srf-3 is a mutant of *Caenorhabditis elegans*, resistant to bacterial infection and to biofilm binding, is deficient in glycoconjugates*

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srf-3 is a mutant of *C. elegans* that is resistant to infection by *Microbacterium nematophilum* and to binding of the biofilm produced by *Yersinia pseudotuberculosis* and *Yersinia pestis*. Recently, SRF-3 was characterized as a nucleotide sugar transporter of the Golgi apparatus occurring exclusively in hypodermal seam cells, pharyngeal cells, and spermaphase. Based on the above observations, we hypothesized that srf-3 may have altered glycoconjugates that may enable the mutant nematode to grow unaffected in the presence of the above pathogenic bacteria. Following analyses of N- and O-linked glycoconjugates of srf-3 and wild type nematodes using a combination of enzymatic degradation, permethylation, and mass spectrometry, we found in srf-3 a 65% reduction of acidic O-linked glycoconjugates containing glucuronic acid and galactose as well as a reduction of N-linked glycoconjugates containing galactose and fucose. These results are consistent with the specificity of SRF-3 for UDP-galactose and strongly suggest that the above glycoconjugates play an important role in allowing adhesion of *M. nematophilum* or *Y. pseudotuberculosis* biofilm to wild type *C. elegans*. Furthermore, because seam cells as well as pharyngeal cells secrete their glycoconjugates to the cuticle and surrounding surfaces, the results also demonstrate the critical role of these cells and their secreted glycoproteins in nematode-bacteria interactions and offer a mechanistic basis for strategies to block such recognition processes.

Caenorhabditis elegans is a genetically and developmentally well characterized organism that has been used as a model to study host-pathogen interactions. Many of these involve carbohydrate recognition, suggesting that chemical analyses of carbohydrate components involved in these processes may be important toward understanding of the molecular basis for these interactions.

The soil is the natural habitat for *C. elegans*, where it comes in contact with commensal and pathogenic bacteria. Among the latter are plant and animal pathogens such as *Erwinia chrysanthemi*, *Agrobacterium tumefaciens*, *Shewanella frigidimarina*, *Photorhabdus luminescens*, *Xenorhabdus nematophilus* (1), *Yersinia pseudotuberculosis* (2, 3), and *Mycobacterium nematophilum* (4). Human pathogens can also infect *C. elegans*, including Gram-negatives, such as *Pseudomonas aeruginosa* (5), *Burkholderia pseudomallei* (6), *Serratia marcesens* (7, 8), and *Yersinia pestis* (2), and gram positives, such as *Streptococcus pneumoniae*, *Enterococcus faecalis*, and *Staphylococcus aureus* (9).

Host-pathogen interactions require bidirectional recognition factors, and often at least one of the components is a glycoconjugate (10). Examples are bacterial toxins such as aerolysin from *Aeromonas hydrophila*, cholera from *Vibrio cholerae*, hemolysin from *Escherichia coli*, and the crystal proteins from *Bacillus thuringiensis* (11). Infection of *C. elegans* by the last bacterium leads to destruction of its intestine. *C. elegans* mutants resistant to this infection have been isolated and are defective in the *bre* gene family, one of which is a homolog of *Drosophila melanogaster* egghead (*egh*) and encodes a GDP-Man;βGalCer β1,4-mannosyltransferase (*bre-3*); another encodes a UDP-GalNAc;β1,4-acetylgalactosaminyltransferase (*bre-4*); and a third is a homolog of *Drosophila brainactin* (*brn*) and encodes a UDP-GlcNAc:Man N-acetylglucosaminyltransferase (*bre-5*) (12, 13). Identification of these BRE activities of *C. elegans* provides striking evidence for the importance of carbohydrates in host-pathogen interactions of this nematode. The BRE proteins appear to be required for sensitivity to crystal proteins and offer a useful model to study invertebrate-host toxin interactions.

Several of the above mentioned pathogens infect *C. elegans* by interacting with the cuticle and surrounding surfaces. Thus, *M. nematophilum* infects the posterior cuticle surface near the anal opening and adjacent rectal tissues, leading to reduced generation times and constipation (4). *Y. pestis* and *Y. pseudotuberculosis* form a biofilm around the head region of *C. elegans*, preventing feeding and leading to starvation. *C. elegans* srf-2, -5, and -3 mutants, which have altered lectin binding affinity to the cuticle surface (2, 3) suggesting changes of oligosaccharides, are resistant to infection by *M. nematophilum*, *Y. pestis*, and *Y. pseudotuberculosis*. srf-3 has recently been cloned and shown to encode a nucleotide sugar transporter for UDP-Gal and UDP-GlcNAc (14). SRF-3: GFP fusion protein shows tissue-specific expression at the spermaphase, hypodermal seam cells, and g1 and g2 glandular cells, with the last two cell types known to play a role in the biosynthesis of the cuticle and surrounding tissues (15). Therefore, one might predict that in srf-3 a subset of glycans are altered, leading to the above described phenotypes.

**EXPERIMENTAL PROCEDURES**

*Isolation of N-Glycans—* The glycoprotein-rich fraction was isolated from 10–15 g of *C. elegans* as previously described (16). Briefly, following treatment of proteins with i-1-tosylamido-2-phenylethyl chloro-
methyl ketone-treated trypsin, N-glycans were released with 8000 units/ml PNGase F (New England Biolabs) overnight at pH 8.5 and 37 °C. Free glycans were separated from the trypptic peptides by solvent precipitation (17) with 50% methanol at pH 5.5 followed by centrifugation at 3500 \times g. The solutions, containing free glycans and some peptides, were subjected to rotary evaporation, and the resulting precipitate was suspended in distilled water and applied to Sep Pak C-18 cartridges. Glycans were collected by elution with distilled water. Absorbed peptides were eluted with isopropyl alcohol, combined with those that had precipitated in 50% methanol, the mixture was dried, and the mixture was digested with PNGase A. The above isolation procedure was then repeated to separate glycans from peptides. Free glycans were quantitated using the phenol sulfuric assay for neutral hexose standardized with mannose (18) except for those released by PNGase A, which were estimated by total ion intensity of MALDI-TOF MS spectra compared with standardized samples.

Isolation of O-Glycans—Subsequent to sequential PNGase F and A release of N-glycans, the above peptide/glycopeptide mixture was subjected to β elimination using standard protocols (19), except that acidic glycans were eluted from AG-1X2 columns with 100 mM ammonium acetate following elution of neutral glycans from the same column with water. Solutions of the glycans were evaporated in a Savant speed vacuum apparatus prior to hydrolysis.

Monosaccharide Analysis—Oligosaccharides were applied to porous graphitized carbon cartridges, washed with 3 ml of distilled water, and eluted with 30% acetonitrile, 0.1% trifluoroacetic acid. Monosaccharide analysis was performed on a Dionex high pH anion exchange chromatography system equipped with a MA-1 column, and content was determined using a linear calibration curve for each monosaccharide as previously described (19).

Mass Spectrometric Analysis—MALDI-TOF MS was performed on a

**TABLE I**

| Measured m/z | Composition |
|--------------|-------------|
| 1171.7       | Man\_GlcNAc3 |
| 1417.0       | Fuc\_Man\_GlcNAc2 |
| 1550.0       | Man\_GlcNAc3 |
| 1724.0       | Fuc\_Hex\_GlcNAc2 |
| 1754.0       | Man\_GlcNAc4 |
| 1784.0       | Man\_GlcNAc5 |
| 1866.4       | Hex\_GlcNAc3 |
| 1929.0       | Fuc\_Gal\_Man\_GlcNAc2 |
| 1988.0       | Man\_GlcNAc3 |
| 2111.5       | Hex\_HexNAc5 |
| 2173.3       | Fuc\_Hex\_HexNAc3 |
| 2192.4       | Man\_GlcNAc2 |
| 2315.8       | Hex\_HexNAc5 |
| 2377.0       | Fuc\_Gal\_Man\_GlcNAc2 |
| 2396.9       | Man\_GlcNAc2 |

* All ions are sodium adducts.

* Glycan molecular ions that are only observed in PNGase A releases and have not been previously reported.

* Glycan molecular ions that are only observed in PNGase A releases and have been previously reported by Haslam et al. (24, 25).

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**FIG. 1.** MALDI-TOF MS analysis of *C. elegans* N2 Bristol- and srf-3 PNGase F-released N-glycans. A, N2 Bristol glycans; B, N2 Bristol in the m/z 1725–2000 region; C, srf-3 glycans; D, srf-3 glycans in the m/z 1725–2000 region; E, comparison of the relative abundances of individual glycan isobars in N2 Bristol and srf-3 glycans. Molecular ions [M + Na]+ are displayed as percentage abundance with S.E. displayed as error bars. The asterisk appears above compounds that were found to be diminished in srf-3 N'-glycans.
The core GalNAc may also be substituted by neutral and acidic residues (23). Gal may be substituted by 35–90 V for MALDI. Nomenclature is that of Domon and Costello (21).

Collision-induced dissociation (CID) fragmentation data were collected using an Applied Biosystems/MDS-Sciex QStar Pulsar quadrupole/orthogonal acceleration TOF mass spectrometer (QqTOF) with nanospray or MALDI (UV laser; nitrogen, 337 nm) sources (Applied Biosystems Inc., Framingham, MA). The MALDI matrix was 2,5-dihydroxybenzoic acid, and typically 50–200 laser shots were summed for each spectrum. The laser power used was 30–33 μJ. Nanospray data was collected using 1-μm nanospray tips, pulled with a Sutter model P-87 micropipette puller. Ion source voltage was 1200–1400 V. Nitrogen was used as the collision gas for MS/MS experiments. The range of operator-controlled collision voltages was 12–50 V for electrospray and was collected using 1-μm nanospray tips, pulled with a Sutter model P-87 micropipette puller. Ion source voltage was 1200–1400 V. Nitrogen was used as the collision gas for MS/MS experiments. The range of operator-controlled collision voltages was 12–50 V for electrospray and was collected using 1-μm nanospray tips, pulled with a Sutter model P-87 micropipette puller. Ion source voltage was 1200–1400 V. Nitrogen was used as the collision gas for MS/MS experiments.

RESULTS

Rationale for Workup Strategy—Based on our knowledge that C. elegans srf-3 mutants are deficient in a Golgi apparatus transporter for UDP-Gal and UDP-GlcNAc, we decided to assess the structures of N- and O-glycans released from protein extracts of whole mixed stages of srf-3 and wild type, parental N2 Bristol strain nematodes. Many of the C. elegans N- and O-glycan structures have been reported, thereby providing the basis for the comparisons of this study (16, 22–28). N-Glycans from C. elegans can be released first with PNGase F, yielding most known N-glycans except those containing α1,3Fuc linked to the reducing end GlcNAc; thereafter, PNGase A treatment releases most remaining N-glycans. Although a subset of the N-glycans containing internally linked Gal are released with both of the above enzymes, the expectation, based on previous studies, was that these species should be enriched in the oligosaccharide fraction released by PNGase A (24, 25). Therefore, significant changes in Gal- or Fuc-containing N- and O-glycans should be observable with this strategy, since previous studies with C. elegans suggest that Fuc addition to glycans often requires the previous addition of Gal (24, 25). N-Glycans containing Golgi type GlcNAc or late additions may also be seen in the above N-glycan fractions.

Previous studies showed that the major C. elegans O-linked glycans contain β1,3Gal bound to core Ser/Thr-linked GalNAc residues (23). Gal may be substituted by β1,2Glc or β1–4,6Glc. The core GalNAc may also be substituted by β1,6Glc or β1,6Gal. Neutral and acidic O-glycans were therefore analyzed based on the prediction that in srf-3 mutants, all of the above O-glycan species may be affected qualitatively and quantitatively.

N- and O-glycans were permethylated prior to MS analysis. This renders oligosaccharides chemically equivalent, with peak intensities generated in the mass spectrometer detector being similar over a broad mass range (29). Using this approach, we found molecular ion intensities to vary within 0.7% S.E. of total spectral peak intensity when analyses were performed in duplicate. This reproducibility was maintained in samples originating from different nematode preparations, as shown below.

PNGase F-released Glycans—In a preliminary study, monosaccharide analyses were performed on a PNGase F-released fraction of N2 wild type and srf-3 nematodes. The molar ratio of GlcN/Mann/Gal/GalNAc/Fuc/Glc was 1:2.0:13.0:0.5:30:10:16 in wild-type and 1:1.6:0.07:trace:0.15:7.0 in srf-3 strains. The Gal, GalN, and Fuc content was lower in srf-3 than in wild type. Comparable differences in N-glycan composition were also observed in wild-type and srf-3 strains (24, 25), and their decrease is in agreement with the reduction in the molar Gal content described above. A slight increase of Hex4HexNAc and complex Hex3HexNAc glycan was seen in srf-3 compared with wild type (Fig. 1E). The peaks at m/z 1905.9 and 1701.8 are most likely those of a glucose-containing polyhexose contaminant (Fig. 1; see below).

When the above N-glycan mixtures were subjected to α1,2-mannosidase digestion, prior to permethylation and MALDI-TOF MS analyses, Hex3HexNAc decreased, whereas Hex5HexNAc increased, in both wild-type and srf-3 (Fig. 2, A and B), demonstrating that abundant molecular ions in both strains are high mannose-containing glycans (Fig. 1 and Table 1). Glycans with m/z 1754.0 and 1929.0, described above, were resistant to the above enzymatic treatment and most likely do not contain terminally linked α1,2-linked Man (Fig. 2A). The majority of Hex3HexNAc, m/z 1754.0 (Fig. 1, B and D), of both strains is resistant to α1,2-mannosidase, demonstrating that it is not Man6GlcNAc, which contains one terminal α1,2Man (Fig. 2, A and B). However, when the mixtures were subjected to jack bean α-mannosidase digestion, the Hex3HexNAc signal intensity was greatly diminished in both strains. High mannose glycans are expected to be digested with α-mannosidase to Man1–6GlcNAc. Since, after this treatment, peak intensity for...
Hex$_3$HexNAc$_2$ remains, it is likely that a significant amount of this compound is derived from the Hex$_2$HexNAc$_2$ after loss of three mannose residues (Fig. 3).

PNGase F glycans released from wild type strains were subjected to reductive amination with 2-aminobenzamide followed by separation on a C-18 column. The Hex$_2$HexNAc$_2$ peak was collected, identified as the major species by MALDI-TOF MS (Fig. 4A, m/z 1539.6), further purified by porous graphite chromatography, and subjected to hydrolysis for monosaccharide analysis. In this case, GlcN, derived from GlcNAc, is underrepresented as a result of derivatization during reductive amination with 2-aminobenzamide of the reducing end. The Hex$_2$HexNAc$_2$ sample yielded Gal:Man in a 1:3 molar ratio (Fig. 4B). A small peak for GlcN was also seen, as well as a major one for Glc, the latter being most likely a contaminant from the polyhexoses (m/z 1133.5 and 1295.5) in Figs. 1 and 4A.

The above results lead us to postulate that the Hex$_2$HexNAc$_2$ was Gal$_1$,Man$_2$,GlcNAc$_2$, with none of the Man being terminally 1,2-linked, and consistent with the retention of the five archeotypal Man residues. A possible linkage sequence may be Man$_1$,$\alpha$(Man$_1$,$\alpha$)Man$_1$,$\alpha$(Gal$_1$,XMan$_1$,$\alpha$)-Man$_3$,$\beta$(1,4)$\beta$(1,6)GlcNAc$_2$. This glycan was then analyzed by CID fragmentation using nanospray on the QoTOF mass spectrometer (Fig. 5).

Abundant product ions corresponding to Y, B, and C fragments are obtained from cleavage of each glycosidic bond in the predicted Man$_1$,$\alpha$(Man$_1$,$\alpha$)Man$_1$,$\alpha$(Gal$_1$,XMan$_1$,$\alpha$)$\beta$Man$_3$,$\beta$(1,4)$\beta$(1,6)GlcNAc$_2$ structure. The $^{3.5}$A$_3$ (m/z 764.80) cross-ring fragment and $^{3.5}$A$_{\alpha\beta}$/$\beta$(m/z 794.31) secondary fragment are consistent with the Man$_1$,$\alpha$(Man$_1$,$\alpha$)Man$_1$,$\alpha$ moiety that was suggested by its sensitivity toward jack bean $\alpha$-mannosidase digestion. Also, the $B_{\gamma}$/$\gamma$(m/z 432.21, $x = \alpha'$ and $\alpha''$) secondary fragment is diagnostic of the disubstituted Man in the same Man$_1$,$\alpha$(Man$_1$,$\alpha$)Man$_1$,$\alpha$ moiety. The $B_{\gamma}$/Y$_{3\alpha}$ (m/z 1084.49) and $B_{\gamma}$/Y$_{5\alpha}$ (m/z 839.4) secondary fragments are diagnostic of the Gal1,Man$_1$,$\alpha$ lower arm. Haslam et al. (24) have predicted the Gal-Man linkage in the likely related Fuc$_2$Gal$_1$,Man$_2$,GlcNAc$_2$ compounds to be Gal1,2Man$_1$3. However, we could not confirm this assignment because the likely predictive cross-ring fragment ions were not unique values.

The Gal$_1$,Man$_2$,GlcNAc$_2$ fragmentation pattern, resistance to $\alpha$1,2-mannosidase, loss of 3Man with jack bean $\alpha$-mannosidase, and presence of Gal led us to postulate its structure as follows: Man$_1$,$\alpha$(Man$_1$,$\alpha$)Man$_1$,$\alpha$(Gal$_1$,Man$_1$,$\alpha$)$\beta$Man$_3$,$\beta$(1,4)$\beta$(1,6)GlcNAc$_2$.

We observed that some of these oligosaccharides are methylated in vivo, leading to their dispersion into a series of minor peaks that cause difficulties in their purification. This may explain our failure to detect these Gal-containing oligosaccharides in our original analysis of C. elegans N-glycans when using high field NMR (16), an inherently insensitive technique. The abundance of Hex$_2$HexNAc$_2$ was slightly increased in srf-3 compared with wild type, demonstrating that the addition of GlcNAc was similar (Fig. 1 and Table II). Previous studies from our own and other laboratories had identified the other PNGase F-released glycans shown in Fig. 1E such as Hex$_3$-$\alpha$HexNAc$_2$-$\alpha$ as Man$_1$,$\alpha$GlcNAc$_2$-$\alpha$ (16, 22, 24, 26).
These permethylated glycans are listed in Table I. As seen in total N-glycans by PNGase F, release by PNGase A yielded Man3–4GlcNAc4–5 and Man7–9GlcNAc2 (16, 22, 24, 25, 30). This Previous studies had shown the latter glycans to be peak containing Gal1Man5GlcNAc2 [M + Na]+ of acidic low neutral PS MS. The matographed by C-18 chromatography and analyzed by MALDI-TOF are decreased in a linear calibration curve for each monosaccharide.

PNGase A-released Glycans—Following the release of glycans by PNGase F, release by PNGase A yielded ~5% of the total N-glycans. The values observed for the molecular ions of these permethylated glycans are listed in Table I. As seen in Fig. 6C, oligosaccharides reported to contain Fuc and Gal (deoxy-Hex3–4HexNAc4–5, indicated by asterisks) (24, 25) are decreased in srf-3 when compared with wild type, whereas high mannose and complex oligosaccharides (Hex3–4HexNAc2 and Hex2–4HexNAc4–5, indicated with crosses) are increased. Previous studies had shown the latter glycans to be Man3–4GlcNAc4–5 and Man2–3GlcNAc2 (16, 22, 24, 25, 30). This shift, which is consistent with the reduced availability of UDP-Gal in these srf-3 mutants, was also observed previously, but less dramatically, in the PNGase F-released fraction (Fig. 1E). The major peaks observed in the PNGase A fraction in both wild type and srf-3 correspond to residual high mannose glycans Man4–5GlcNAc2 that were not completely released by PNGase F. Overall, in the above studies, the ion intensity is shifted toward GlcNAc-modified glycans; therefore, although SRF-3 transports UDP-GlcNAc, it is unlikely that the availability of this nucleotide sugar was reduced for the biosynthesis of PNGase A-releasable glycans in srf-3 mutants.

Acidic O-Glycan Fraction—Acidic O-glycans were eluted from an AG-1X2 column with 100 mM ammonium acetate following neutral O-glycan elution with water. The total amount of acidic O-glycans of srf-3 was ~3-fold less than wild type (106 versus 298 nmol/g) as determined by a phenol sulfating assay. Most molecular ions detected in this fraction indicated the presence of HexA (Fig. 7 and Table I), consistent with the composition of GlcA-containing glycosidase previously reported for C. elegans (23).

Monosaccharide analyses were performed on fractions from both strains and were normalized to Gal. The molar ratio of Gal/Fuc/Glcn/MAN/GlcA/Glc was 1:0.45:0:07:0.20:5.94 in srf-3 glycans and 1:0.11:0.14:0:04:0.50:11.60 in wild type. Increases in Fuc and GlcN and decreases in GlcA and Glc were seen in srf-3 compared with wild-type. MALDI-TOF MS analysis of the permethylated glycans revealed that in srf-3, HexAHex2HexNAc4–5 [M + Na]+ (m/z 1364.68) was below detection limits, and that of HexAHex2HexNAc4–5 [M + Na]+ (m/z 1160.69) was reduced compared with wild type (Fig. 7). MALDI QqTOF MS/MS fragmentation analysis of the [M + Na]+ m/z 1160.69 isobar provided evidence of four branch isomers (Fig. 8A). Abundant Y ions were seen for all structures due to loss of the terminal Hex, or GlcA substitutions. The presence of Structure I was indicated by the observation of Y1, Z1, B2, C2, and Z1, product ions at m/z 520.31, 502.30, 663.32, 681.33, and 924.5, respectively. These ions define the core region and provide strong evidence that two branches substitute the reduced HexNAc, one containing a GlcA and two Hex residues, whereas the other contains a single Hex residue. The larger branch contains a 3,6-disubstituted Hex as indicated by the presence of the 2,4A2 at m/z 329.16. The presence of Structures IIA and B was supported by the presence of core region ions B2 and C2 at m/z 867.49 and 885.41. The presence of di- or trisubstituted Hex of Structures IIA and B was suggested by the presence of the B2/Y1 and B1/Y1 (where x represents β or γ) secondary fragments at m/z 635.34 and 649.34. Structure IIB is consistent with Y1a and Z1a fragments at m/z 738.38 and 720.34. Both can be envisioned as related to compounds already reported in C. elegans (23). Structure III was indicated by the presence of Z1b and Y1b ions at m/z 720.3 and 738.4, which are consistent with structures having linear HexA. Further evidence of Structure III is seen by the presence of a 0.2 Hex cross-ring fragment at m/z 781.42, which indicates the presence of Hex that is monosubstituted with GlcA.

MALDI QqTOF MS/MS fragmentation analysis of permethylated HexAHex2HexNAc4–5 [M + Na]+ (m/z 1364.68) is shown in Fig. 8D. The presence of a trisubstituted branch is supported by the presence of the B2 and C2 ions at m/z 867.47 and 885.39, the absence of B-type and C-type fragments at m/z 738.38 and 720.34, and also by the presence of secondary fragments B2/Y2 and B2/Z2, seen at m/z 649.29 and 667.32 (where x represents α or α′). The identical compound has been reported in C. elegans (23).

Based on the above results and previous studies, the above glycans have the Galβ1,3GlcA high core and probably have the branching patterns shown in Fig. 9. Guerardel et al. (23) have shown that the core GalNAc can also be substituted with β,1,6Gal. We are not certain of the identity of the terminal Hex substitution in HexA in structures IIA and B but have represented it here as Gal. However, it is possible that Gal or Man substitutes. Thus, the reduced overall amount of acidic O-glycans and the loss of more highly processed GlcA-containing glycoforms are consistent with the decreased availability of UDP-Gal in srf-3.

Neutral O-Glycan Fraction—Neutral O-linked glycans, released from srf-3, were 21 nmol/g, whereas those of wild type were 30. Preliminary monosaccharide analysis of the neutral O-glycan fraction of both strains was also performed. The molar ratio of Fuc/Gal/MAN/Glc was 1:2.29:0.39:10.1 in srf-3 strain versus 1:1.69:0.39:10.7 in wild-type glycans. The profiles were similar, although srf-3 glycans were increased in Gal likely due to a shift in Gal/Glc containing branch isomers. Virtually no GalNol was detected from the material released from the reducing end, most likely the result of its breakdown during the preparation of monosaccharides. Fig. 10, A and B, and Table II
FIG. 5. CID nanospray QqTOF MS/MS analysis of *C. elegans* permethylated Gal$_1$Man$_1$GlcNAc$_n$. The origin of key fragments is shown in the upper panel. Y, B, and C ions are indicated by broken, thick broken, and solid lines. Key secondary and cross-ring fragments are also indicated. Nomenclature is that of Costello and Domon *et al.* (21).
show the molecular ions detected, whereas the histogram in Fig. 10C shows that virtually no differences in O-linked glycans were found between wild type and srf-3 except for a slight loss of Fuc-containing oligosaccharides.

Molecular ions detected at m/z 1146.6 and 942.6 are assigned as [M + Na]+ Hex$_3$HexNAc$_1$ol, consistent with Glc$_1$Gal$_1$GalNAc$_1$ol, previously reported in C. elegans (23). We cannot, however, rule out the presence of other isobaric compounds. Low abundance molecular ions were also detected at m/z 1166.8, 1320.8, and 1524.8, consistent with components containing deoxy-Hex$_1$Hex$_1$HexNAc$_1$ol, which have not been reported previously in C. elegans.

### DISCUSSION

In this study, we have found that C. elegans srf-3 mutants that are defective in a Golgi apparatus transporter for UDP-Gal and UDP-GlcNAc have major reductions in O- and N-linked glycans compared with wild type nematodes. Acidic O-linked glycans containing GalNAc at their reducing end and GlcA at the nonreducing end were decreased by 65%. The oligosaccharide structures observed here were consistent with previous studies by Guerardel et al. (23) showing acidic O-linked species such as Glc$_2$Gal$_1$GalNAc$_1$, containing β1,3Gal linked to the reducing end of GalNAc in these nematodes. N-Glycans containing Fuc$_1$Gal$_2$Man$_3$GlcNAc$_2$ were also decreased compared with wild type nematodes.

Two important conclusions can be drawn from the above results: 1) the glycoconjugate species decreased in these mutants are most likely involved in the previously described interactions between the surface of wild type nematodes and the pathogenic bacteria M. nematophilum and the secreted biofilms of Y. pseudotuberculosis and Y. pestis; and 2) because all of the above glycans contain Gal, our findings are consistent with the substrate specificity of the SRF-3 transporter, resulting in a decrease of UDP-Gal supply to the Golgi apparatus lumen.

These results also strongly imply an important biological role for oligosaccharides of glycoproteins secreted to the surface coat and cuticle by seam cells and pharyngeal g1 and g2 cells, where the SRF-3 is localized. These oligosaccharides most likely play a critical role in recognition between nematodes and pathogenic bacteria. The srf-3 mutant used in this study, srf-3(yj10), has a G to A transition that creates a stop codon in exon 5 (of six) and is expected to terminate the protein at amino acid 234 instead of 328 as in the wild type protein, and the variant protein is thereby lacking the putative three most carboxyl terminal transmembrane domains. Is this protein a functional null? Whereas we cannot predict this with complete certainty, we did observe that, when the mutation is placed in the context of a genetic deficiency, srf-3(yj10;D)22, the resulting phenotype was no more severe than the homozygous srf-3(yj10),

### Table II

| Measured m/z | Composition $^{a,b,c}$ |
|-------------|-------------------------|
| 942.6       | Hex$_3$HexNAc$_1$       |
| 1116.6      | Fuc$_1$Hex$_3$HexNAc$_1$|
| 1146.6      | Hex$_3$HexNAc$_1$       |
| 1160.7$^*$  | Hex$_3$HexNAc$_1$GlcA$_1$|
| 1290.8      | Fuc$_1$Hex$_3$HexNAc$_1$|
| 1320.8$^*$  | Hex$_3$HexNAc$_1$GlcA$_1$|
| 1364.7$^*$  | Fuc$_1$Hex$_3$HexNAc$_1$|
| 1524.8      | Hex$_3$HexNAc$_1$       |

$^a$ Some undermethylated forms were also seen.

$^b$ All ions are sodium adducts.

$^c$ Charged glycans.

FIG. 6. MALDI-TOF MS analysis of C. elegans N2 Bristol and srf-3 PNGase A-released N-glycans. A, N2 Bristol glycans; B, srf-3 glycans; C, comparison of the relative abundances of individual glycan isobars in N2 Bristol and srf-3 glycans. Molecular ions are displayed as percentage abundance with S.E. displayed as error bars. The asterisk appears above compounds that were found to be diminished in srf-3 N-glycans. Compounds that are increased in srf-3 are indicated with a cross.

FIG. 7. MALDI-TOF MS analysis of C. elegans N2 Bristol and srf-3 acidic O-glycans. A, N2 Bristol glycans; B, srf-3 glycans.
FIG. 8. CID MALDI QoTOF MS/MS spectrum of *C. elegans* permethylated acidic O-glycans. A, four possible isobaric structures of the composition GlcA1Gal1-2 Glc1-2GalNAc1-ol [M + Na]+ (m/z 1160.58) are predicted. The CID MS/MS spectrum and product ion assignments for GlcA1Gal1-2 Glc2-3GalNAc1-ol [M + Na]+ (m/z 1364.68) are shown in B. The origins of key fragments are shown in the derived structures in Fig. 9.
suggesting that the allele is a functional null (14).

Why was there no obvious decrease of the Golgi apparatus-type addition of GlcNAc to the glycoconjugates, although SRF-3 also transports UDP-GlcNAc? Whereas we do not have a conclusive answer, two possibilities arise. The *C. elegans* genome contains 16 putative nucleotide sugar transporters; we know that at least two other transporters can use both UDP-Gal/UDP-GlcNAc as substrates.2 Whereas their tissue location has not yet been established, it is possible that one or both of them is also required for the supply of UDP-GlcNAc in seam cells and g1 and g2 pharyngeal cells or other organs that may contribute to glycoprotein secretion to the surface coat and cuticle. It is also possible that different UDP-Gal/UDP-GlcNAc transporters have different relative substrate affinities and that a transporter other than SRF-3 has high affinity for UDP-GlcNAc and therefore allows an adequate supply of this nucleotide sugar into the Golgi lumen for the GlcNAc addition to oligosaccharides destined to the surface coat and cuticle.

The discussion of the above described glycans of *srf-3*, in comparison with wild type, as well as the absence of infection of *srf-3* mutants by *Yersinia* (3) and *M. nematophilum* (4) suggests a critical role for these glycans in the recognition between *C. elegans* and the above pathogens. However, it is also possible that proteoglycans and glycolipids play an important role in the interactions between the above bacteria and *C. elegans*.

The underlying molecular mechanism for the aberrant wheat germ agglutinin binding phenotype of the *srf* mutants is not known. Two models have been advanced (31): 1) wheat germ agglutinin binding targets that are normally masked in wild-type worms are unmasked in the *srf* phenotype; 2) the defects are due to altered glycoprotein processing. Both of these models are consistent with our results. Unmasking of wheat germ agglutinin epitopes may be the result of the observed drastic decrease of O-glycans and the slight increase of GlcNAc-

2 P. Berninsone and C. B. Hirschberg, unpublished observations.
containing complex N-glycans resulting from altered glyconjugate processing. In another study, it was found that a wild-type L1-specific O-glycanase-sensitive O-glycoprotein detected by M38 monoclonal antibody is heterochronically expressed in L2–L4 stages of srf(yj43) but not expressed in srf-3; the latter is epistatic to the former, since in srf-3 srf(yj43) binding of M38 is eliminated (32). These observations are consistent with the role of SRF-3 UDP-Gal/UDP-GlcNAc transport and the observed decrease of O-glycans in srf-3.

The α1,2fuc-containing nonreducing ends of the fucosylated N-glycans (24, 25) that are decreased in srf-3 mutants are similar to those of the Lewis b and Lewis y antigens. These have been shown to be targets for bacterial invasion processes including those between Helicobacter pylori BabA-Lewis b and SabA-Sialyl Lewis X-mediated gastric mucosa adhesion (33, 34) as well as P. aeruginosa infection in cystic fibrosis and in chronic bronchitis (34, 35).

The types of oligosaccharides described in these studies also allow some speculations regarding biosynthetic pathways with caveats that will be outlined further below. The occurrence of Gal$_1$Man$_5$GlcNAc$_2$ in srf-3, as shown in Figs. 1, B and D, and 4A, suggests that it may represent an intermediate in the biosynthesis of Fuc$_1$–Gal2Man$_5$GlcNAc$_2$ observed in Fig 1, A–D, and Fig. 6, A–C. It may be that the latter structures cannot be synthesized due to an inadequate supply of UDP-Gal by SRF-3. This may prevent the subsequent action of galactosyltransferases and fucosyltransferases that have been previously described in C. elegans (36).

Man$_3$–GalNAc$_3$–5, observed in Fig. 1, was not decreased in srf-3. Thus, Golgi type GlcNAc addition must have occurred, suggesting, as previously mentioned, that wild type cells express another transporter capable of UDP-GlcNAc transport or that some glycans are derived from cells other than those having SRF-3. An intriguing observation is that N-acetylgalactosaminyltransferase I orthologues gly-12 and gly-13 are expressed in the hypodermis, where SRF-3 is normally expressed (28, 37, 38). This glycosyltransferase catalyzes the first committed step in complex N-glycan biosynthesis by adding GlcNAc in a β1,2-linkage to the lower arm α1,3Man of Man$_5$GlcNAc$_2$ to yield Man$_5$GlcNAc$_4$. This is the same site of the Gal addition proposed here to yield Gal$_1$Man$_5$GlcNAc$_2$. Therefore, N-acetylgalactosaminyltransferase I and the galactosyltransferase may compete for the same Man$_5$GlcNAc$_2$ substrate; this in turn might explain the increase in Man$_3$–GalNAc$_3$–5 seen in the srf-3 mutant as the availability of UDP-Gal is diminished.

Nevertheless, as mentioned above, because the multiple cell types secrete different glycans and the possibility exists that they may have several nucleotide sugar transporters for the same substrate as well as multiple glycosyltransferases of similar substrate specificities, it is not possible at this time to have a full understanding of the biosynthesis of the described glycan.

The acidic O-glycans detected in C. elegans are novel in structure; this, together with their drastic decrease in srf-3 and resistance of the mutant toward adhesion of biofilm produced by Yersinia and infection by M. nematophilum, suggests a possibly important role for the above glycans in nematode-bacterial recognition and biofilm adherence. The most studied biofilms are those produced by Pseudomonas, and among its major components are alginates, linear polymers containing acetylated and nonacetylated guluronic acid and mannuronic acid, both sugars that are similar to glucuronic acid (39). Biofilm gelation requires calcium-coordinated intermolecular bonds between the hexuronic acid residues (39). Y. pestis contains homologs of P. aeruginosa alg-D, -A, and -C, genes required for the formation of alginates, as well as a protein with weak homology to alg-G, which encodes a C-5 polynmannurane epimerase. It is therefore conceivable that calcium coordination or other interactions occur between alginates of Yersinia and the glucuronic acid-containing glycans of C. elegans. Interestingly, Pseudomonas sp. NCIMB 2021 adhesion to an alginate hydrogel withstands greater shear stress than that formed on agarose, chitosan, or PVA-SbQ (39). Whereas we do not yet know the structure and biological relevance of biofilms produced by Yersinia, nor whether they contain alginates, further studies on this subject should be of major help in understanding the mechanism of nematode-biofilm adhesion.

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