesses a host of genes involved in glycosylation similar to those found in mammals (Schachter, 2004). Because of the ease with which a researcher can carry out *C. elegans* genetic experiments, the animal provides an excellent system for studying the function of specific glycosylation genes and specific disorders that may result from the lack of these genes (Schachter, 2004). Studies of *C. elegans* Bt resistance mutants has led to the explanation and detailed characterization of intestinal glycolipids (Griffitts *et al.*, 2005). It was demonstrated that the carbohydrate portion of arthroseries glycolipids found in the *C. elegans* intestine are essential for intoxication and bind Cry5B—that is, they act as toxin receptors. The sequence of carbohydrates and their linkages were delineated for three Cry5B-binding glycolipids, with the most complex one containing 11 sugar residues. Further work demonstrated that terminal galactose residues play a particularly important role in the binding of Cry5B to glycolipids (Griffitts *et al.*, 2005). The techniques used in this research demonstrate the value of *C. elegans* in studying glycobiology. Following are the procedures used to isolate and characterize glycolipids from *C. elegans*.

Isolation of Glycosphingolipids from *C. elegans* by Svennerholm Partitioning

This method of purification involves using chloroform, methanol, and water to purify glycolipids from homogenized worm pellets. This is achieved by extracting lipid material into two chemical phases (Svennerholm and Fredman, 1980). The upper phase, which is more hydrophilic, attracts glycolipids with more polar carbohydrate structures. The hydrophobic lower phase collects simple glycolipids and other nonpolar lipids, which have less complexity or no sugar structure. After separation, the upper phase solution is depleted of any nonlipid contaminants by applying the samples to a small hydrophobic column (Schnaar, 1994). The samples purified off of the column are dried down and resuspended in methanol for further analyses (Schnaar and Needham, 1994). For example, the glycolipids from a wild-type *C. elegans* population can be compared with the glycolipids from a

population lacking the *bre-2* gene. After these comparative studies, specific glycolipid species (e.g., those absent in the mutant animals but present in wild-type animals) may be subjected to further purification and analyses. Processing of a 600-μl worm pellet will result in an upper phase volume of 300 μl and a lower phase volume of 600 μl.

Section 1: Preparing Worms

To prepare a useful volume of sample, six 100-mm ENG plates should be made for each strain to be processed. Each plate should be spread with 200 μl of fresh overnight culture of *E. coli* strain JM103. These plates should be allowed 48 h to dry and grow a healthy lawn at room temperature.

The six seeded ENG plates (see preceding) are inoculated with approximately 2000 mixed life stage worms from the specific strain of interest. These 2000 worms can be collected from typical 60-mm maintenance plates. The worms are washed from the maintenance plates with 1.5 ml of H_2O and transferred to a 15-ml conical tube (Corning #430052). The residual OP50 is washed from the worms by performing three washes (see Notes below). Each wash consist of adding 8 ml of water to the worms, gently inverting several times, centrifuging at 500*g* for 45 sec in a swinging bucket clinical centrifuge, and aspirating the supernatant. The density of worms is estimated by counting the number of worms in several 5-μl amounts on an NG plate using a dissecting microscope. An appropriate amount is then pipetted to place 2000 worms on each of the 100-mm plates.

Notes: Mixed life stages are used here to ensure that glycosphingolipids that may only be produced during specific life stages are not excluded. Completely starved worms are avoided because of the possibility of altered glycosphingolipid expression resulting from starvation.

After inoculation of a 100-mm ENG plate with 2000 mixed stage worms, the plates are grown for approximately 4 days or more at 20°, depending on the growth rate of the specific strain, until the worm population is nearly starved (see Notes). Plates that are close to starving can be easily identified by a visible wave of worms forming at the edge. After the wave forms, the plate will starve within approximately 8 h at 20° as the wave slowly closes in on the center of the plate. After the plate has reached a point near starvation, the worms can be washed from the plates (see Notes). Using 3 ml of water for each plate, soak the worms for 10 min. The suspended worms can be transferred to a 15-ml conical tube (Corning #430052). Spin and wash the worms three times with chilled water as described for the initial inoculation. After the final spin, remove as much of the supernatant as possible and flash freeze the pellet in liquid nitrogen (see Notes). The pellets can be stored at –80° until

the time of purification. The typical size of the worm pellet is between ~0.5 and ~1 ml.

Notes: Do not try to hasten the growth by incubating at 25°, because the increase in temperature may also contribute to variances in glycolipid expression. Worms are harvested at a point near starvation to maximize the amount of worm material collected while minimizing the quantity of bacteria to wash away before purification. Even if you are performing the purification on the same day as the worm collection, you should flash freeze the pellets in liquid nitrogen to aid in weakening worm cuticle.

Section 2: Lipid Extraction

Thaw the previously frozen pellets at room temperature. When purifying glycolipids from more than one strain, it is helpful to adjust all pellets to equal volumes for steps involving centrifugation. The volume is adjusted by removing a small amount of material from slightly larger pellets. After adjusting, add three pellet volumes of sterile double distilled water (ddH_2O) to each pellet and transfer it to a 10-ml glass conical vial (Pierce # 13225). Sonicate the samples for 30 sec at 8 watts (power dial between 4 and 5) using a microtip on a sonicator (Fisher model #60). Keep the samples on ice during the sonication. Verify complete disruption of the sample by tilting the vial and looking for whole worms under a dissecting microscope. If whole worms are still visible, repeat the sonication.

After the sonication, confirm the pellet volumes and adjust all samples to be equal by adding sterile ddH_2O. After confirming the volume of each sample, add 2.67 homogenate volumes of methanol to each sample and briefly vortex (see Notes). At this point it is no longer necessary to keep the sample on ice. Allow the samples to incubate at room temperature for 5 min. After the incubation, add 1.33 homogenate volumes of chloroform and mix by gently inverting several times (see Notes). This mixture contains a final ratio of 4:8:3 (chloroform/methanol/water).

After adding the chloroform and methanol, the samples are incubated at 37° for 2 h. During the 2 h of incubation, mix the samples by inverting several times every 15 min.

On completion of the incubation, centrifuge the samples at 1400*g* for 5 min using a swinging bucket clinical centrifuge. Using a 25-ml glass pipette, transfer the supernatant to a new 30-ml glass centrifuge tube (Kimble HS No. 45600-30, Cap No. 24-400). Be careful not to disturb the pellet when transferring the supernatant. Add 0.173 volumes of water to each of the supernatants, cap, and mix by inverting several times (see Notes). After mixing the samples, centrifuge them at 1400*g* for 5 min. At this point, two

distinct layers should be visible in the sample tubes. The top layer (upper phase) contains glycolipids with polar oligosaccharide structures, which are soluble in the more aqueous solution, whereas the bottom layer contains less substituted lipids, phospholipids, and cholesterol (Schnaar, 1994).

Notes: Methanol added = 2.67 × (original wet pellet volume + 3 pellet volumes of water). Chloroform added = 1.33 × (original wet pellet volume + 3 pellet volumes of water). After adding the chloroform and methanol, the samples are stable enough to be left at 4° until time permits the completion of the protocol. Water added = 0.173 × (the volume transferred to the 30-ml glass tube).

Section 3: Purification

To remove remaining contaminants from the upper phase glycolipids, it is necessary to perform reversed-phase chromatography. This process removes nonlipid contaminants while concentrating glycolipids (McCluer *et al.*, 1989; Schnaar, 1994; Williams and McCluer, 1980). This process can be performed with a Sep-Pak + cartridge (Millipore Corp Product #–WAT036810) and a 5-ml glass syringe (BD #512471) for applying sample to the cartridge. After applying the sample, it is washed, eluted, and dried down to concentrate the sample. Three solvent mixtures are required for this protocol: 2:43:55 (chloroform/methanol/water), methanol, and 1:1 (chloroform/methanol).

After the 30-ml tubes have been centrifuged to produce two distinct layers, they may remain at room temperature during the purification. Transferring of sample and all solvents should be performed with glass pipettes to reduce product loss. After adding the first solution to the cartridge (see Notes), air must not be passed over the cartridge during the procedure. Flow rate should be a fast drip. One Sep-Pak + column contains enough substrate to efficiently purify approximately 8 ml of upper phase glycolipids. Collect all waste solution in a large beaker in a fume hood.

The purification cycle begins by pushing 4 ml of 1:1 chloroform/methanol over the column at the pace of a fast drip using a 5-ml syringe (approximately 2 drops/sec). At the end of every solution addition, be sure to stop before air is pushed into the column to maintain substrate homogeneity. Continue the equilibration by adding 4 ml of methanol to the column. After the methanol has been pushed through the column, add 4 ml of 2:43:55 (chloroform/methanol/water) to finish the equilibration. The next step requires a clean test tube in which your first run of sample can be collected. Every 4-ml portion of upper phase should be run over the column twice to ensure a maximum yield. Add 4 ml of the first upper phase sample. Push the sample through the column while collecting the run through in a clean test tube. Place the first run through aside while proceeding to the first wash step. Wash the

sample by applying 4 ml of 2:43:55 chloroform/methanol/water. The run through for this step can be collected in the waste container. The washed sample can now be eluted into a clean 10-ml glass conical vial (Pierce # 13225). Elute the sample by adding 4 ml of 1:1 chloroform/methanol. Allow the first five water-rich drops from the previous wash to run into the waste container. Elute the rest of the solution into the conical vial. Your purified sample should emerge as a white precipitate between the first 10 and 20 drops of eluted solution. This is the one step in which you may push the eluting solution through with air to completely evacuate the column. This completes the first half of one cycle. The second half of the cycle involves following the same procedure, except the sample run-through saved in the test tube is applied to the column. During this half of the cycle, the sample run-through can be collected in the waste beaker. Eluting the remaining sample into the conical vial completes one full cycle. One column is good for purifying 8 ml of raw sample. The columns should be switched when either changing strains or 8 ml of raw sample has been run through the column twice.

Purified upper-phase lipids can be dried down by gently blowing a continuous stream of nitrogen gas over the sample while it sits on a 45° heating block. The lower phase can be collected by drawing it from the bottom of the 30-ml glass tube with a glass pipette to transfer it to a separate conical vial. The lower phase sample should also be dried down using a stream of nitrogen. Samples may be damaged if they are left on the heating block too long after they have completed drying.

Section 4: Suspension of Dried Samples

The upper and lower phase dried samples are suspended in a volume of solvent proportional to the original worm pellet volumes. Upper phase samples are dissolved in a volume of methanol, which is equal to one half the original worm pellet volume. To completely dissolve the sample, it may be necessary to scrape it from the sides using a glass Pasteur pipette. Vortexing or sonicating in a bath-type sonicator in addition to gentle heating (in a 45° water bath) may also help dissolve the sample (Schnaar, 1994). Remaining white precipitate can be discarded, because it likely consists of protein impurities. Lower phase samples can be dissolved in a volume of 1:1 chloroform/methanol, which is equal to the volume of the original worm pellet. Vortexing is typically sufficient to completely dissolve the sample.

Analysis of Glycolipids by Thin Layer Chromatography

Analysis of both upper and lower phase glycolipids can be accomplished by resolving the samples on thin layer chromatography (TLC) plates.

This system involves using solvent solutions to separate different glycolipid species on the basis of polarity (Schnaar and Needham, 1994). The sample is applied to a thin layer of silica substrate. The silica is a polar substrate, which binds samples applied to the TLC plate. Sample resolution is achieved through the use of specifically prepared solvents. These solvents participate in polar interactions with the glycolipids targeted for resolution. The mobility of glycolipids on silica TLC plates is generally inversely proportional to the number of monosaccharide residues in the head group, although non-carbohydrate polar substitutions can change mobility dramatically (Schnaar and Needham, 1994).

After resolving glycolipid samples by TLC, the resolved band pattern produced can be visualized by staining. One common method used for staining glycolipids involves the use of an orcinol-sulfuric acid test (Schnaar and Needham, 1994; Svennerholm, 1956). Orcinol is a general carbohydrate stain, which is capable of detecting neutral sugar residues in quantities as low as 500 pmol (Schnaar and Needham, 1994). In the interest of studying how specific glycolipids may interact with other molecules, fixing the resolved samples to a TLC plate makes it possible to probe them with labeled proteins much like a Far Western blot (Magnani *et al.*, 1987; Schnaar and Needham, 1994). In studies involving the *Bt* crystal toxin Cry5B, this overlay approach has been used to determine that glycolipids directly bind Cry5B. Overlay methods have also been used in competition studies to evaluate the specific properties of the identified receptors (Griffitts *et al.*, 2005). This information was later used to target specific glycolipids for further analysis and help determine which monosaccharides play an important role in the binding interaction (Griffitts *et al.*, 2005).

Section 1: Resolving Sample by TLC

C. elegans glycolipids can be resolved using Merck glass-backed silica-60 High-Performance Thin Layer Chromatography (HPTLC) plates (Fisher # M5631-5), a 1-μl Hamilton syringe (Fisher # 14824200) for loading sample, solvents, and a developing tank (Fisher # K416180-0000). The solvent used to resolve upper phase glycolipids contains 4:4:1 (chloroform/methanol/water), and the solvent used to resolve lower phase glycolipids contains 45:18:3 (chloroform/methanol/water). The silica plate can be prepared by lightly marking with a pencil to label and evenly space multiple samples. Sample lines should be approximately 1 cm long and at least 1 cm from the bottom or either edge of the plate. Sample lines should be spaced at least 0.1 cm apart. An additional mark can also be made 4.5 cm from the bottom as a point of termination for solvent migration (Fig. 2). This will permit the

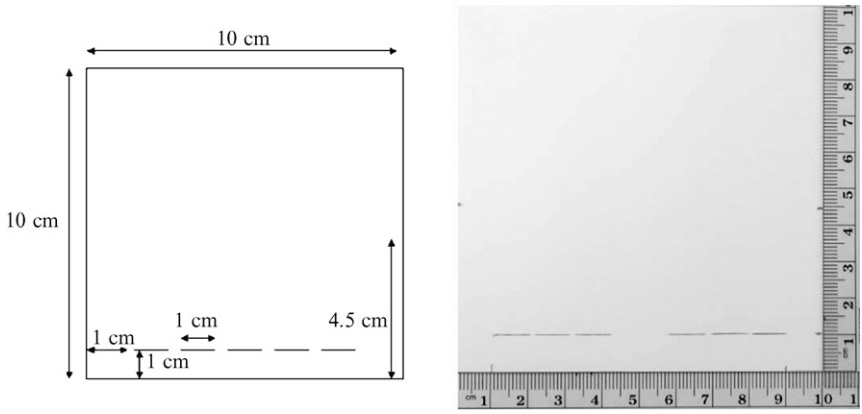

FIG. 2. Pencil mark layout for a 10 × 10-cm TLC plate.

use of two ends of a 10 cm by 10 cm silica plate. If greater sample resolution is required, mark the termination point 1 cm from the top of the plate. After the plate has been labeled, it should be dried in an oven at 125° for 10 min before applying sample (Schnaar and Needham, 1994).

While drying the plate, resolving solutions should be added to the developing tank at a volume of approximately 20 ml (0.5-cm depth). The tank should be allowed to equilibrate with the solvent solution for at least 30 min at room temperature before developing the plate (Schnaar and Needham, 1994). After the silica plate has cooled, thin lines of sample can be added just above the pencil guidelines. A 1-μl Hamilton syringe can be used to apply the sample. The sample should be applied in a thin line to achieve better resolution of closely spaced glycolipid bands. A 5-μl load is typically sufficient to produce a visible band pattern after orcinol staining. After applying the sample, allow the sample to dry on a heat block at 45°. The dried silica plate can then be placed in the equilibrated resolving chamber. Quickly replace the airtight cover and allow the solvent front to travel ~4.5 cm. The plate is then dried on a heat block at 45°. The plate is now ready for staining.

Section 2: Orcinol Staining Resolved Glycolipids

Equipment required for this protocol includes a fine mist sprayer (Fisher # K422530-0125), a sprayer box, a fume hood, and a drying oven. Chemicals required for this protocol include orcinol (Sigma # O-1875), methanol (EMD # MX0485-7), water, and sulfuric acid (Fisher # S79200MF).

The orcinol solution is made in the reservoir portion of the glass sprayer, which should be wrapped with aluminum foil to prevent the degradation of the orcinol by light exposure; 40 ml of orcinol solution can be made as follows: In a fume hood, add 200 mg of orcinol followed by 25 ml of methanol and 5 ml of water directly to the flask sprayer reservoir. Gently mix the three components by swirling the flask. Then 10 ml of sulfuric acid should slowly be added to the mix while gently swirling (taking appropriate precautions when working with the strong acid). The acid must be added slowly because of the heat-producing chemical interaction between the acid and aqueous solutions. After mixing all of the components and allowing the sprayer flask to cool, the sprayer may be assembled. The orcinol solution can be kept in a dark area at 4° for a period of approximately 7 days (Schnaar and Needham, 1994).

The application of the orcinol staining solution should be done in a fume hood while using a sprayer box. Apply air to the sprayer at a pressure that produces a fine and even mist. Evenly spray the plate with multiple passes, but stop before the plate shows signs of visible moisture. Immediately move the plate to the drying oven at a temperature of approximately 125° and allow it to dry for no more than 10 min. After drying, resolved banding patterns should be visible on the surface of the plate (Fig. 3). The plate should immediately be scanned or photographed, because the stain will begin fading soon after being removed from the oven (Schnaar and Needham, 1994).

Section 3: Preparing Protein Probe for Overlay Analysis

Cry5B is labeled by biotinylation before probing glycolipids to test for direct binding. The labeling reaction is performed by combining the protein of interest with a fourfold to sixfold molar excess of N-hydroxysuccinimidyl

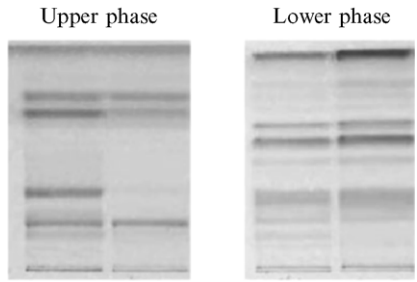

FIG. 3. Thin layer chromatography of upper and lower phase samples from two different strains of *C. elegans*. The solvent used to resolve the upper phase glycolipids consisted of 4:4:1 (chloroform/methanol/water). The solvent used to resolve the lower phase sample consisted of 45:18:3 (chloroform/methanol/water). After samples were resolved, staining was performed with an orcinol-sulfuric acid spray. The two lanes for both plates represent glycolipids from N2 and *bre-2*(*ye31*) animals, respectively, from left to right.

esters of biotinamidocaproate (Sigma #B-2643). After combining in an appropriate buffer, incubate for 2 h at 20°. Excess label can be removed by desalting with P-6 resin beads (Bio-Rad # 150-0738) equilibrated in the protein's final suspension buffer. A buffer consisting of 20 m*M* HEPES at a pH of 8.0 was used for labeling and desalting Cry5B (Griffitts *et al.*, 2005).

Biotinylated lectins may also be used as protein probes for overlay analysis. There are many lectins that bind specific sugar residues. These can be used to obtain a general analysis for the exposed structure of glycolipid samples resolved by TLC. The only potential difference in protocol for using a lectin versus Cry5B as a probe is the buffer used. In either case, a buffer specific to the protein or lectin may be used to encourage target binding to the glycolipids.

Section 4: Preparing Resolved Samples and Protein Overlay Analysis

Preparation of glycolipid samples for overlay analysis first involves resolving the samples on an HPTLC plate as described in Section 1. The resolved glycolipid samples can be fixed to the plate using a solution of 0.02% polyisobutylmethacrylate (PIBM) (Aldrich # 18154-4) in hexane (Fisher # H303-1) after the running solvent has been completely evaporated (Griffitts *et al.*, 2005; Schnaar and Needham, 1994).

All steps involving hexane are performed in a fume hood. Before fixing, the TLC plate is equilibrated in a plastic tray (w × h × d 7.5 × 2.5 × 11 cm) containing 40 ml hexane for a period of 60 sec. The plate is then quickly transferred to a second tray containing 40 ml 0.02% PIBM in hexanes for 60 sec. After 60 sec, move the plate to a 45° heat block where the residual hexane is completely evaporated. The fixed plate is now ready to be blocked in a 0.5% bovine serum albumin (BSA), phosphate-buffered saline (PBS) (136 m*M* NaCl, 2.6 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4) with 0.02% Tween-20.

The volume of buffer for this protocol was designed for a 5 cm by 10 cm plate in a plastic tray; 200 ml of block solution (180 ml H_2O, 20 ml 10× PBS pH with HCl to 7.2, 160 μl 25% Tween-20, 1 g BSA) is sufficient for completing this protocol. The initial block requires 10 ml of blocking buffer and 30 min on a rocker at room temperature. The plate can then be probed with the labeled protein in block solution. When probing with labeled Cry5B, the crystal toxin is added to 10 ml of block solution to a concentration of 11 n*M* (Griffitts *et al.*, 2005). After adding probe, the plate should be incubated on a rocker for 2 h at room temperature. When probing is complete, remove the solution and add 10 ml block solution and rock for 1 min. Wash the plate a second time with 10 ml of block solution for 5 min. After completing the washes, a streptavidin-linked alkaline phosphatase

solution can be added to the plate. Components to make this solution can be obtained from a Vetastain Kit (Vector Labs # AK-5000). The two components of this kit include streptavidin and biotinylated alkaline phosphatase. The kit components are diluted 150 fold when added (40 μl each) to a tube containing 6 ml of block solution. Mix by gently inverting the tube several times before adding it to the plate. Allow the plate to incubate in the solution on a rocker for 1 h at room temperature. After removing the alkaline phosphatase solution, wash the plate three times for 1 min in 10 ml of clean blocking buffer. The specific glycolipids that bind with the labeled protein can be revealed by a chemical color reaction.

The color reaction is initiated by submerging the plate in a solution of 5-Bromo-4-Chloro-Indolyl-Phosphatase (BCIP) and Nitro blue Tetrazolium (NBT) diluted in alkaline phosphatase (AP) buffer (100 m*M* Tris pH 9.5, 100 m*M* NaCl, 50 m*M* $MgCl_2$). Add 30 μl each of BCIP and NBT, in order, to 5 ml of AP buffer. After the washes, move the plate to a new tray and submerge it in 5 ml of the color reaction solution. Allow the plate to rock for 10 min or until stained bands become clearly apparent (Griffitts *et al.*, 2005). After achieving the desired amount of staining, the plate can then be washed with water and dried for scanning or photography (Fig. 4).

Section 5: Polystyrene Microplate Overlay

Semiquantitative analysis of Cry toxin-binding affinity with different *C. elegans* strain upper phase samples can be performed through the use of a polystyrene microplate overlay analysis.

Upper phase glycolipids from mixed life stage worm pellets in which the total number of worms can be quantitated by sampling worm quantities in several small aliquots. The glycolipids are dried down and dissolved in a

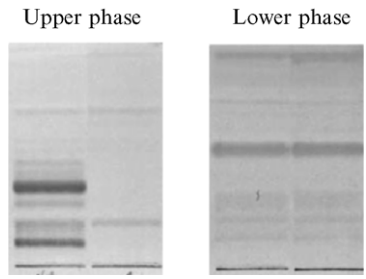

FIG. 4. Cry5B overlay analysis of upper and lower phase glycolipid samples from two different *C. elegans* strains. The two samples were resolved as described in Fig. 3. The plates were fixed with 0.02% PIBM in hexane before being probed with biotinylated Cry5B. The two samples and their order are the same as described in Fig. 3.

solution of 1:1 (methanol/water). Solution volumes, representative of specific numbers of worms, are then transferred to a 96-well polystyrene microtiter plate (Costar #9017, medium binding). This solution is allowed to evaporate at room temperature for a period of 135 min. Any remaining solution is removed and replaced with blocking solution (42 m*M* Na_2HPO_4, 85 m*M* NaCl, 1 m*M* $MgSO_4$, 0.2% defatted BSA) and allowed to block for 30 min. The wells can then be probed with biotinylated toxin (~20 n*M*) in blocking solution for 1 h at room temperature. The wells are then washed twice with blocking buffer and incubated with the alkaline phosphatase solution (Vector Labs # AK-5000), made as described in section 4 , in blocking buffer for 45 min. Wells were then washed twice with BSA-free block solution and once with water. *P*-nitrophenyl phosphate (PnPP) (Sigma #N9389) is then added at a concentration of 1 mg/ml in PnPP buffer (50 m*M* HCO_3, .5 m*M* $MgCl_2$, pH 10). After positive control (wild-type glycolipids at the highest concentration) wells reach an OD405 of 1, the color reaction can be stopped by adding 3 *M* NaOH at a 1:5 ratio to each well. OD405 measurements can then be taken for all wells.

Isolation of Specific Glycosphingolipid Components

To further investigate the structure and function of a specific glycolipid species, it is necessary to separate it from all other isolates collected during the bulk purification described previously. Because any specific glycolipid species will make up only a small portion of the total extracted sample, the purification procedure must be scaled up. The glycolipid extraction protocol for *C. elegans* should be scaled up to accommodate a 10–20 g worm pellet (approximately 10 150-mm ENG plates). The purification of a specific glycolipid species uses the same principles as resolving glycolipid samples on a TLC plate. This method involves using a silica gel column for separating specific species that are eluted by use of a gradient of solvents. Solvent ratios can be predetermined using TLC to identify a specific solution that is capable of providing adequate resolution for the target species. Because of the possibility that multiple species may have different carbohydrate structures while still migrating very close together, the isolation of one specific glycolipid species cannot be guaranteed. In cases involving species that are difficult to separate, additional methods may be required for completing purification (Schnaar, 1994).

Section 1: Preparation of a 4-ml Silica Gel Column

A silica gel column can be prepared using a 4-ml glass column (Pierce #20055), silica gel (Sigma #227196), and a small amount of clean sand

(Sigma #274739). Approximately 1 g of silica is used for every 10 g of wet worm pellet. The silica should be dried in an oven at 125° before being resuspended in 45:18 (chloroform-methanol); 2 g of the silica slurry can then be added to a column with the provided end cap in place; 2 mm of clean sand is added on top of the silica to help prevent surface disruption when adding solvent and sample to the column. Wash the column with 45:18 (chloroform-methanol) at 30 ml/g of dry silica. To prevent air from entering the column, stop the flow of solvent at a point above the silica at the end of every step.

Section 2: Sample Preparation and Glycosphingolipid Component Purification

Desalted upper phase sample should be dried down and resuspended in an equal volume of 45:18 (chloroform/methanol) before loading it onto the column. The sample should be loaded, using a Pasteur pipette, onto the column at a flow rate of less than 1 ml/min to permit adequate time for substrate binding to occur. To ensure the column is capable of retaining the entire sample, it may be necessary to analyze the flow-through by TLC. After loading the sample, wash the column with several column volumes of 45:18 (chloroform/methanol). After washing the sample, a multistep elution can be performed with multiple solutions with different chloroform/methanol/water ratios. The best ratios for purification of specific glycolipids can be predetermined by TLC analysis. With respect to wild-type *C. elegans*, specific upper phase components can be well separated using a gradient from 30:30:3 to 30:30:8 (chloroform/methanol/water) over 20 column volumes. This is achieved by making six solvent solutions that all differ by 1 part water (30:30:3-8). The amount of each solution used should be split evenly among the 20 column volume fractions (~8 ml each). Start the gradient with the least polar solvent (30:30:3) and increase by one part water with each additional step in the gradient. All fractions are checked by TLC and pooled by the specific component targeted for isolation. After completing the purification, pooled fractions are dried down and resuspended in methanol (Griffitts *et al.*, 2005; Schnaar, 1994).

Section 3: Enzymatic Release of a Purified Glycolipid Carbohydrate

After a specific glycolipid species has been purified, the carbohydrate portion can be isolated from the lipid by ceramide glycanase (Calbiochem #219484); 1 unit of ceramide glycanase from *Macrobdella decora* is capable of releasing carbohydrate from approximately 100 nmol of purified glycolipid. Purified sample can be prepared for this reaction by drying down and suspending in ceramide glycanase buffer (0.1% taurodeoxycholate, 30 m*M*

sodium acetate at pH 5.0). Add ceramide glycanase at a ratio of 1 unit per 100 nmol of purified glycolipid. Incubate the reaction at 37° for 24–36 h. TLC can be used to check for complete digestion every 12 h. When carbohydrate has been completely released, a dark band will remain at the origin when resolved with 4:4:1 (chloroform/methanol/water) (see Notes) (Griffitts *et al.*, 2005; Schnaar and Needham, 1994).

When the reaction is complete, the sample can be purified from the lipid portion with a Sep-Pak + cartridge (Millipore Corp Product #–WAT036810). The cartridge is primed by adding, in order, 4 ml chloroform, 4 ml methanol, and 4 ml water. After priming, the sample is added and eluted off the column with water in approximately 12 200-μl fractions. TLC can be used to check for the presence of released carbohydrate in each fraction. All fractions containing carbohydrate can be pooled and dried under nitrogen (Griffitts *et al.*, 2005; Schnaar and Needham, 1994).

Notes: 2:1 (isopropanol/water) can also be used as a resolving buffer for the detection of carbohydrates. The polar sample is capable of further migration and resolution when using a more hydrophilic buffer.

Section 4: Determination of Carbohydrate Concentration

The concentration of a purified carbohydrate sample can be determined using a 2% anthrone solution in ethyl acetate. In a spectrophotometer-compatible glass test tube, add 400 μl of sample diluted in water to 100 μl 2% anthrone and 1 ml sulfuric acid (Fisher #S79200MF) for a total volume of 1.5 ml. Gently swirl until the ethyl acetate is hydrolyzed and a floc of anthrone appears. Swirl more rapidly to dissolve the anthrone and allow the solution to develop for 10 min. After the solution has developed, check the optical density of the sample at 620 nm (Loewus, 1951).

The sample OD can be compared with a series of known standards near a similar concentration. A standard curve can be produced using three concentrations of glucose. A typical carbohydrate sample can be quantitated using a glucose standard curve, which includes 0.4 μg, 1.6 μg, and 4.0 μg in the 400-μl sample portion (Loewus, 1951).

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