Resistance to Bacillus thuringiensis Toxin in Caenorhabditis elegans from Loss of Fucose*

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A mutation in the Caenorhabditis elegans bre-1 gene was isolated in a screen for Bacillus thuringiensis toxin-resistant (bre) mutants to the Cry5B crystal toxin made by B. thuringiensis. bre-1 mutant animals are different from the four other cloned bre mutants in that their level of resistance is noticeably lower. bre-1 animals also display a significantly reduced brood size at 25 °C. Here we cloned the bre-1 gene and characterized the bre-1 mutant phenotype. bre-1 encodes a protein with significant homology to a GDP-mannose 4,6-dehydratase, which catalyzes the first step in the biosynthesis of GDP-fucose from GDP-mannose. Injection of GDP-fucose but not fucose into C. elegans intestinal cells rescues bre-1 mutant phenotypes. Thus, C. elegans lacks a functional fucose salvage pathway. Furthermore, we demonstrate that bre-1 mutant animals are defective in production of fucosylated glycolipids and that bre-1 mutant animals make quantitatively reduced levels of glycolipid receptors for Cry5B. We finally show that bre-1 mutant animals, although viable, show a lack of fucosylated N- and O-glycans, based on mass spectrometric evidence. Thus, C. elegans can survive with little fucose and can develop resistance to crystal toxin by loss of a monosaccharide biosynthetic pathway.

The crystal (Cry)3 proteins made by Bacillus thuringiensis are naturally occurring agents that are used for the control of insects that eat crops and carry disease (1). Cry proteins have been used for over 50 years as an environmentally safe and effective alternative to synthetic pesticides. One attractive feature of Cry proteins is their nontoxicity toward mammals and other vertebrates (2). Consistent with this lack of mammalian toxicity, several of the receptors for Cry proteins have been characterized and encode invertebrate-specific glycolipids and/or an insect family of cadherins (3). Because of their efficacy against invertebrates and safety toward vertebrates, Cry proteins are widely used worldwide as topical sprays on crops, as topical sprays to kill mosquitoes and black flies that carry disease, and as transgenes expressed in plants as an environmentally friendly alternative to chemical pesticides (4, 5). In the year 2005, over 26 million hectares of B. thuringiensis transgenic corn and cotton were planted (6). In addition, B. thuringiensis crystal proteins are now also being explored for their possible use in the control of nematode parasites (7, 8).

In our efforts to gain insight into the important question of how invertebrates develop resistance to Cry proteins, we isolated mutations in five Caenorhabditis elegans genes that result in resistance to the crystal protein, Cry5B (9). Four of these bre genes have been cloned and characterized. These genes, bre-2, bre-3, bre-4, and bre-5, encode glycosyltransferase genes that catalyze the addition of monosaccharides onto invertebrate-specific glycolipids (10–12). The resulting oligosaccharide chain is a receptor for the Cry protein (11). Thus, loss of any one of these genes results in loss of the receptor for the toxin and a high level of resistance.

Here we clone the fifth bre gene, bre-1, and characterize its phenotype. bre-1 mutant animals are resistant to Cry5B but at a lower level than the other bre mutants. Mutation of the bre-1 gene comes at a significant cost to the animal and includes a small brood size at 25 °C. bre-1 encodes a protein with significant homology to GDP-mannose 4,6-dehydratase (GMD), a cytosolic enzyme involved in the biosynthesis of GDP-fucose. This inferred enzymatic function is supported by fucose rescue experiments that also demonstrate that C. elegans lacks an alternative pathway for production of GDP-fucose, the fucose salvage pathway. bre-1 mutant animals show qualitative and quantitative defects in the production of glycolipid receptors for Cry5B, explaining its resistance phenotype. bre-1 mutant animals have overall very low levels of fucose and appear to be lacking fucosylated N- and O-glycans. C. elegans can evidently survive with little protein fucosylation.

EXPERIMENTAL PROCEDURES

C. elegans Maintenance and Microscopy—C. elegans N2 and other strains were maintained on standard NG plates spread with the Escherichia coli strain OP50 (13). bre-1(ye4) was outcrossed six times to N2 prior to the work conducted here. All nematode assays were carried out at 20 °C unless otherwise noted. Nematodes were mounted for microscopy on 2% agar.

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3 The abbreviations used are: Cry, crystal; GMD, GDP-mannose 4,6-dehydratase; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PNGase, peptide-N-glycosidase; RNAi, RNA interference; dsRNA, double-stranded RNA; PnPP, p-nitrophenyl phosphate; LC, lethal concentration.
Cry5Bm was found to be the only gene capable of rescue (8/8 lines). For final rescue experiments, animals were placed on a isopropyl β-D-thiogalactoside/carbenicillin NG plate spread with a 60/40 dilution of E. coli expressing Cry5B(without mutation):E. coli transformed with vector alone. cDNA was isolated from total bre-1(ye4) RNA and used for sequencing the bre-1(ye4) gene. SL-1 was used as the leading primer for 5’ sequencing, and oligo(dT) was used as the reverse primer for 3’ sequencing.

Cry5B Lethal Concentration Assay—Cry5B was purified from B. thuringiensis using a sucrose gradient as described (15) and solubilized in 20 mM Hepes (pH 8.0) just prior to setting up the lethality assay. The Cry5B lethality assay was performed as described (16). Cry5B was tested with N2 and bre-1(ye4) worms at the L4 stage using a range of Cry5B from 0.312 to 120 μg of Cry5B/ml. Worm viability was scored after 8 days at 20 °C. Each individual assay was set up in triplicate for each concentration of Cry5B. Three independent individual assays were performed. Probit analysis (17) was used to calculate the lethal concentration (LC) of Cry5B that killed 50 and 90% of the worms (LC50 and LC90, respectively). The mean values from the individual assays were compared using a paired t test to determine statistical significance. A probability value of less than 0.05 was set as significant. For graphical representation of the lethality assays, a nonlinear regression analysis was performed with GraphPad Prism (GraphPad Software, San Diego).

RNA-mediated Interference (RNAi) by Injection—20 rrf-3(pk1426) L4 worms were plated and incubated 12 h for each of the injection samples. After the 12-h incubation, 10 worms were injected for each of the injection samples. Injections were performed as described previously. Each injected worm was plated individually and allowed 24 h of recovery time at 15 °C. At this point the worms were transferred to new individual plates and incubated at 20 °C for an additional 24 h. Progeny obtained more than 48 h after injection were allowed to grow to the L4 stage at which point 20 were transferred to plates seeded with E. coli expressing Cry5Bm. After an additional 48 h, the response to toxin was observed. In addition, three L4 worms were picked out to individual plates for each injection sample and tested for brood size. The brood size assay was performed
as described above. The double-stranded RNA (dsRNA) fragments were made from RNA purified from N2 worms. The dsRNA fragments were amplified using T7-linked primers (see Table 2) with an Ambion T7 polymerase kit. dsRNAs were injected at a concentration of 2 mg/ml.

**Fucose Rescue Experiments**—12 worms were injected for each sugar. *bre-1*(ye4) and *bre-2*(ye31) L4 worms were injected with 2.5 mM solutions of either L-fucose (F1395; Sigma) or buffer and incubated with an alkaline phosphatase solution in tissue staining buffer for a period of 2 h. The remaining steps were the same as those performed for the *Cry5B* overlay experiments. After 48 h worms were mounted and imaged as described above. This experiment was independently repeated three times.

**Preparation of Lipids and TLC Analyses**—Lipids were prepared, separated by TLC, and probed with biotinylated *Cry5B* as described (11, 18).

**Ulex europaeus Fucose-binding Lectin Overlay**—Plates of resolved glycolipids were fixed with polyisobutylmethacrylate and blocked in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.02% Tween 20 for 30 min. After blocking, the plate was washed for 1 min and again for 5 min with a tissue-staining buffer (10 mM Hepes (pH 7.5), 0.15 M NaCl). The plate was then probed with a 10 μg/ml solution of biotinylated *U. europaeus* lectin (B-1065; Vector Laboratories) in tissue staining buffer for a period of 2 h. The remaining steps are the same as those performed for the *Cry5B* overlay experiments (11).

**Analysis of Glycolipid Affinity for *Cry5B***—Glycolipids were purified as described (11) from mixed stage worm pellets in which the total number of worms was quantitated by sampling worm quantities in several small aliquots. Upper phase glycolipids were dissolved in a solution of 1/1 (methanol:water). Solution volumes, representative of a specific number of worms, were then transferred to a 96-well polystyrene microtiter plate (Costar 9017, medium binding). This solution was allowed to equilibrate at room temperature for a period of 135 min. Any remaining solution was removed and replaced with blocking solution (42 mM NaH₂PO₄, 85 mM NaCl, 1 mM MgSO₄, 0.2% defatted bovine serum albumin) and allowed to block for 30 min. The wells were then probed with 22 nm elastase-activated, biotinylated *Cry5B* in blocking solution for 1 h at room temperature. *Cry5B* protoxin was activated and labeled as described (11). The wells were then washed twice with blocking buffer and incubated with an alkaline phosphatase solution in blocking buffer for 45 min. Wells were washed twice with bovine serum albumin-free block solution and once with water. *p*-Nitrophenyl phosphate (PNPP) was then added at a concentration of 1 mg/ml in PNPP buffer (50 mM HCO₃, 0.5 mM MgCl₂ (pH 10)). After positive control wells reached an *A*₄₀₅ of 1, 3 M NaOH was added to each well to stop the color reaction. Three control wells were used to determine the *A*₄₀₅ at three 10-min time points (10, 20, and 30 min) during the PNPP incubation. Control wells generally reached an *A*₄₀₅ of 1 after 30 min with PNPP. *A*₄₀₅ measurements were then taken for all wells. Background was determined by setting up duplicate wells with a 100-fold excess of unlabeled activated toxin for all worm numbers. Each condition was represented by three wells in each of three replicates.

**Monosaccharide Analysis of Worm Pellets**—Large populations of worms were grown on 100-mm ENG plates spread with *E. coli* strain OP50. Before the populations starved, they were washed from the plates with water and transferred to a Falcon tube producing a pellet volume of ~1 ml. Collected worm pellets were washed eight times with water. Pellets were then treated using standard protocols (40).

**Structural Analysis of Glycoproteins**—Worm pellets were acquired as described above under “Monosaccharide Analysis of Worm Pellets.” The worm pellets were sonicated in an extraction buffer consisting of 0.5% (w/v) cetyltrimethylammonium bromide and 0.1 M Tris (pH 7.4). Material for analysis was extracted for an additional 24 h on a rocker at 4 °C. Solid debris was removed by centrifugation at 1400 × g for 10 min. Detergent removal was extended by extensive dialysis against 50 mM ammonium bicarbonate buffer (pH 7.6). After dialysis the samples were lyophilized. Reduction and protection of the disulfide bridges of the extracted proteins were carried out as described (19). The reduced carboxymethylated proteins were trypsinized and digested with PNGase F (EC 3.5.1.52; Roche Applied Science) as described (20). Glycopeptides remaining after PNGase F digestion were further digested with PNGase A (EC 3.5.1.52; Roche Applied Science), in ammonium acetate buffer (50 mM, pH 5.0), for 16 h at 37 °C using 0.2 million units of the enzyme. The reaction was terminated by lyophilization, and the products were purified on a C18 cartridge. O-Linked oligosaccharides were liberated from glycopeptides after PNGase F digestion and further digested with PNGase A by reductive elimination (400 μl of 1 M NaBH₄ in 0.05 M NaOH at 45 °C for 16 h) and desalted through a Dowex 50W-X8 (H) column. Excess borates were removed by coevaporation with 10% (v/v) acetic acid in methanol under a stream of nitrogen.

**Chemical Derivatization for MALDI-MS**—Pernethylation using the sodium hydroxide procedure was performed as...
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described (19). After derivatization, the reaction products were purified on a Sep-Pak C18 (Waters) as described (19). MALDI data were acquired using a PerSeptive Biosystems Voyager-DETM STR mass spectrometer in the reflectron mode with delayed extraction. Derivatized glycans were dissolved in 10 μl of methanol, and 1 μl of dissolved sample was premixed with 1 μl of matrix (2,5-dihydrobenzoic acid) before loading onto a target plate.

Structural Analysis of Freed Glycans from Glycosphingolipid—Upper phase glycosphingolipids were collected from bre-1 worms as described under “Preparation of Lipids and TLC Analyses.” Upper phase glycosphingolipid head groups were enzymatically released as described (11). Pure bre-1 glycans in water were then lyophilized, permethylated, and analyzed by MALDI-MS as described above.

RESULTS

A single allele of bre-1, bre-1(ye4), was isolated in genetic screens for C. elegans mutants resistant to Cry5B toxin made by the invertebrate Gram-positive pathogen B. thuringiensis (9). In the same screens, multiple mutant alleles in each of four other genes, bre-2, -3, -4, and -5, were also isolated. Resistance of bre-1(ye4) animals relative to wild-type animals can be readily seen at a 60% dose of Cry5B-expressing E. coli (Fig. 1, A and B). As demonstrated previously (9), resistance of bre-1 mutant animals is less than that of animals mutant for the other four bre genes, such as bre-2 (Fig. 1C).

Based on LC90 and LC50 values, bre-1(ye4) mutant animals are 3.2- and 2.2-fold, respectively, more resistant to purified Cry5B than wild type (Fig. 1D; p = 0.036 for LC90 and 0.0173 for LC50). bre-1(ye4) mutant animals also produce small brood sizes at 25 °C (Table 3). There thus appears to be a steeper cost to the nematode for mutation of the bre-1 gene when compared with most of the other bre mutants (Table 3). Interestingly, bre-3 mutant animals also display low progeny production at 25 °C (Table 3). The lower level of resistance and the brood size defects associated with the bre-1 mutant suggested that the bre-1 gene could encode a protein that is dissimilar to the four other bre genes, all of which encode glycosyltransferases.

The bre-1 gene was mapped using the bre-1(ye4) allele in standard three-factor and single-nucleotide polymorphism techniques to an interval covered by three cosmids on chromosome IV. All three cosmids were injected into bre-1(pk1426) animals, and only one, C53B4, resulted in rescue of bre-1 resistance to toxin phenotype. Injection of PCR fragments that contain all open reading frames, including their putative promoters and 3′-untranslated regions on C53B4, identified C53B4.7 as the only rescuing gene (Fig. 2A). Further confirmation that C53B4.7 and bre-1 are synonymous is provided by the following facts: 1) injection of double-stranded C53B4.7 RNA into rrf-3(pk1426) animals recapitulates the bre-1 resistance to toxin phenotype (Fig. 2B; rrf-3(pk1426) animals show normal response to Cry5B (21) and are more sensitive to dsRNA), and 2) bre-1(ye4) animals show a mutation of Gly217 to Glu in the C53B4.7 open reading frames (Fig. 2C). Injection of the C53B4.7 fragment also rescues the reduced brood size phenotype associated with bre-1(ye4) (not shown).

bre-1 encodes a putative enzyme with significant homology to GMD. This enzyme has been characterized in humans as working upstream of GDP-keto-6-deoxymannose-3,5-epimerase-4-reductase (FX protein) to convert GDP-mannose into GDP-fucose (22). Human GMD maintains 64% amino acid identity to the BRE-1 protein (Fig. 2C). Wormbase shows two alternatively spliced variants for bre-1, C53B4.7a and C53B4.7b. We confirmed both of these variants by sequencing reverse transcription-PCR products from wild-type RNA. C53B4.7b is 15 amino acids longer than C53B4.7a at its N terminus, and the first eight amino acids of C53B4.7a differ as well. Otherwise,
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![Bre-1 (ye4) rescued with C53B4.7]

**Figure 2.** BRE-1 is encoded by the C. elegans gene C53B4.7. A, L4-staged bre-1(ye4) worms transformed with C53B4.7 were grown on E. coli transformed with empty vector (left panels) or on 60% E. coli expressing Cry5B (right panels). The animals are rescued back to full susceptibility. B, RNAi of the bre-1(ye4) gene (C53B4.7) and its nearest homologue F56H6.5 and its open reading frames (C53B4.7 and F56H6.5) either by feeding dsRNA to rrf-3(pk1426) animals or injection of dsRNA into the gonad of C. elegans animals does not result in resistance to Cry5B (Fig. 2B). In addition, co-injection of F56H6.5 and C53B4.7 dsRNAs into rrf-3(pk1426) animals qualitatively resulted in the same level of resistance to Cry5B as injection of C53B4.7 dsRNA alone (Fig. 2B). These results suggest that F56H6.5 does not play an important role in facilitating intoxication by Cry5B. It should be noted that most genome-wide RNAi screens have reported no abnormalities for RNAi of C53B4.7 or F56H6.5, although occasional phenotypes such as larval lethality and larval arrest have been noted (24, 25). However, these screens did not focus in detail on any individual gene (as we have done here), and these studies list any phenotype even if it appears in only 10% of the animals. In all our feeding or injection RNAi experiments, we have not noted any significant phenotype for either of two genes other than Cry5B resistance and low brood size for C53B4.7.

**GDP-fucose Rescue of bre-1(ye4)-mediated Resistance—**If bre-1 is a functional GMD and the toxin resistance defect in the bre-1(ye4) mutant is because of a lack of fucose, then the supplementation of fucose into the cells targeted by the toxin should rescue the bre-1(ye4) toxin resistance phenotype. Similar experiments have been successfully performed in mice where it has been shown that supplementation of fucose into the diet of FX-defective mice (FX is the enzyme directly downstream of GDP-fucose) rescues defects resulting from lack of fucose (26). The fucose presumably enters the cells via a fucose-specific transporter that has been reported in several types of mammalian cells (27, 28).

Repeated attempts to rescue bre-1(ye4) defects by supplementation of fucose in the medium used to grow C. elegans failed to succeed. We hypothesized that the problem with the rescue experiment might reside in the lack of the salvage pathway that is responsible for converting free fucose from the environment to usable GDP-fucose (29). This process involves a fucokinase and GDP-fucose pyrophosphorylase (30). Indeed, a search of the C. elegans genome failed to show any homologues of these proteins in the nematode. Supplementation of the diet with GDP-fucose did not work in attempted experiments presumably because of the absence of a GDP-fucose transporter into C. elegans cells.

We therefore modified our rescue approach. We directly injected the intestines of bre-1(ye4) and bre-2(ye31) L4 worms with either L-fucose or GDP-fucose. The intestine is the anatomical focus of action of Cry5B toxin (10, 12). After a 3-h recovery at 15 °C, these worms were transferred to plates with and without toxin. Injection of L-fucose or GDP-fucose did not harm the health of N2, bre-1(ye4), or bre-2(ye31) animals in the absence of toxin (Fig. 3, A–E, left panels). In contrast, bre-1(ye4) animals were intoxicated similar to wild type in the presence of toxin when injected with GDP-fucose but not L-fucose (Fig. 3, right panels).
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Bre-1( ye4) is because of defects in production of glycolipids. Comparison of orcinol-stained upper phase glycolipids isolated from wild-type and bre-1( ye4) mutant animals demonstrate that bre-1( ye4) animals are indeed defective in the production of some highly polar glycolipid species (Fig. 4A). Glycolipid species D is a notable exception in that it is still produced in normal abundance in bre-1( ye4) animals. However, this species does not contain fucose, and so its presence in the bre-1 mutant is expected (11). There are at least two new species of glycolipid that appear in bre-1( ye4) animals (Fig. 4A).

To see if these glycolipid defects translate into defects in binding Cry5B toxin, activated Cry5B was biotinylated and used in overlay binding experiments as described previously (11). Consistent with the results found with orcinol-stained glycolipids, three dominant Cry5B-binding glycolipid species present in wild type, B, C, and F, are absent in bre-1( ye4) animals (Fig. 4B). Both species B and C are known to contain fucose; the structure of species F is not known (11). Cry5B-binding glycolipid species E is present in the mutant (albeit at reduced levels), but this species normally lacks fucose so its presence in bre-1( ye4) is expected. At least two poorly resolved new glycolipid bands produced by bre-1( ye4) bind Cry5B. These bands might each represent a defucosylated B, C, or F glycolipid species that retains some binding activity.

To confirm that glycolipids from bre-1( ye4) animals lack fucose, we probed upper phase glycolipids from wild-type and bre-1( ye4) mutant animals with U. europaeus agglutinin I (UEA-1) that recognizes terminal fucose residues linked to galactose via an α-2 linkage (contained in both species B and C). In wild type animals, glycolipid species C is the predominant UEA-1-binding species (Fig. 5, A and B). Several less abundant and less polar UEA-1 binding species are also detected. Although glycolipid species B also contains terminal fucose, it does not bind UEA-1 or does so poorly. This result is interesting because the only difference between bands B and C is one terminal galactose on band B. UEA-1 may be sensitive to a structural change caused by the addition of the terminal galactose to band B, which could make fucose less accessible. In glycolipids from bre-1( ye4) animals, the major (band C) and minor UEA-1-binding species are no longer present (Fig. 5B).

Although there are still some Cry5B-binding glycolipids in bre-1( ye4) mutant animals, qualitatively there appear to be less total receptors for Cry5B in the mutant relative to wild-type animals (Fig. 4B). To confirm this result quantitatively, total upper phase glycolipids from increasing numbers of wild-type (N2), bre-1( ye4), and bre-3( ye28) animals were isolated, immobilized in polystyrene wells, and probed with biotinylated Cry5B. The results were as predicted based on their resistance.

A–C, right panels). In other words, bre-1( ye4) animals can be rescued back to wild type with injection of GDP-fucose. Rescue of the low brood phenotype at 25 °C is also achieved with injection of GDP-fucose (not shown). bre-2( ye31) animals are not rescued back to toxin susceptibility when injected with either 1-fucose or GDP-fucose because they are not defective in fucose biosynthesis (Fig. 3, D and E, right panels). These data are consistent with bre-1 encoding a functional GMD and with C. elegans lacking a functional fucose salvage pathway.

Bre-1( ye4) Animals Are Defective in the Production of C. elegans Glycolipid Receptors for Cry5B—Because at least two of the glycolipid receptors for Cry5B contain two terminal fucose residues (11), we hypothesized that the resistance phenotype of
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FIGURE 5. *bre-1*(*ye4*) animals are deficient in fucosylated glycolipids. A, orcinol-stained plate of N2 wild-type and *bre-1*(*ye4*) glycolipids that were resolved in tandem with the plate in B, B, fucose-binding lectin overlay of N2 and *bre-1* glycolipids. This shows that *bre-1* glycolipids do not bind the fucose-specific lectin, whereas band C and other glycolipids from N2 do bind the lectin.

FIGURE 6. *bre-1*(*ye4*) animals show reduced Cry5B binding to *C. elegans* glycolipids. Total glycolipids from N2 wild-type (diamonds), *bre-1* (squares), and *bre-1*(*ye28*) (triangles) animals were tested for their ability to bind biotinylated Cry5B using an enzyme-linked immunosorbent assay. The number on the x axis refers to the number of worm equivalents from which glycolipids were isolated for that particular well.

FIGURE 7. Total monosaccharide analysis of N2 wild-type, *bre-1*, and *bre-3* whole worm pellets. These results represent the average of two trials. Glucose is shown on the right at a different scale.

We have demonstrated previously that *C. elegans* protein-linked N- and O-glycans are rich in fucosylated structures (11).

We show intermediate levels of toxin resistance, have total glycolipids that show intermediate levels of binding relative to wild type (high levels of toxin binding) and *bre-3* animals (very low levels of toxin binding) (Fig. 6).

*bre-1* Mutant Animals Show Severe Reductions in Fucose and in Fucosylated N- and O-Glycans—To determine the effects of the *bre-1*(*ye4*) mutation on the production of fucose, we performed monosaccharide analyses of wild-type, *bre-3*(*ye28*), and *bre-1*(*ye4*) mutant animals (Fig. 7). Although *bre-1* mutant animals display relatively normal levels of galactose, GalNAc, and GlcNAc, they contain no detectable fucose. Thus *bre-1* plays a major role in the production of GDP-fucose in *C. elegans*, consistent with it encoding a functional GMD.

**DISCUSSION**

The *bre-1* gene, which mutates to resist Cry5B crystal toxin, encodes a putative GMD, the first enzyme in the biosynthesis of cellular GDP-fucose. The evidence that BRE-1 is the major functional GMD in *C. elegans* is as follows: 1) BRE-1 shows extensive (64% amino acid identity) identity with human GMD; 2) loss of BRE-1 results in a loss of detectable fucose on proteins as determined by monosaccharide analysis and by mass spec-
FIGURE 8. MALDI mass spectra of permethylated N- and O-glycans from N2 and bre-1 worms. The N-glycans of N2 and bre-1 worm glycoproteins were released from tryptic glycopeptides by sequential digestion with PNGase F and PNGase A, and O-glycans were subsequently released by reductive elimination. Released glycans were permethylated and screened by MALDI-MS. A–C, N2 PNGase F, PNGase A, and O-glycans; D–F, bre-1 PNGase F, PNGase A, and O-glycans.

TABLE 4
Assignments of molecular ions observed in MALDI spectra of permethylated N- and O-glycans of N2 and bre-1 worms

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FIGURE 9. MALDI mass spectrum of permethylated bre-1 upper phase glycolipid-derived glycans. The glycan component of upper phase bre-1 glycolipids were enzymatically released, permethylated, and analyzed by MALDI mass spectrometry.

tive in the production of C. elegans polar glycolipids; some polar glycolipids appear to be fully absent (e.g. species B and C), whereas some polar glycolipid species appear to be reduced in level (e.g. species E). Because polar glycolipids serve as receptors for Cry5B (11), the reduction in number and/or affinity of glycolipid species that bind Cry5B toxin in the bre-1 mutant explains the basis of resistance. They also explain the lower level of resistance seen in bre-1 animals relative to the other bre mutants; the binding of Cry5B to glycolipids found in the bre-1 mutant is quantitatively less than that found in wild-type animals but greater than what is seen in the bre-3 mutant, which disrupts the production of all polar arthroseries glycolipids in C. elegans (11). Thus, Cry5B toxicity correlates with the amount of...
binding to glycolipids. This binding is heavily dependent on the presence of terminal galactose residues (11), which may explain why the absence of fucose is not sufficient for achieving full resistance to Cry5B. In contrast to galactose, fucose is not capable of competing with receptor in Cry5B binding experiments. Because of the fact that galactose is still present in bre-1, it is possible that the remaining toxin-binding bands found in bre-1 glycolipids may be altered forms of the primary binding bands B and C found in N2, as suggested in Fig. 9. Although we cannot be sure that the ye4 allele represents a complete loss of function, it seems to represent at least a strong reduction of function allele because of the following: 1) ye4 mutation is in an amino acid residue that is conserved in GMDs, 2) RNAi leads to a similarly penetrant phenotype, and 3) bre-1(ye4) in trans to a deletion allele qualitatively leads to the same level of resistance as the homozygous mutant (not shown).

This report is the first to study the fucose biosynthetic pathway and GMD in C. elegans. Because of the importance of fucosylation, GMD itself is an important enzyme studied in a wide variety of organisms. Biochemical deficiency of GMD activity in humans is associated with leukocyte adhesion deficiency II, a rare genetic disease characterized by immunodeficiency and severe mental and growth retardation (33). GMD in Arabidopsis thaliana is encoded by two genes, GMD1 and GMD2 (MIR1), and loss of GMD2 leads to reduced tensile strength of elongating stem segments and slight dwarfism (34). GMD, along with the fucose salvage pathway, also plays an important role in the ability of the bacterial symbiont Bacteroides to colonize the mammalian intestine (35), and a mutation in Pseudomonas fluorescens GMD was shown to be deficient in biofilm formation and attenuated for virulence in a Drosophila model system (36). Our data also demonstrate that C. elegans lacks a functional fucose salvage pathway to convert 1-fucose to GDP-fucose because injection of 1-fucose is not able to rescue bre-1 mutant phenotypes (in contrast to injection of GDP-fucose). It was suggested that Drosophila melanogaster also lacks a functional fucose salvage pathway because no detectable homologues of the enzymes involved in this pathway could be discerned from its genome (37).

These data show a unique variant on the mechanism of resistance to crystal toxin via biosynthesis of a monosaccharide. All previous data in insects and nematodes point to either mutation in proteases required to process crystal toxin or mutation in genes that directly encode the receptor (3). Here, resistance is caused by mutation of a gene that indirectly influences the production of receptor. It is conceivable that many other similar mutations will be found in the future. In addition to the resistance defect, bre-1 mutant animals show a significant reduction in brood size at 25 °C. Based on the glycan analysis of bre-1 mutant animals, it is plausible that the reduction in brood observed is because of the absence of fucosylated glycan critical to the development of the germ line and/or proper egg fertilization. Fucosylated glycan has been shown to be critical in mammalian fertility (38). Interestingly, bre-3 mutant animals also display a reduced brood size at 25 °C suggesting that bre-3 is involved in making glycolipid species that is made important for fertility but that does not involve bre-2, -4, or -5 (e.g. Manβ1–4Glc-ceramide).

There is evidence for two interesting feedback mechanisms in the bre-1 mutant. First, the level of at least one nonfucosylated glycolipid (e.g. species E) is reduced in bre-1 mutant animals. Thus, the lack of fucosylation somehow feeds back onto the overall production of glycolipids. Furthermore, the severe reduction in fucose appears to result in a moderate increase in the amount of total cellular mannose. Because GDP-mannose is the precursor for GDP-fucose, this result is expected because it might be predicted that there would be an accumulation of mannose.

Fucose is also recognized by a functioning mammalian immune system as part of an epitope for IgE antibodies attacking the parasitic helminthes Hemonchus contortus in infected sheep (39). The core α1–3 fucosylated N-glycan functioning as an epitope in this study has also been found in C. elegans (39). Based on this information alone, a fucose-deficient C. elegans mutant such as bre-1 could be an important tool in the study of the IgE immune response to parasitic nematodes.

The reduction in total fucose levels and fucosylated N- and O-glycans in the bre-1 mutant is striking and dramatic. Fucose levels are reduced to undetectable levels in the mutant, and fucosylated N- and O-glycans are missing by mass spectroscopy analyses. There is no precedent for such a severe level of fucose depletion leading to such a mild phenotype in an animal. The FX mouse, which is defective for the second enzyme in the fucose biosynthetic pathway, has been found to be incapable of survival without being provided an outside source of fucose (26). As noted above, defects in GMD in humans can lead to leukocyte adhesion deficiency type II, which is a human disorder characterized by growth and mental retardation in addition to immune problems stemming from a defect in leukocyte rolling during inflammation. The lack of a fucose salvage pathway is consistent with these data; the nematode has apparently evolved to survive with very little fucose and fucosylated proteins.

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Addendum — The protein encoded by the bre-1 gene has been shown recently to have the predicted enzymatic activity for GMD (41).