Loss of srf-3-encoded Nucleotide Sugar Transporter Activity in Caenorhabditis elegans Alters Surface Antigenicity and Prevents Bacterial Adherence

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During the establishment of a bacterial infection, the surface molecules of the host organism are of particular importance, since they mediate the first contact with the pathogen. In Caenorhabditis elegans, mutations in the srf-3 locus confer resistance to infection by Microbacterium nematophilum, and they also prevent biofilm formation by Yersinia pseudotuberculosis, a close relative of the bubonic plague agent Yersinia pestis. We cloned srf-3 and found that it encodes a multitransmembrane hydrophobic protein resembling nucleotide sugar transporters of the Golgi apparatus membrane. srf-3 is exclusively expressed in secretory cells, consistent with its proposed function in cuticle/surface modification. We demonstrate that SRF-3 can function as a nucleotide sugar transporter in heterologous in vitro and in vivo systems. UDP-galactose and UDP-N-acetylglucosamine are substrates for SRF-3. We propose that the inability of Yersinia biofilms and M. nematophilum to adhere to the nematode cuticle is due to an altered glycoconjugate surface composition of the srf-3 mutant.

To counteract infections, all higher organisms have evolved sophisticated immunological defenses. Although adaptive immunity is specific to vertebrates, the mechanisms of innate immunity are ancient in evolutionary terms and have been highly conserved during evolution (1). This suggests that studying their role in diverse species may yield key insights into the evolutionary origins and molecular mechanisms of the mammalian innate immune system. Therefore, vertebrate as well as invertebrate model systems have been established to understand the underlying mechanisms of innate immunity.

The use of Caenorhabditis elegans as a host model system for innate immunity was first demonstrated for the human opportunistic pathogen Pseudomonas aeruginosa (2). In the short time since then, C. elegans has been established as a model system for studying infection by a wide variety of pathogens (3). Unlike P. aeruginosa, Yersinia pestis and Yersinia pseudotuberculosis do not colonize the intestinal tissues of C. elegans but instead generate a sticky biofilm on the exterior of the animal’s head that impairs feeding, leading to growth delay or larval arrest (4). This phenomenon is a model for bubonic plague transmission, because biofilm formation by Y. pestis requires putative polysaccharide biosynthetic genes that are also required for flea infection and transmission of the pathogen by flea bites (5). A biofilm is defined as a community of bacteria enclosed in a self-produced exopolysaccharide matrix that adheres to a biotic or abiotic surface. Biofilm formation by pathogens is of great clinical importance, because bacteria embedded in biofilms have been shown to be more resistant to antibiotics, to components of the host immune system, and to removal by mechanical forces (6).

A novel C. elegans pathogen has been described recently (7). Microbacterium nematophilum adheres to the rectum of wild type animals, inducing a localized nonlethal response and causing a swelling of the underlying hypodermal tissue (deformed anal region (Dar) phenotype). In the same study, several mutants with altered surface antigenicity (srf mutants) were found to be resistant to infection by M. nematophilum. Altering surface antigenicity is an important mechanism by which parasitic nematodes can evade the host immune system, and C. elegans has been used as a model to understand the factors required for changing the surface composition (8, 9). srf-1 was identified as a surface polymorphism in variants of C. elegans that failed to bind a polyclonal antiserum raised against the adult cuticle of wild type N2 worms (10). Additional srf mutations were identified in a screen for mutants showing altered surface binding of antisera. It has been proposed that
srf-3 animals have lost surface components, thereby exposing antigenic determinants that are hidden in wild-type animals (11). srf-3 mutants were also found in a screen for ectopic binding by wheat germ agglutinin (12). srf-3, srf-8, and srf-9 have extensive pleiotropic defects and show no resistance to infection by M. nematophilum, suggesting that the mutations are involved in biological processes distinct from that of other srf genes. In contrast, srf-2, srf-3, and srf-5, which all show no visible alterations in morphology or behavior, are resistant to infection by M. nematophilum (7).

Here we show that the resistance of srf-3 animals to infection by M. nematophilum and to biofilm formation by Y. pseudotuberculosis is due to the failure of the bacteria or their secreted products to adhere to the animals’ cuticle. We cloned srf-3 and found that it codes for a nucleotide sugar transporter (NST) of M. nematophilum by screening a SalI site upstream and an EcoRV immediately downstream of the ORF M02B1.1 in pBY1454 (17). p426GPD was digested with XbaI, and pBY1866 was digested with XbaI, and p426GP was digested with SpeI; fragments were ligated to create pBY1822 (VSYSFR-3). For generating constructs allowing expression in C. elegans, SRF-3 was fused with a VSV-G tag at the C terminus by PCR mutagenesis and cloned into BluescriptII SK-.

EXPERIMENTAL PROCEDURES

Strain Maintenance and Genetics—All C. elegans strains were grown as described (13). Transgenic animals were constructed as described (14). Injection of dsRNA was done as described (15). Staining for β-galactosidase activity was done as previously described (16). The following strains were used: LGII srf-3[J4H26] (17), LGII unc-11(Hed44) (18), LG IV dpy-20(e1282) unc-30(e191), srf-3[yj10], unc-31(e169) srf-3[yj10] (19), unc-31(e169) srf-3[yj10] lev-1(e211) (19); SU93 (jcs1) (20), and dF22/ nT1 IV +nTIV (21). The following yeast strains were used: Saccharomyces cerevisiae, PRY225 (ura3–52, lys2–801am, ade2–1020, his3, leu2, trpl1); Kleuyveromyces lactis, RL3 (Mat a, ura3, nnn-2, arg4-101, pdr1) (22). Mapping of srf-3 was carried out as follows. From unc-31(e169) srf-3[yj10] (+/+ + lev-1[e211]) parents, zero Lev Srf non-Unc and seven Lev non-Srf mutants were identified first by assignment to linkage group IV and then by complementation tests. All alleles except brf were tested for complementation of the reference allele srf-3[yj10], using both the visible Bus phenotype and the Srf phenotype as judged by FITC-wheat germ agglutinin (Vector Laboratories) binding (12). srf-3(brf) was tested for complementation of srf-3(yj10) in the Yersinia bioluminescent formation assay. For cloning srf-3 by rescue, we injected srf-3(yj10) unc-30(e191)+/+ animals with cosmids or subclones, using co-injected pBY1153 (sel-12:egfp) at a concentration of 25 ng/μl as a transformation marker. The progeny of transgenic Unc animals were grown and tested for the Dar phenotype on plates that had been seeded with a mixture of 99.9% Escherichia coli OP 50 and 0.1% M. nematophilum CBX102. All injection mixtures were supplemented to a DNA concentration of 100 ng/μl with Bluescript II SK-(Stratagene). Biofilm formation was assayed as follows. Five adult worms per plate were allowed to lay eggs for 2–4 h on plates containing Y. pseudotuberculosis YP31. After removing the worms, the plates were incubated at 20 °C for 2 days or 15 °C for 4 days, and the number of L4 larvae was compared with the number of total worms. Because the biofilm blocks feeding and causes larval arrest or growth delay, this assay serves as an indirect measurement of biofilm attachment.

Plasmid Construction—To identify the open reading frame sufficient to provide srf-3 activity, the rescuing cosmids M02B1 (line 1 at the top of Fig. 3A) were cloned with PacI, and the 27-kb backbone was ligated, creating the plasmid pBY1453 (line 6). A 4.6-kb (line 2) and a 7-kb (line 3) PstI fragment of M02B1 were ligated into BluescriptII SK- (Stratagene), generating the plasmids pBY1451 and pBY1452, respectively. A 13-kb AatII fragment derived from M02B1 was ligated into BluescriptII SK- (Stratagene), creating the plasmid pBY1454 (line 4). pBY1508 was constructed by deleting 3.2 kb with Eco52I, which removed the ORF M02B1.1 in pBY1458 (line 5). pBY1458 was digested with SapI to generate pBY1509, which carries a 4.2-kb deletion in the ORF ZK896.9 (line 8). To tag SRF-3 at the N terminus with GFP, 2.5-kb of srf-3 promoter sequence was PCR-amplified with primers that introduced a SalI site upstream and an EcoRV immediately downstream of the ATG. This PCR product was inserted into pPD118.15 (SalI- and Acc65I-blunted), creating the plasmid pBY1603. The SRF-3 coding region plus 1.7-kb of untranslated sequence was PCR-amplified and cloned via NheI and AatII into pBY1603, resulting in the plasmid pBY1605. To tag SRF-3 at the C terminus with β-galactosidase carrying a nuclear localization signal, the srf-3 genomic region, including 2.5-kb of promoter sequence, was PCR-amplified. The primers were designed so that the stop codon was removed, and S6T sites were introduced at both ends of the PCR product. The PCR product was inserted into pDPD95.57, creating pBY1907. For heterologous expression of srf-3, the DNA was fused by PCR mutagenesis with a sequence encoding an 11-amino acid VSV-G tag and cloned into BluescriptII SK-, creating the plasmid pBY1820 (VSV tag at the N terminus, VSYSFR-3). From pBY1820 the cDNA was cloned via XbaI to pDNA3.1, creating pBY1823 (VSYSFR-3). To ligate VSYSFR-3 to p426GP, pBY1820 was digested with XbaI, and p426GP was digested with SpeI; fragments were ligated to create pBY1822 (VSYSFR-3). For generating constructs allowing expression in K. lactis, SRF-3 was fused with a VSV-G tag at the C terminus by PCR mutagenesis and cloned into BluescriptII SK- (pBY1819). SRF-3VSV was PCR-amplified with primers introducing S6T sites immediately upstream of the ATG and downstream of the stop codon. The PCR product was ligated to Xhol-digested pE4 vector, resulting in the plasmid pBY1866 (SRF-3VSV).

The co-injection marker pBY1153 was cloned as follows. The sel-12 cDNA in pBY895 (22) was replaced with EGFP coding sequence on a Smal/NotI fragment from pEFGP-N1 (Chontech). The resulting construct, pBY1153 (sel-12:egfp), drives expression of egfp under the control of the sel-12 promoter. To express the srf-3 cDNA under the control of the srf-3 regulatory sequences, the 3′-untranslated region was amplified with PCR using the primers 5′-GATCTCGAGATCCGCGGCCTG-3′ and 5′-GATCTCGAGATGGCGCGGCTG-3′ and cloned into BluescriptII SK-.

Cloning of the srf-3 cDNA—To confirm the exon-intron boundaries of srf-3, three independent reverse transcription reactions (primer RT1, AAATATTTAAAAGACGAGCA; RT2, TGATAAATTTAATCAGAAGT; RT3, GCAATTTTTTAAAGGCGTTACT) were performed on RNA prepared from a population of mixed staged N2 animals. PCR was performed using the primers 5′-GATCTCGAGATCCGCGGCCTG-3′ and 5′-GATCTCGAGATGGCGCGGCTG-3′. The PCR product was digested with XbaI and cloned into BluescriptII SK-. The resulting plasmid was co-injected with the plasmid encoding the srf-3 coding region, and the srf-3 transgene was identified by the DNA sequence of a PCR product that contained gGAGCTACCAGGCGTATCAGCGGGAGAGTTTGAGATG-3′ and 5′-GATCTCGAGATCCGCGGCCTG-3′ and cloned into BluescriptII SK-.

Molecular Biology—DNA sequences of srf-3 alleles were determined directly from PCR-amplified DNA. The site of the Tc1 insertion in the c2789 allele was identified by the DNA sequence of a PCR product obtained with Tc1-specific primers combined with SRF-3-specific primers. For the analysis of SRF-3 localization, 5 ng/μl pBY1605 was co-

FIG. 1. srf-3 animals are resistant to infection by M. nematophilum and to biofilm formation of Y. pseudotuberculosis. Photoregraphs of the head region of N2 (A) and an srf-3 (D) animal grown on plates containing Y. pseudotuberculosis. Normarski differential interference contrast (DIC) photoregraphs of the tail region of an N2 (C) and an srf-3 (D) animal grown on plates containing M. nematophilum.
for cloning of the constructs presented in this study can be obtained measured with a Tecan microplate reader. Sequences of primers used FITC (EY Laboratories) in 0.9% NaCl, 0.5 mM CaCl₂ and incubated for approximately 5 h at 30 °C. Surviving cells were cloned and grown at 30 °C in complete medium containing Geneticin (G418; 0.4 mg/ml). Stained plates were photographed with white light illumination. By staining with methylene blue in 50% methanol. Stained plates were photot assay and analysis of the samples was carried out as previously described (7), using dye purchased from Molecular Labs (Leiden, The Netherlands).

Nucleotide Sugar Transport Assay—The theoretical basis for the translocation assay of nucleotide sugars into Golgi apparatus enriched vesicles has been described previously (27). The nucleotide sugar transport assay and analysis of the samples was carried out as previously described (28). To determine radioactivity, liquid scintillation spectrometry was used. Radionucleotides were purchased from PerkinElmer Life Sciences and American Radiolabelled Chemicals (St. Louis, MO).

Generation of Stable Madin-Darby Canine Kidney (MDCK) RCAr Transfectants and Determination of Ricin Resistance—MDCK RCar cells were transfected in OPTI-MEM (Invitrogen) medium with 1 μg of plasmid DNA using Lipofect (Invitrogen) for 6 h and then grown for 72 h in complete medium (minimal essential medium containing 10% fetal calf serum and antibiotics). Cells were trypsinized and plated at low density in complete medium containing Geneticon (G418; 0.4 mg/ml). Surviving cells were cloned and grown at 30 °C at variable concentrations of ricin (RCA II (E.Y. Laboratories Inc., San Mateo, CA)) in microtiter plates to determine resistance. Cell survival was determined by staining with methylene blue in 50% methanol. Stained plates were photographed with white light illumination.

Cell Surface Labeling of K. lactis—K. lactis cells transformed with pE4-srf-3vsv or vector alone were grown at 30 °C in SCM-URA medium and then washed three times with 0.9% NaCl, 0.5 mM CaCl₂. Approximately 5 × 10⁷ of cells was resuspended in 100 μl of 0.5 mg/ml OSEF FITC (EY Laboratories) in 0.9% NaCl, 0.5 mM CaCl₂ and incubated for 1 h at 30 °C with shaking. Samples were washed three times and resuspended in 0.9% NaCl, 0.5 mM CaCl₂. Flourescence at 535 nm was measured with a Teco microplate reader. Sequences of primers used for cloning of the constructs presented in this study can be obtained upon request.

RESULTS

srf-3 Animals Are Resistant to Infection by M. nematophilum and to Biofilm Formation by Y. pseudotuberculosis—Wild-type worms grown on plates containing M. nematophilum CBX102 became constipated due to a postanal swelling and, as a consequence, feed less. This resulted in a 20% slower growth rate compared with a resistant srf-2 mutant grown under the same conditions (7). When grown on M. nematophilum, srf-3(yj10) animals were also found to be resistant to infection. The tails of the srf-3 animals grown on this pathogen were indistinguishable from tails of animals grown on E. coli OP50, the normal food, indicating that srf-3 mutants strongly suppressed the Dar phenotype (Fig. 1). This resistance was not allele-specific, since additional srf-3 alleles were isolated in mut-7 and ethylmethanesulfonate screens for mutants resistant to M. nematophilum infection (Bus, bacterial unswollen). With the notable exception of e2797, all srf-3 alleles tested did not exhibit a Dar phenotype when grown under standard conditions (20 °C) on plates containing M. nematophilum (Table I and Fig. 1). At 15 and 20 °C, srf-3(e2797) animals showed a Dar phenotype that was weaker than that exhibited by wild-type animals, but at 25 °C, animals were indistinguishable from other srf-3 alleles. Thus, the Bus phenotype of e2797 is temperature-sensitive.

We also examined the progeny of wild-type and various srf-3 alleles on plates containing M. nematophilum (Table II). When grown on a lawn of E. coli OP50, the number of progeny of all tested srf-3 alleles was in the range of or slightly lower than that of wild type. In contrast, on plates containing 0.1% M. nematophilum, the brood size of srf-3 mutants was unaffected, whereas the number of progeny produced by wild type worms was reduced by 50% (Table II).

All srf-3 alleles were also resistant to biofilm formation by Y. pseudotuberculosis. When we grew wild-type animals on a lawn of Y. pseudotuberculosis, a close relative of the plague agent Y. pestis, the heads of the animals became surrounded by a biofilm (Fig. 1), which blocks feeding and inhibits larval development (4, 29). No bacteria could be detected in the intestine or in other interior tissues, suggesting that biofilm production represents the only sign of infection by this bacterial species. In contrast, all srf-3 alleles were resistant to Yersinia biofilm formation (Fig. 1 and Table III), including e2797 which, as with M. nematophilum, displayed a temperature-sensitive phenotype. A novel srf-3 allele, br6, was identified in a direct screen for animals resistant to Yersinia biofilm formation.

M. nematophilum and Y. pseudotuberculosis Biofilms Cannot Adhere to the Cuticle of srf-3 Animals—There are two obvious mechanisms that would result in the resistance of C. elegans to infection by these diverse bacterial species. First, adherence to the cuticle surface could be inhibited in the mutant, preventing colonization by M. nematophilum and biofilm formation by Yersinia. Second, bacterial adherence might not be prevented, but alteration of secondary (signaling) mechanisms could prevent anal swelling or the generation of a bio-

Table I

Penetrance of Dar phenotype in N2 and srf-3 animals on plates containing M. nematophilum

| Wildtype (N2) | yj10 | e2797 | e2680 | e2797 | e2680 | br6 |
|--------------|------|-------|-------|-------|-------|-----|
| 25 °C 100 (n=322) | 0 | (n=238) | 0 | (n=279) | 0 | (n=135) |
| 20 °C 100 (n=403) | 0 | (n=543) | 0 | (n=343) | (n=460) | (n=135) |
| 15 °C 100 (n=183) | 0 | (n=253) | 0 | (n=385) | 0 | (n=280) |

Table II

Fecundity of N2 and different srf-3 alleles on plates containing standard OP50 as food source and on plates containing M. nematophilum

| Strain | E. coli OP50 | E. coli OP50 + 0.1% M. nematophilum |
|--------|-------------|-----------------------------------|
| N2     | 310 ± 05 (n=15) | 144 ± 09 (n=16) |
| srf-3(yj10) | 285 ± 09 (n=20) | 260 ± 07 (n=20) |
| srf-3(e2798) | 248 ± 09 (n=18) | 259 ± 06 (n=20) |
| srf-3(e2680) | 262 ± 20 (n=17) | 241 ± 19 (n=20) |
| srf-3(e2797) | 272 ± 08 (n=18) | 231 ± 13 (n=20) |
| srf-3(e2689) | 253 ± 12 (n=18) | 286 ± 08 (n=20) |

2 M. J. Gravato-Nobre, unpublished data.
3 C. Darby, unpublished results.
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Given is the percentage of total worms that are L4s after 2 days (20 and 25 °C) or 4 days (15 °C) (see also “Experimental Procedures”). The values below show the average of three independent experiments.

| T      | Wild type (N2) | yj10 | e2789 | e2689 | e2797 | e2689 | br6 |
|--------|---------------|------|-------|-------|-------|-------|-----|
|        | %             | %    | %     | %     | %     | %     | %   |
| 25 °C  | 7.9 (n = 764) | 7.2  | 56.7  | 99.5  |       |       |     |
| 20 °C  | 3.4 (n = 641) | 99.7 | 100   | 1.4   | 99.4  | 99.4  |     |
| 15 °C  | 0 (n = 395)   |      |       | 1.8   |       | 98.9  |     |

**Table III**

Growth of N2 animals and srf-3 animals on *Y. pseudotuberculosis*

Fig. 2. Fluorescence and DIC photographs of srf-3 (A) and N2 (B) animals grown on plates containing *M. nematophilum* and subsequently stained with Syto 13. The content of the white frame is shown in higher magnification in C. The white bar represents 50 μm.

film. For example, it was recently shown that inactivation of a MAPKK pathway can alter the *C. elegans* immune response (30). To distinguish between these possibilities for *M. nematophilum*, srf-3 mutants and wild-type worms were grown on plates containing the pathogen and subsequently incubated with the dye Syto13, a nucleic acid vital stain, under conditions that stain bacteria preferentially. In wild-type *C. elegans*, fluorescent *M. nematophilum* that have infected the anal region are clearly visible. In contrast, srf-3 mutants do not show any staining, indicating that *M. nematophilum* is not able to adhere to the cuticle of srf-3 mutants (Fig. 2). Since the case of *Yersinia*, bacteria do not adhere directly even to wild-type animals; adherence is always mediated by biofilm polysaccharide (4). There is also no evidence for signaling between *M. nematophilum* and *C. elegans*. In summary, resistance against two unrelated bacterial strains that use different pathogenic strategies can be conferred by mutations in a single factor, srf-3.

**Molecular Identification of srf-3**—In order to identify the mechanism that confers resistance against bacterial infection, we cloned srf-3. Initial mapping placed srf-3 on chromosome IV to the right of unc-22 (11). Three-factor crosses mapped srf-3 between unc-31 and lev-1, near unc-31 (Fig. 3A). Injection of cosmids from this region into srf-3 mutants revealed that M02B1 rescued the resistance to infection in transgenic offspring of injected worms (data not shown; see also “Experimental Procedures”).

A subclone with only one open reading frame, M02B1.1, was sufficient to rescue susceptibility to infection by *M. nematophilum* and biofilm formation of *Y. pseudotuberculosis* in all srf-3 alleles tested (see Fig. 3B). RT-PCR and subsequent sequencing identified a 987-bp srf-3 cDNA (Fig. 4 for exon-intron boundaries; see “Experimental Procedures” for details). In *C. elegans*, the 5’-end of many mRNAs begins with a 22-nucleotide splice leader sequence added by trans splicing (31). The srf-3 transcript we found was exclusively splice leader sequence 1 (SL-1)-spliced and therefore most likely includes the true 5’-end of the mRNA. Injection of double-stranded RNA prepared from this cDNA into *srf-3(pk1426)*, a strain with increased sensitivity to RNA interference, rendered the progeny resistant to infection by *M. nematophilum*. Furthermore, expression of the srf-3 cDNA under the control of 2.5 kb of 5’ and 3’ genomic elements restored *M. nematophilum* infection in srf-3 animals (data not shown), indicating that the isolated cDNA codes for a functional protein and suggesting that the construct contains the entire regulatory region of the gene.

We subsequently sequenced all six srf-3 alleles. All of them carried mutations in the open reading frame (Fig. 4). The mutations observed in yj10, br6, e2789, and e2689 would lead to a severe truncation of the encoded protein and are most likely null alleles. This is supported by the fact that, when srf-3(e2789) was placed over a deficiency, the srf-3(e2789)/sdF22 phenotype was not more severe than that of the homozygous srf-3(e2789) mutant.

srf-3 encodes a 328-amino acid type III transmembrane protein that, as shown in an alignment with proteins from *Drosophila melanogaster*, *Homo sapiens*, and *Schizosaccharomyces pombe* (Fig. 4B), is similar to NSTs. These proteins function as antiprotomers, exchanging a nucleotide sugar for the corresponding nucleoside monophosphate in the lumen of the endoplasmic reticulum (ER) or the Golgi apparatus. Transport of nucleotide sugars into the Golgi apparatus lumen provides the donor substrate for glycosyltransferases and is therefore necessary for the subsequent addition of sugars to proteins, lipids, and glycosaminoglycans (32). SRF-3 is most similar to the subfamily of UDP-galactose and UDP-N-acetylgalactosamine (UDP-GalNAc) transporters.

**SRF-3 Is Expressed in Seam Cells, Glandular Cells g1g2, and Spermatheca**—To determine the cellular expression pattern of srf-3, constructs were generated, which fused GFP to the N terminus of SRF-3 and β-galactosidase to the C terminus.

4 Tan, L., and Darby, C. (2004) *J. Bacteriol.* **186**, in press.
The complete somatic expression pattern of SRF-3, since the fusion construct rescued the resistance of srf-3 to M. nematophilum. To test whether SRF-3 expression is altered upon infection, we infected worms carrying srf-3::gfp with M. nematophilum. Pattern and intensity of the GFP signal in infected worms were indistinguishable from those in uninfected worms.

srf-3 animals have been shown to be very fragile, indicated by a low survival rate after mechanical penetration during microinjection. In addition, unlike wild type, srf-3 dauers larvae are SDS-sensitive (11). The observed expression in the lateral seam led us to speculate that this phenotype may be due to a defect in the function of the seam cells that are responsible for secreting the protective cuticle. In order to test seam cell morphology in the mutants, we crossed srf-3 with animals carrying an integrated array of ajm-1::gfp, a marker staining the apical borders of the C. elegans epithelium (20). ajm-1::gfp expression in srf-3 animals was indistinguishable from that in N2 animals (data not shown), suggesting that at least the number and shape of seam cells are unaffected in srf-3 mutants.

SRF-3 Transports UDP-Galactose and UDP-N-Acetylglucosamine—To determine whether SRF-3 is indeed a Golgi apparatus NST, we tested its ability to transport different nucleotide sugars. The specificity of NSTs cannot reliably be deduced from the protein sequence alone but has to be determined experimentally (33). For example, the MDCK cell UDP-GlcNAc transporter is 53% identical to the UDP-Gal transporter and 40% identical to the murine CMP-sialic acid transporter, yet each has a highly individual substrate specificity (24). Similarly, SRF-3 not only shares 61% amino acid identity with a Drosophila UDP-Gal/UDP-GalNAc transporter but also has 60% identity with a murine CMP-sialic acid transporter. However, based on a mass spectrometry analysis, C. elegans does not use or contain sialic acid (34). To identify substrates for SRF-3, we measured in vitro the transport of radiolabeled nucleotide sugars into Golgi apparatus enriched vesicles. These were prepared from S. cerevisiae expressing srf-3 with an N-terminally fused VSV epitope coding sequence (vsrf-3). The same approach had been previously used to determine the substrate specificity of SQV-7 (28). Prior to vesicle isolation, it was verified, by immunoblotting, that the vsrf-3 fusion gene is expressed in yeast (Fig. 6A).

VSVSRF-3 was exclusively detected in the membrane fraction of the S. cerevisiae extracts and comigrated with carboxyl anhydrate (29 kDa), which is consistent with the predicted fusion protein size of 32 kDa. In a control experiment for the integrity of the prepared vesicles, we used the known capabilities of yeast cells to transport UDP-glucose into the lumen of Golgi apparatus and ER. Vesicles derived from strains expressing vsrf-3 and vesicles with vector alone transported UDP-glucose in a comparable manner, suggesting that both preparations were of equivalent quality (Fig. 6B). UDP-Gal and UDP-GlcNAc were transported in a temperature-dependent manner into vesicles isolated from yeast cells expressing vsrf-3 but not into vesicles isolated from yeast cells transformed with the vector alone (Fig. 6B). In contrast, UDP-GalNAc, UDP-glucuronic acid, GDP-fucose and CMP-sialic acid were not transported under the conditions of the experiment.

To further analyze the transport characteristics of SRF-3, we quantitated the rate of transport at different nucleotide sugar concentrations. UDP-Gal and UDP-GlcNAc were transported in a saturable manner with an apparent $K_m$ of 5.9 and 20 μM, respectively (Fig. 6C). SQV-7, a recently identified C. elegans nucleotide sugar transporter with multisubstrate specificity, showed a $K_m$ of 4 μM for UDP-glucuronic acid, 8.7 μM for UDP-galactose, and 3.9 μM for UDP-GlcNAc (36).
UDP-GalNAc, and 4.6 μM for UDP-Gal (28). Therefore, the measured $K_m$ values are in close agreement with those of previously described nucleotide sugar transporters. In summary, we conclude that SRF-3 is able to transport UDP-Gal and UDP-GlcNAc in vitro.

In a parallel approach, the in vivo substrate specificity of SRF-3 was determined by its ability to phenotypically correct mutants previously characterized as defective in transport of either UDP-Gal or UDP-GlcNAc. For UDP-Gal transport measurements in vivo, the MDCK cell line RCAr was chosen on the basis of its defective UDP-Gal transport and its resistance to the toxin ricin, which recognizes terminal galactose of oligosaccharides (35). RCAr cells grow at ricin concentrations 10 times higher than wild-type MDCK cells. It was shown that this phenotype is linked to a Golgi apparatus-localized UDP-Gal transporter, and this system has been used to find substrates of previously unknown nucleotide sugar transporters (28). MDCK RCAr cells were transfected with a construct coding for an N-terminal VSV-tagged SRF-3 (VSVSRF-3) to test whether the SRF-3 transporter can restore ricin sensitivity (Fig. 7A). MDCK RCAr cells, transfected with vector alone, grew at all tested concentrations up to 3 ng/ml, whereas both SRF-3 and SQV-7 (used as a control) transfected cells were only viable up to a concentration of less than 0.25 ng/ml ricin. These data demonstrated that SRF-3 UDP-Gal transport capabilities restored the ricin sensitivity of MDCK RCAr cells by restoring galactosylated oligosaccharides on their cell surface.

Previously, a K. lactis mutant (KL3) with a defect in a UDP-GlcNAc transporter was described (36). This mutant lacks $N$-acetylglucosamine in its outer mannan chains, leading to a differential binding of Griffonia simplicifolia II (GSII) lectin (37). A mammalian UDP-GlcNAc transporter was cloned by transforming K. lactis KL3 with a MDCK cell-derived cDNA library and subsequent labeling with FITC-conjugated GSII lectin (24). This result also showed that the K. lactis mutant could be used to test heterologous proteins for their UDP-GlcNAc transport activity. We transformed K. lactis KL3 cells with a construct expressing srf-3. In contrast to the KL3 mutant, the cells expressing srf-3 showed increased lectin labeling, indicating that SRF-3 was also able to transport UDP-GlcNAc in K. lactis cells (Fig. 7B) and restore cell surface $N$-acetylglucosaminylation of proteins and lipids. In summary, our data demonstrate that SRF-3 is a nucleotide sugar transporter that is able to transport both UDP-galactose and UDP-$N$-acetylglucosamine in vitro and in vivo.
**DISCUSSION**

We show in this study that two different species of bacteria, *M. nematophilum* and *Y. pseudotuberculosis*, fail to adhere to the cuticle of *srf-3* mutants, suggesting that this accounts for the resistance of *srf-3* to bacterial infection. This is a novel type of resistance mechanism, because pathogens such as *P. aeruginosa* or *Salmonella typhimurium* do not adhere to the cuticle but instead kill *C. elegans* by colonization and accumulation in the intestine (38). We identified *srf-3* as a nucleotide sugar transporter capable of transporting both UDP-Gal and UDP-GlcNAc, a combination of substrate specificities not previously described. The mechanism of *srf-3* resistance and molecular identity of the gene suggests that a defect in UDP-Gal and UDP-GlcNAc transport into the Golgi apparatus might alter glycosylation and/or secretion of host components involved in bacterial adhesion.

All *srf-3* alleles that we analyzed carried mutations in the coding region expected to result in a truncated or aberrant protein. *e2797*, the temperature-sensitive mutant, carries a mutation in a splice acceptor site (Fig. 4). *e2680* carries a glycine to glutamic acid substitution in the conserved VGGLSVA motif of the predicted transmembrane domain 6 (Fig. 4). A similar mutation was found in CHO cells of the complementation group Lec8, which harbors a glycine to aspartic acid substitution at position 281 (39). Experiments with chimeras, in which segments of a human UDP-Gal and a human CMP-sialic acid transporter were exchanged, indicated that the C-terminal domain of NSTs is involved in generating an active transport site and might participate in the process required to translocate nucleotide sugars across the membrane (40). Thus, in the case of *e2680*, the transporter may be correctly targeted and may even recognize its substrate but fails to translocate it across the membrane.

Transport of nucleotide sugars into the Golgi apparatus is necessary for the subsequent addition of sugars to proteins, lipids, and glycosaminoglycans. The SRF-3::GFP signal indeed indicates an ER or Golgi apparatus localization of the transporter, which is consistent with its proposed function in glycosylation. Since the *K. lactis* UDP-GlcNAc and the MDCK UDP-Gal transporter have been shown to function in the Golgi apparatus (41, 42), SRF-3 has to localize to the same compartment in these heterologous systems in order to be able to rescue the corresponding MDCK or *K. lactis* mutants. This is further supported by the fact that SRF-3 lacks the consensus sequence for a C-terminal ER retention signal KXXX. In many nematodes the collagenous cuticle is covered by an amorphous layer, the surface coat. In parasitic nematodes, this surface coat has been shown to be rich in carbohydrates that are highly immunogenic and play a critical role in the interaction with the host (43). The dysfunction of the SRF-3 NST could result in global underglycosylation and/or aberrant secretion of surface components, leading to the exposure of antigenic determinants that are usually embedded or hidden. This is supported by radioiodination analysis and carbohydrate labeling experiments, which revealed the absence of various components in the cuticle of *srf-3* animals (11, 44, 45).
adult-specific, hypodermally expressed seam cells play an important part in cuticle synthesis at each molt and served as a positive control. The UDP-Gal transport activity of SQV-7 has been described previously in UDP-GlcNAc transport transformed with pE4 vector alone (28, 55). C50F4.14 was shown to transport GDP-Fuc (56), and SRF-3 transports UDP-Gal and UDP-GlcNAc (this work).

In contrast to sqv-7 mutants, srf-3 animals show only a mild locomotion phenotype, which suggests that the traction between the worm and the agar substrate is altered. However, by visual inspection, srf-3 worms are superficially almost indistinguishable from wild-type animals. The mutant animals even seem to benefit from their resistance to some pathogens, which they may actually encounter in their natural habitat. Even on an ultrastructural level, there is no morphological difference in the cuticle as judged by standard transmission electron microscopy of srf-3 animals. Therefore, it is feasible that the transport activities provided by SRF-3 can in part be fulfilled by other, yet unidentified, transporters. However, a mass spectrometry analysis of glycans composition revealed that the abundance of complex glycans containing galactose was severely reduced in C. elegans srf-3 as compared with wild-type animals, indicating that a loss of srf-3 function leads to consequences on the molecular level in vivo. In addition, closer examination reveals that srf-3 does serve an important function during cuticle formation/modification. srf-3 animals are fragile when mechanically manipulated (e.g. by microinjection), show increased susceptibility to drug treatment, and as dauer larvae are SDS-sensitive (11, 12, 57). Furthermore, srf-3 animals were found to be more susceptible to trapping by Duddingtonia flagrans, a nematode predatory fungus, which suggests a protective function of this gene with respect to natural enemies (58). This last observation is supported by results from parasitic nematodes, which show that the cuticle of C. elegans LPG2, has a defect in a GDP-mannose transporter (54). srf-3, Surface Antigenicity, and Bacterial Adherence.

Glycoconjugates represent an extremely abundant component of the cell surface. Therefore, it does not seem surprising that the majority of described binding sites for microbes on host cells are complex carbohydrates found on glycoproteins, glycolipids, and proteoglycans. To further understand the interaction between C. elegans and M. nematophilum/Y. pseudotuberculosis, it would be interesting to identify the structures that are recognized and bound by these pathogens or their secretions. These two bacterial species are not closely related and therefore might use different mechanisms and different host structures for their interaction with worms. This suggests that either there is commonality in the structure recognized by these two bacterial species or that the missing sugars affect the

\[ \text{Function of the observed expression in the spermatheca is currently unknown. The fact that srf-3 hermaphrodites are almost as fertile as wild-type worms shows that the spermatheca functions normally with respect to sperm preservation, sperm function, and allowing dislodged sperm to recolonize after ovulation.} \]

There are examples of the pathophysiological relevance of NSTs. A human congenital disorder of glycosylation called leukocyte adhesion deficiency syndrome II or CDG type IIc leads to immunodeficiency and severe mental and psychomotor defects and was linked to a defect in GDP-fucose transport (52). In the protozoal parasite Leishmania donovani, which causes visceral leishmaniasis, a mannose-rich lipophosphoglycan (LPG) was shown to be important for virulence; one avirulent mutant, LPG2, has a defect in a GDP-mannose transporter (53). The C. elegans genome contains 16 NST-like proteins, of which only three have been described in detail (54). SQV-7, the null phenotype of which is embryonic lethal, is a transporter with multisubstrate specificity (28, 55). C50F4.14 was shown to transport GDP-Fuc (56), and SRF-3 transports UDP-Gal and UDP-GlcNAc (this work).

Glycoconjugates play an important role in the generation of the cuticle and the surface coat. The lack of srf-3 expression in the excretory system, which in T. canis has been implicated in the secretion of surface antigens, suggests that, at least in C. elegans, this organ may not be involved in regulating surface composition. In support of this suggestion, none of the exc mutants (51), which have an abnormal or a defective excretory cell, showed a Srf phenotype as judged by FITC-wheat germ agglutinin labeling. The function of the observed expression in the spermatheca is currently unknown. The fact that srf-3 hermaphrodites are almost as fertile as wild-type worms shows that the spermatheca functions normally with respect to sperm preservation, sperm function, and allowing dislodged sperm to recolonize after ovulation.

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6 J. Castano, D. Gibson, and S. Politz, unpublished data.

7 J. Cipollo, personal communication.
display of more terminal saccharide units. Since both UDP-
galactose and UDP-N-acetylgalcosamine are substrates for
SRF-3 transport, many glycoconjugate structures could be affec-
ted in the mutant. The identification and characterization of
srf-3 can now be used as a starting point to exploit the experi-
mental advantages of the model system C. elegans to identify
host factors required for pathogen adherence, a crucial step
in the establishment of most infections. Furthermore, this system
can provide a genetic tool to examine factors important in
regulating surface composition in nematodes.

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REFERENCES
1. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999)
   Science 284, 1313–1318
2. Mahajan-Miklos, S., Tan, M. W., Rahme, L. G., and Ausubel, F. M. (1999)
   Proc. Natl. Acad. Sci. U. S. A. 96, 3527–3532
3. Alegado, R. A., Campbell, M. C., Chen, W. C., Slutz, S. S., and Tan, M. W.
   (1999) Nature 397, 881–883
4. Blaxter, M. L. (1993) Biochim. Biophys. Acta 1178, 291–303
5. Mendoza De Gives, P. M., Davies, K. G., Clark, S. J., and Behnke, J. M. (1999)
   Exp. Parasitol. 88, 36–42
6. Costerton, J. W., Stewart, P. S., and Greenberg, E. P. (1999) Science 284, 16283–
   16300
7. Edwards, M. K., and Wood, W. B. (1983) J. Biol. Chem. 258, 16283–16288
8. Blaxter, M., Page, A. P., Hamilton, A. J., and Maizels, R. M. (1992) Exp. Parasitol.
   71, 295–307
9. Albertson, D. G., and Thomson, J. N. (1976) J. Comp. Neurol. 174, 539–551
10. Oelmann, S., Stanley, P., and Gerardy-Schahn, R. (2001) J. Biol. Chem. 276,
    26291–26299
11. Buechner, M., Hall, D. H., Bhatt, H., and Hedgecock, E. M. (1999) Dev. Biol.
    211, 393–404
12. Hwang, H. Y., and Horvitz, H. R. (2002) Cell 110, 559–571
13. Berninsone, P. M., and Hirschberg, C. B. (2000) Exp. Parasitol. 71, 295–307
14. Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros, V. (1991) EMBO J.
    10, 227–231
15. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C.
    C. (1998) Nature 391, 844–848
16. Moynihan, D., Muller, R., and Funk, M. (1995) Gene (Amst.) 156, 119–122
17. Guillen, E., Abeijon, C., and Hirschberg, C. B. (1998) Proc. Natl. Acad. Sci.
    U. S. A. 95, 7888–7892
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A
    Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor,
    NY
19. Grzegorczyk, R. D., and Woods, R. A. (2002) Methods Enzymol. 350, 87–96
20. Perez, M., and Hirschberg, C. B. (1987) Methods Enzymol. 138, 709–715
21. Berninsone, P., Hwang, H. Y., Zentseva, I., Horvitz, H. R., and Hirschberg,
    C. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3738–3743
22. Buechner, M., Hall, D. H., Bhatt, H., and Hedgecock, E. M. (1999) Dev. Biol.
    211, 393–404
23. Mendoza De Gives, P. M., Davies, K. G., Clark, S. J., and Behnke, J. M. (1999)
    Parasitology 119, 95–104
24. Segawa, H., Kawakita, M., and Ishida, N. (2002) Eur. J. Biochem. 299, 128–138
25. Mierta, N., Ishida, N., Hoshino, M., Yamauchi, M., Hara, T., Ayusawa, D., and
    Kawakita, M. (1996) J. Biochem. (Tokyo) 120, 236–241
Loss of srf-3-encoded Nucleotide Sugar Transporter Activity in Caenorhabditis elegans Alters Surface Antigenicity and Prevents Bacterial Adherence

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