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Glycosylation Genes Expressed in Seam Cells Determine Complex Surface Properties and Bacterial Adhesion to the Cuticle of *Caenorhabditis elegans*

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ABSTRACT

The surface of the nematode *Caenorhabditis elegans* is poorly understood but critical for its interactions with the environment and with pathogens. We show here that six genes (*bus-2*, *bus-4*, and *bus-12*, together with the previously cloned *srf-3*, *bus-8*, and *bus-17*) encode proteins predicted to act in surface glycosylation, thereby affecting disease susceptibility, locomotory competence, and sexual recognition. Mutations in all six genes cause resistance to the bacterial pathogen *Microbacterium nematophilum*, and most of these mutations also affect bacterial adhesion and biofilm formation by Yersinia species, demonstrating that both infection and biofilm formation depend on interaction with complex surface carbohydrates. A new bacterial interaction, involving locomotory inhibition by a strain of *Bacillus pumilus*, reveals diversity in the surface properties of these mutants. Another biological property—contact recognition of hermaphrodites by males during mating—was also found to be impaired in mutants of all six genes. An important common feature is that all are expressed most strongly in seam cells, rather than in the main hypodermal syncytium, indicating that seam cells play the major role in secreting surface coat and consequently in determining environmental interactions. To test for possible redundancies in gene action, the 15 double mutants for this set of genes were constructed and examined, but no synthetic phenotypes were observed. Comparison of the six genes shows that each has distinctive properties, suggesting that they do not act in a linear pathway.

ALL eukaryotic genomes include many genes encoding enzymes and transport proteins that act in the synthesis of complex carbohydrates and the glycosylation of proteins and lipids. Therefore, the glycome is evidently important, but ascribing biological function to these genes is often difficult in view of the many possible roles for glycosylation in cell signaling, development, structure, physiology, and defense. The scale of this problem is evident in the genome of the nematode *Caenorhabditis elegans*, which is one of the smallest and most intensively studied of animal genomes, but still contains at least 100 genes that encode proteins predicted to act in carbohydrate synthesis and/or modification, few of which have any firmly established biological function (for general review, see Berninsone 2006). In some cases, the predicted biochemical activities for nematode glycoslation genes have been demonstrated *in vitro* or by expression in heterologous systems, but such data are rarely very informative as to what processes they actually control or execute *in vivo*.

Specific biological functions have been ascribed to some of the carbohydrate-modifying enzymes in the worm, notably in various aspects of cell migration, but for the most part RNA interference (RNAi) knockdown or deletional knockout of the corresponding genes has relatively minor effects. Two sets of glycosylation genes that have better defined and more important roles in *C. elegans* biology are the *sqv* and *bre* genes. The eight *sqv* genes are all involved in chondroitin biosynthesis, and mutants in any of these genes exhibit a distinctive abnormal vulval morphogenesis phenotype ("squashed vulva") together with maternal-effect lethality caused by defects in the fluid-filled space between the embryo and eggshell (Herman and Horvitz 1999; Hwang *et al.* 2003). The five *bre* genes are required for synthesis of glycosphingolipids, and four of them encode glycosyltransferases that act consecutively to build up carbohydrate chains (Griffitts *et al.* 2001, 2003). Mutants in these genes are viable but resistant to killing by certain Cry toxins produced by *Bacillus thuringiensis* because the glycosphingolipid on the luminal surface of intestinal cells acts as the receptor for these toxins. The *bre* genes also affect developmental signaling by the Notch/LIN-12 pathway (Katic *et al.* 2005).

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Earlier work has shown that some of the glycosylation genes in *C. elegans* play important roles in determining the surface properties of *C. elegans* and also affect susceptibility to certain bacterial pathogens. The first of these to be studied, *srf-3*, encodes a UDP-sugar transporter that is required for normal surface antigenicity (Höflich *et al.* 2004). Mutants of *srf-3* are resistant to infection by the coryneform pathogen *Microbacterium nematophilum*, which is able to cause disease by adherence to rectal cuticle. *M. nematophilum* elicits a characteristic swollen tail, or Dar (Deformed Anal Region) phenotype, in infected worms, and the *srf-3* mutants exhibit a Bus (Bacterially Un-Swollen) phenotype (Hodgkin *et al.* 2000). Similarly, *srf-3* mutants fail to form a bacterial biofilm on the head when grown on lawns of Yersinia spp. bacteria (the Bah, or Biofilm Absent on Head, phenotype) (Darby *et al.* 2002, 2007). The same two bacterial resistance phenotypes were observed in mutants of *bus-17*, which encodes a predicted galactosyltransferase (Yook and Hodgkin 2007). This gene is required for cuticle integrity because mutants exhibit hypersensitivity to agents such as alkaline hypochlorite (bleach) (Gravato-Nobre *et al.* 2005).

Subsequently, we found that the *bus-8* gene, encoding another predicted glycosytransferase, plays two essential roles in the life of the worm: it is required during embryogenesis to enable ventral enclosure movements, and it is also required postembryonically at each molt cycle to permit successful shedding and replacement of cuticle, as well as to maintain the physical integrity of the cuticle (Partridge *et al.* 2008). Both viable and lethal alleles of *bus-8* have been identified; weak mutants of *bus-8* such as *e2698* are viable and resistant to *M. nematophilum* (Bus phenotype), but they still permit formation of Yersinia biofilms, in contrast to other mutants of this type. Another essential carbohydrate-modifying gene required for cuticle integrity is *glf-1*, encoding UDP-galactopyranose mutase (Novelli *et al.* 2009). Available mutants in this gene are barely viable, making it difficult to assess interactions with *M. nematophilum* or Yersinia, but a *glf-1* hypomorph (made by partial rescue of a lethal *glf-1* mutant with a Leishmania-derived transgene) is resistant to *M. nematophilum* infection, indicating that this gene, like *bus-8*, is essential both for surface barrier formation and for bacterial surface infection.

Selections and screens for resistance to *M. nematophilum* have therefore provided a powerful means for identifying *C. elegans* mutants with altered surfaces to which these bacteria are unable to adhere. Many *bus* genes have been defined by mutation, but have not yet been studied at the molecular level (Gravato-Nobre *et al.* 2005). It seemed likely that additional *bus* genes would be involved in carbohydrate biochemistry, and indeed we report here the identification and analysis of three further *bus* genes with predicted roles in surface glycosylation (*bus-2*, *bus-4*, and *bus-12*). We compare their properties with the previously identified *srf-3*, *bus-8*, and *bus-17* and demonstrate a diversity of phenotypic effects on bacterial susceptibility, cuticle integrity, locomotion, and male/hermaphrodite mate recognition. This contrasts with the largely uniform phenotypes observed for the *sqv* and *bre* gene sets.

MATERIALS AND METHODS

Nematode growth conditions and strains: Standard procedures for *C. elegans* culture and genetics were employed (Brenner 1974; Sulston and Hodgkin 1988). Methods for infecting *C. elegans* with *M. nematophilum* were as previously described (Gravato-Nobre *et al.* 2005). Mutations examined were the following: for *bus-2*—*e2676*, *e2677*, *e2687*, *e2705*, *e2776*, *e2780*, *e2781*; for *bus-4*—*e2693*, *e2700*, *e2752*, *e2788*, *e2803*, *br4*; for *bus-12*—*e2740*, *e2974*, *e2975*, *e2976*, *e2977*, *e2978*, *e2979*, *br5*; for *bus-8*—*e2698*; for *bus-17*—*e2800*, *e2693*; and for *srf-3*—*yj10*, *e2689*. Other alleles utilized were the following: *sqv-7(n2839)*, *rrf-3(pk1426)* (LGII); *unc-119(ed3)* (LGIII); *fem-1(hc17)*, *dpy-20(e1282)*, *unc-26(e205)* (LGIV); and *xol-1(y9)* (LGX). Deletion alleles obtained from the *C. elegans* Gene Knockout Consortium were the following: *ZK896.9 (ok3050)*, *K06H6.3(ok3012)*, *F44C8.7(ok2206)*, *T21B6.5(ok3220)*, and *C50F4.14(ok2860)*. Strain RB1727, homozygous for *F44C8.7(ok2206)*, was found to exhibit temperature-sensitive lethality, but out-crossing showed that this lethality was caused by an unlinked background mutation rather than by the *ok2206* deletion.

Noncomplementation screen: Additional alleles of *bus-12* were sought by means of a noncomplementation screen: wild-type (N2) males were mutagenized with 0.47 mM ethyl methanesulfonate according to a standard protocol (Brenner 1974) and mated with females of the strain CB6662, genotype *fem-1(hc17) bus-12(e2740) unc-26(e205); xol-1(y9)*. Mated females were placed in sets on mixed bacterial lawns (*Escherichia coli* OP50/*M. nematophilum* CBX102), and progeny were screened for rare Bus animals. These were picked and allowed to self-fertilize; new alleles were isolated as homozygotes by utilizing the flanking wild-type alleles of *fem-1* and *unc-26*.

Double-mutant construction: The following 15 double mutants, defective in two different predicted surface glycosylation genes, were constructed and their phenotypes were examined: *bus-2(e2687) bus-4(br4)* (CB6605), *bus-2(e2687) srf-3(yj10)* (CB6857), *bus-2(e2687) bus-12(e2977)* (CB6868), *bus-2(e2687), bus-8(e2698)* (CB6824), *bus-2(e2687); bus-17(e2800)* (CB6848), *bus-4 (br4) srf-3(yj10)* (CB6493), *bus-4 (e2700) bus-12(e2977)* (CB6863), *bus-4(e2700); bus-8(e2698)* (CB6859), *bus-4 (e2700); bus-17(e2800)* (CB6832), *srf-3(yj10) bus-12(e2977)* (CB6858), *srf-3(yj10); bus-8(e2698)* (CB6831), *srf-3(yj10); bus-17(e2800)* (CB6860), *bus-12(e2977); bus-8(e2698)* (CB6864), *bus-12(e2977); bus-17(e2800)* (CB6866), *bus-8(e2698) bus-17(e2800)* (CB6865), and *sqv-7(n2839); bus-12(br5)* (CB6656). In addition, a double mutant of *bus-12* and the related gene *sqv-7* was constructed: [CB6656, *sqv-7(n2839); bus-12(br5)*].

Simple crosses followed by segregation and progeny testing were used for constructing most of these double mutants. Doubles between closely linked genes were made by using linked visible markers. For example, the double mutant *srf-3(yj10) bus-12(e2977)* was constructed by first making two different double mutants, *srf-3(yj10) unc-26(e205)* and *dpy-20(e1282) bus-12(e2977)*. Males of the latter strain were crossed with hermaphrodites of the former strain to yield phenotypically wild-type *srf-3* + + *unc-26*/+ *dpy-20 bus-12* + males. These males were crossed with *dpy-20 unc-26* hermaphrodites, and

rare non-Dpy non-Unc cross-progeny were picked, most of which proved to have the genotype *dpy-20 unc-26 / srf-3 bus-12*. Unmarked homozygous *srf-3 bus-12* hermaphrodites were obtained from the next generation. Crosses with appropriate test males confirmed the genotypes of this and the 15 other strains.

Reporter constructs: The following reporter constructs and corresponding transgenic lines were constructed: for *bus-2*, CB6616 = *bus-2(e2687); eEx621[bus-2p::bus-2::GFP(pEntry)]* (3.77 kb upstream, 2.5-kb coding region, 23-bp 3′ UTR) and CB6846 = *unc-119; bus-2(e2677); eEx677[bus-2::mCherry; unc-119(+)]*; primers K08D12.5_forward (CATCAACTGA TAAGTTGTTGATATTGTTGTAAA) and K08D12.5_reverse (GGTGAAAGTAGGATGAGACAGCGGCAAAAAAATCCATC AAGATCGCCACC). The following are operon constructs that were examined as multicopy transgenic arrays, so the expression level of the fluorescent reporter is unlikely to be affected by any inefficiency in productive splicing of the *bus-2* transcript (see RESULTS): for *bus-4*, CB6612 = *bus-4(e2693); eEx619[bus-4p::bus-4::GFP; rol-6(dm)]* and CB6817 = *bus-4(e2700); eEx683[bus-4(+)::mCherry; sur-5::GFP]* (2.99 kb upstream, 3.38-kb coding region); primers T22B11.2_forward (CCAATGCACCAAAACTCCCAACC) and T22B11.2_reverse (GGTGAAAGTAGGATGAGACAGCGGATTGTTTTCTGCCC ACCCTGTCG); for *bus-12*, CB6630 = *bus-12(br5); eEx628[bus-12(+); sur-5p::GFP]* (4.5-kb JC8.12 rescuing fragment); CB6655 = *unc-119(ed3); eEx640 [bus-12p::bus-12::dsRedII; unc-119(+)]* (1.3 kb upstream, 2.85 kb coding region, 160-bp 3′ UTR); primers JC8.12P_forward (GTGAAGCTCTGG GAAGAGGACT) and JC8.12P_reverse (TAGACCGACTAGA ATCAAAATCGG).

***bus-2* RNA isolation and cDNA synthesis:** Total RNA from mixed stage wild-type worms was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. Samples were purified by chloroform-isoamyl alcohol extraction and ethanol precipitation and subsequently treated with DNaseI to remove any residual DNA. cDNA synthesis was performed in the first step, using 1 μg of total RNA per 20-μL reaction and primed with oligo(dT) and SuperScript III (Invitrogen) according to the manufacturer's protocol. In the second step, PCR was performed using *bus-2*-specific primers and Phusion High-Fidelity DNA polymerase (NEB). The following oligos were used: *bus-2* cDNA_forward (ATAGTTTCCCGACGGAT TCCAGTT) and *bus-2* cDNA_reverse (ATACGGATCCCACC ACCTGCATAG). No evidence was obtained for a variant isoform recently predicted by WormBase, BUS-2B, which lacks six amino acids in exon 1 (aa 34–39).

Microscopy: Living animals were mounted on 2% agar pads containing 4% (v/v) propylene phenoxytol or 1 mM sodium azide in M9 and examined under Nomarski differential interference contrast microscopy (Zeiss Axioplan2 microscope with Axiocam). To visualize bacterial colonization in the rectum, worms were stained with the live-cell nucleic acid stain SYTO 13 (Molecular Probes) as previously described (HODGKIN *et al.* 2000).

Hurdles assay: Assay plates were prepared by painting 5.5-cm NGM plates with three stripes of *B. pumilus* CBX120 and one stripe of *E. coli* OP50, using an inoculating loop, as shown schematically in Figure 7B. Plates were incubated for 48–72 hr at 25° to create sticky bands of *B. pumilus*, each ~4 × 50 mm in extent. Twenty young adult worms of each genotype to be tested were placed at one end of the plate, opposite to the *E. coli* stripe and outside the first *B. pumilus* stripe. After 10–20 hr at room temperature, plates were examined and the worms that had accumulated in the *E. coli* stripe by swimming through the *B. pumilus* "hurdles" were counted. Worms placed directly in the *E. coli* stripe tended to remain there, indicating that this is their preferred location.

Mating contact assay: For each test, a 25-μl drop of a stationary *E. coli* OP50 suspension was applied to the center of a 5.5-cm NGM plate and incubated overnight, creating a 10-mm circular spot of bacteria. Four egg-laying adult hermaphrodites (wild type or mutant) then were placed on the spot, followed by eight wild-type (N2) males picked at 1 day post L4 molt, a stage at which male mating efficiency is maximal (HODGKIN and DONIACH 1997). After equilibration for 1–3 hr at 22°, contact between hermaphrodites and males was scored at 1-min intervals over two sessions of 25 min, separated by 2–4 hr. Hermaphrodites were scored positive if a male was sliding his tail over a female's body or had achieved vulval location. A score of 0–4 was noted at each time point, giving a maximum score of 200 (= 100% occupancy) for the total of 50 min assayed. Each "snapshot" measurement required <5 sec and allowed seven or eight plates to be scored in parallel, thereby avoiding extensive and tedious behavioral monitoring. All the hermaphrodites and males usually remained in the spot of bacteria or close to it for the duration of the assays. Tests in which any worms wandered away from the spot for long periods were omitted from analysis. Under these conditions, wild-type hermaphrodites experience mating contacts for less than half the time (average 44% occupancy), indicating that the number of males is far from saturating.

RESULTS

Molecular identification and characterization of *bus-2*: Mutants in the *bus-2* complementation group were frequently recovered after selection for resistance to *M. nematophilum* and found to exhibit a non-infectable phenotype, as previously reported (GRAVATO-NOBRE *et al.* 2005). No *bus-2* mutants were recovered in corresponding screens for Bah phenotype mutants, which fail to support biofilm formation by Yersinia spp. but tests on existing *bus-2* mutants revealed that they had a Bah phenotype as well as a Bus phenotype (DARBY *et al.* 2007). Genetic mapping together with analysis of transposon-induced mutations of *bus-2* established that *bus-2* corresponds to *K08D12.5*, encoding a predicted galactosyltransferase enzyme (PALAIMA *et al.* 2010). Sequencing of EMS-induced alleles revealed missense changes in all cases (Figure 1). Rescue experiments, in which the Bus (*M. nematophilum* resistant) phenotype was converted back to the wild type (sensitive to *M. nematophilum*) by transgenes expressing *K08D12.5*, provided further confirmation (Figure 2C).

Scrutiny of the possible coding sequence for *bus-2/K08D12.5* revealed an anomaly, as noted elsewhere (PALAIMA *et al.* 2010): the protein sequence based on the gene model in WormBase (referential data freeze WS200) can be improved in terms of alignment with other glycosyltransferases, including the *Caenorhabditis briggsae* ortholog of *bus-2*, by replacing the 19-aa WormBase exon 3 with a different 43-aa exon 3. The region concerned (exon 2 to exon 5) was amplified from a *C. elegans* cDNA preparation, and sequencing demonstrated the presence of the 43-aa exon 3 and its predicted junction with exon 4. However, correct removal of intron 3 must have entailed use of a GG 5′

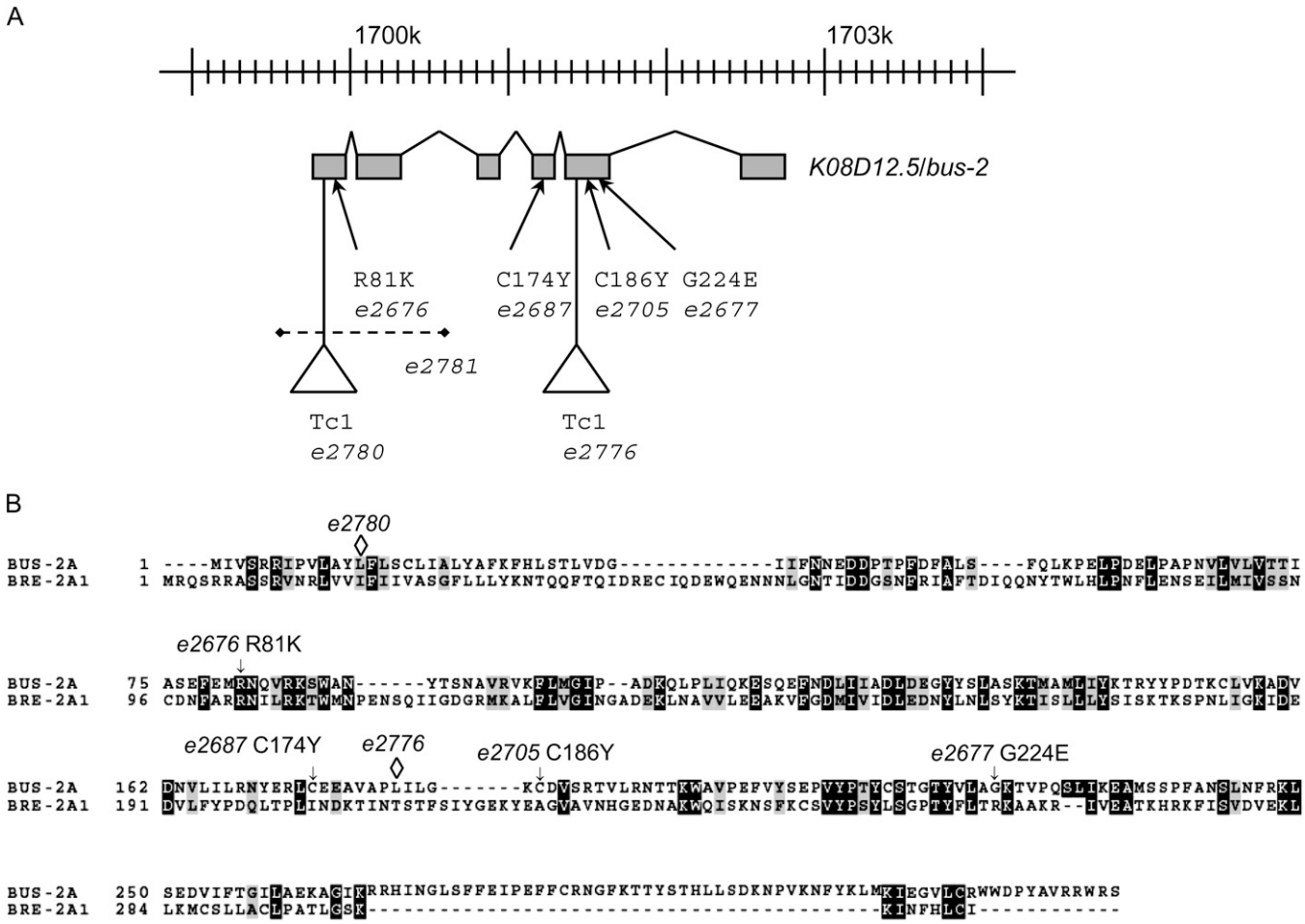

FIGURE 1.—Structure and mutations of *bus-2*. (A) Exon structure and localization of mutations, plotted against genomic coordinates of chromosome IV. The exact structure of allele *e2781* is uncertain (exons 1 and 2 could not be amplified, while other exons appeared normal). (B) Sequence alignment of BUS-2A with the glcosyltransferase BRE-2A.1, which is the most similar to BUS-2 of the multiple isoforms encoded by *bre-2*. Sequence alterations in *bus-2* mutants are indicated. A minor isoform, BUS-2B, which lacks the hexapeptide GIIFNN at the end of exon 1, has been reported by WormBase.

splice donor in contrast to the normal GU or (rarely) GC 5′ splice donor (FARRER *et al.* 2002), which is why WormBase and other gene predictions failed to include this exon. The corresponding genomic region was repeatedly sequenced during molecular identification of the various mutant alleles, and the GG donor sequence was confirmed. Use of GG as a 5′ intron signal has not previously been commented on in *C. elegans*, which is surprising; moreover, the *C. briggsae* ortholog of *bus-2* has a conventional GU at this site. However, there appears to be at least one other *C. elegans* gene, *B0244.4*, which is predicted to contain a 5′ GG donor in its third intron, and in fact it contains an identical junction sequence to that in *bus-2* (TAGgggagtttt). Such a junction is nevertheless likely to result in very inefficient splicing, and the *bus-2* mRNA abundance is substantially lower than for comparable genes (see supporting information, Figure S1). Unusual post-transcriptional events have also been inferred for the predicted glycosyltransferase gene *bus-8*, which appears to depend on translational frameshifting for proper expression (PARTRIDGE *et al.* 2008).

Probable biochemical properties of BUS-2 are reported at greater length elsewhere (PALAIMA *et al.* 2010). It is predicted to be a galactosyltransferase, and plausible orthologs can be found in the genomes of all Caenorhabditis species so far sequenced (see WormBase). Alignment with one of the galactosyltransferases encoded by *bre-2* is shown in Figure 1B.

The seven mutant alleles of *bus-2* all have very similar phenotypes, and two of the transposon alleles are simple insertions of Tc1 into exons 1 and 5, respectively, which are expected to result in a null phenotype. The second of these, *e2776*, has therefore been used in an extensive characterization of altered glycosylation patterns in *bus-2* worms (PALAIMA *et al.* 2010). The EMS alleles are all missense changes in conserved regions of the predicted protein; the existing *bus-2* reference allele, *e2687*, causes a Cys-to-Tyr change in one of these conserved regions and has been used for most of this work as a presumed null or severe hypomorph.

Expression patterns for *bus-2*: Expression patterns for *bus-2* have been briefly reported elsewhere (PALAIMA

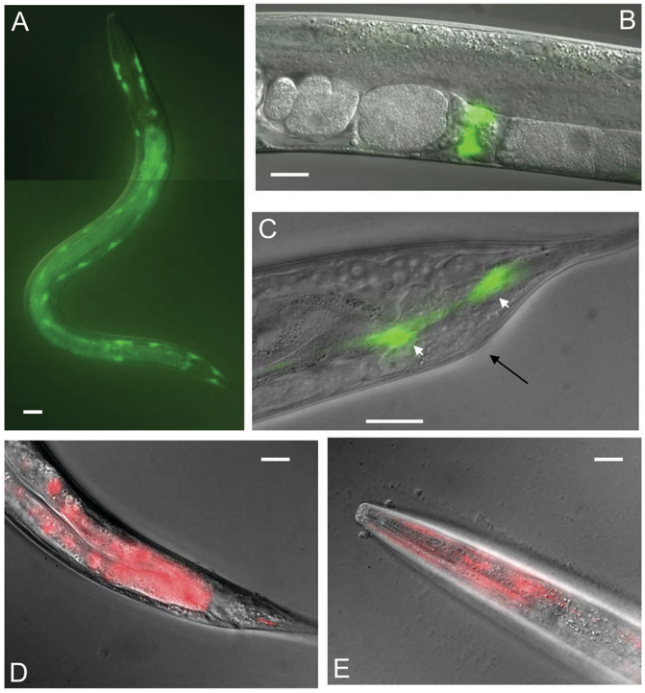

Figure 2.—Expression patterns for *bus-2*. (A–C) Expression from the rescuing operon construct *bus-2*::GFP in a *bus-2(e2687)* background, strain CB6616. (D and E) Expression from the rescuing operon construct *bus-2*::*mCherry* in a *bus-2(e2687)* background and corresponding mCherry construct, strain CB6846. (A) Whole adult worm, showing seam-cell expression. (B) Detail of oviduct, showing strong spermathecal expression. (C) Detail of tail after exposure to *M. nematophilum*, illustrating tail-swelling (arrowed) to demonstrate functional rescue of *bus-2* activity; seam-cell expression is visible (white arrowheads). (D) Adult posterior region, showing intestinal expression. (E) Adult head region, showing head muscle expression. Scale bars: ~25 μm.

et al. 2010) and are described in more detail here. The expression pattern of *bus-2* was initially examined using an operon construct for *bus-2*, with a wild-type *bus-2* gene upstream of a separate GFP cistron. This construct rescued *bus-2* function, as assayed by the restoration of susceptibility to *M. nematophilum*, with concomitant tail swelling in the presence of this pathogen (Figure 2C). Strong expression was observed in larval and adult seam cells and also in the hermaphrodite spermatheca (Figure 2, A–C). Expression in the intestine was difficult to score owing to tissue autofluorescence, which interferes with detection of GFP. Therefore, an equivalent mCherry reporter was constructed and transformed into *bus-2* worms. Examination of these animals confirmed the seam-cell expression and revealed significant expression in the posterior intestine, as well as in head muscles (Figure 2, D and E). The intestinal expression is consistent with detectable alterations in intestinal lectin staining, which are described elsewhere (Palaima *et al.* 2010).

Molecular identification and characterization of *bus-4*: Mutations of *bus-4* were recovered in screens for both Bus and Bah mutants (Gravato-Nobre *et al.* 2005; Darby *et al.* 2007). The genetic map position for *bus-4* was determined using SNP markers (data not shown), and a plausible candidate locus, *T22B11.2*, was identified within the probable interval for this gene. PCR amplification across *T22B11.2* demonstrated the presence of transposon insertions in both *mut-7*-induced alleles of *bus-4*, and subsequent sequencing of EMS and ENU alleles showed that these also carried lesions in *T22B11.2*. Transgene rescue experiments provided further confirmation of the gene identity. All six mutant alleles have similar phenotypes and are likely to be severely hypomorphic or null for gene activity on the basis of the mutational lesions (Figure 3).

T22B11.2/bus-4 encodes a predicted galactosyltransferase, but in a different subfamily from *bus-2*. Also in contrast to *bus-2*, which lacks any paralogs, there are nine fairly closely related homologs of *bus-4* in the *C. elegans* genome, all of which encode predicted galactosyltransferases. The most similar of these, *C16D9.6* (BLAST score 2e-66 relative to *bus-4*), has a genomic location very close to that predicted for the functionally similar gene *bus-6*, but it does not correspond to this gene (D. O'Rourke and J. Hodgkin, unpublished results). The more distantly related gene *C38H2.2* (BLAST score 2e-34 relative to *bus-4*) has been investigated as the apparent ortholog of human T-synthase, which is responsible for synthesis of the common core 1 O-glycan structure by catalyzing addition of galactose to GalNAc GalNAcα1-Ser/Thr. Appropriate galactosyltransferase activity was demonstrated by expressing *C38H2.2* in insect cells by Ju *et al.* (2006). Alignment of BUS-4 and C38H2.2 is shown in Figure 3B. Given the sequence similarity among these genes, it seems likely that *bus-4* also encodes a galactosyltransferase. However, another related gene in this group (*sqv-5*) encodes the nematode chondroitin synthase, acting to transfer both glucuronic acid and *N*-acetylgalactosamine to build up chondroitin sulfate chains (Hwang *et al.* 2003); therefore, other possible enzymatic activities cannot be excluded for BUS-4.

Expression patterns for *bus-4*: The expression pattern for *bus-4*, as determined by GFP and red fluorescent protein (RFP) reporter fusions, was similar to that of *bus-2*. Major expression was observed in the seam cells throughout development (Figure 4, A–C). However, expression appears to start earlier than for *bus-2* because strong fluorescence is seen in the seam cells of late embryos (Figure 4A) whereas embryonic expression was not seen for *bus-2*. The reporters used were rescuing operon constructs that were able to confer susceptibility to infection by *M. nematophilum* (Figure 4C). As with *bus-2*, significant intestinal expression could be detected using an RFP reporter (Figure 4D), although this could not be convincingly seen with the GFP reporter. Minor expression was seen in some other

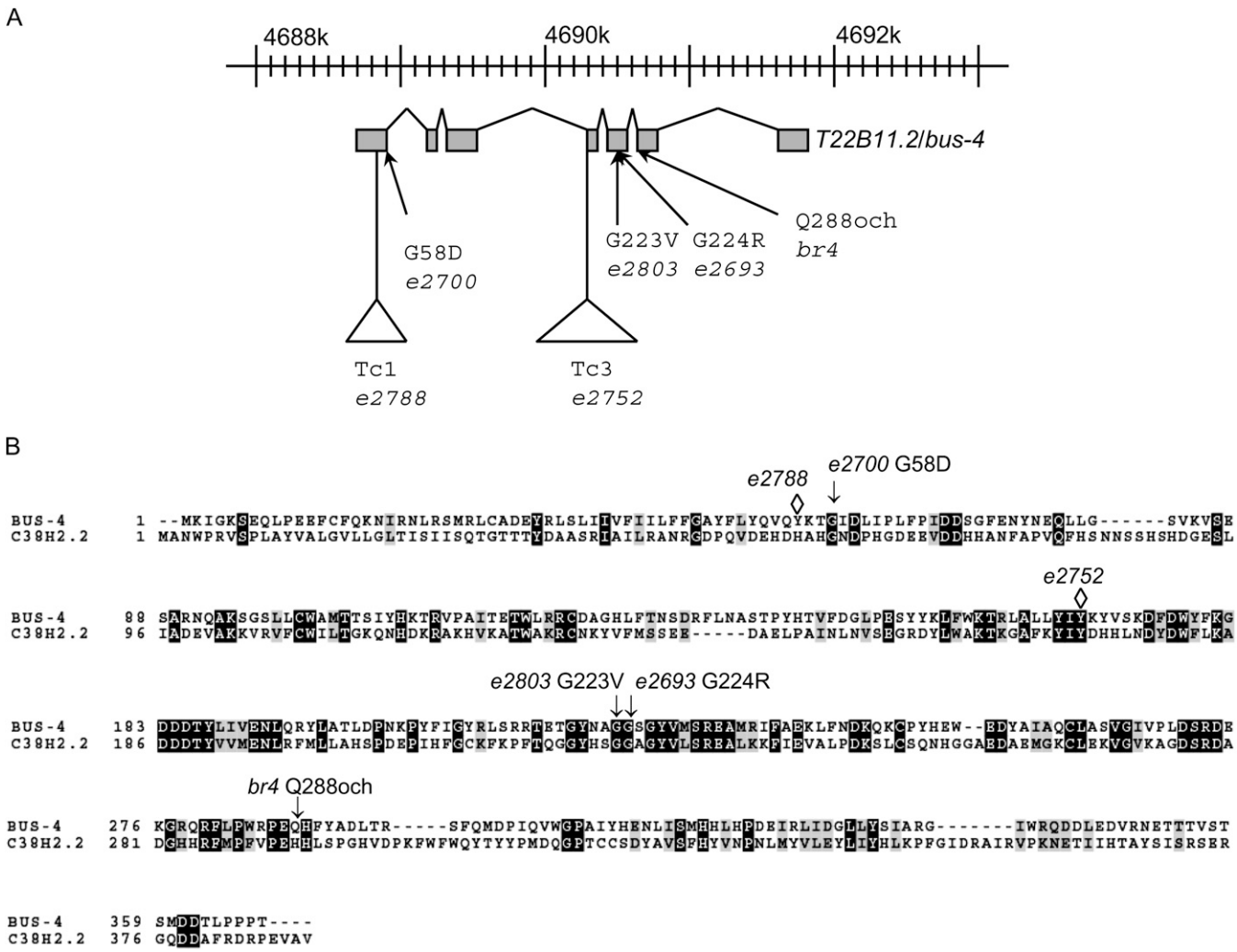

FIGURE 3.—Structure and mutations of *bus-4*. (A) Exon structure and localization of mutations, plotted against genomic coordinates of chromosome IV. (B) Sequence alignment of BUS-4 with the glycosyltransferase encoded by *C38H2.2*. Sequence alterations in *bus-4* mutants are indicated.

tissues such as the anterior pharynx (Figure 4F) but not in the spermatheca, in contrast to *bus-2*.

Molecular identification and characterization of *bus-12*: Single mutations of *bus-12* were recovered in screens for both Bus and Bah mutants (GRAVATO-NOBRE *et al.* 2005; DARBY *et al.* 2007). Previous work had defined the map position of *bus-12* on LGIV at ~ +8.5 cM; further crosses demonstrated very tight linkage to the gene *unc-26* at position +8.52 cM. Cosmid and fosmid rescue experiments using clones in this region, together with sequence analysis of candidate genes, indicated that *bus-12* corresponds to the gene *JC8.12* (Figure 5).

The first allele identified for *bus-12*, *e2740*, has a perceptibly weaker Bus phenotype than that of allele *br5*, and significant rectal accumulation of the pathogen can be seen in some infected *bus-12(e2740)* worms (Figure 6A), but never in *bus-12(br5)* worms (Figure 6B). PCR experiments and sequencing indicated the *e2740* allele, induced by *mut-7*, is a duplication of *JC8.12*, with two defective copies. One contains a Tc1 transposon insertion, and the other an insertion of four nucleotides in the fifth exon, and it seems possible that one or both copies may still supply some residual activity. In contrast, the allele *br5*, induced by ENU and isolated on the basis of a Bah phenotype (DARBY *et al.* 2007), was found to be a deletion affecting both *bus-12* and the gene immediately adjacent to its left, *ttr-45*. The null phenotype of *bus-12* was therefore uncertain, and additional alleles of *bus-12* were therefore sought by means of a noncomplementation screen (see MATERIALS AND METHODS). This yielded six new alleles of the gene, all of which had a Bus phenotype similar to that of the deletion allele *br5*. Sequencing showed that all six were different molecular lesions affecting *bus-12* alone (Figure 5A). Moreover, expression of transgenes expressing wild-type *bus-12*, but not *ttr-45*, were capable of rescuing susceptibility to *M. nematophilum* (Figure 6C). Therefore, the loss of *ttr-45* activity does not contribute to the bacterial resistance

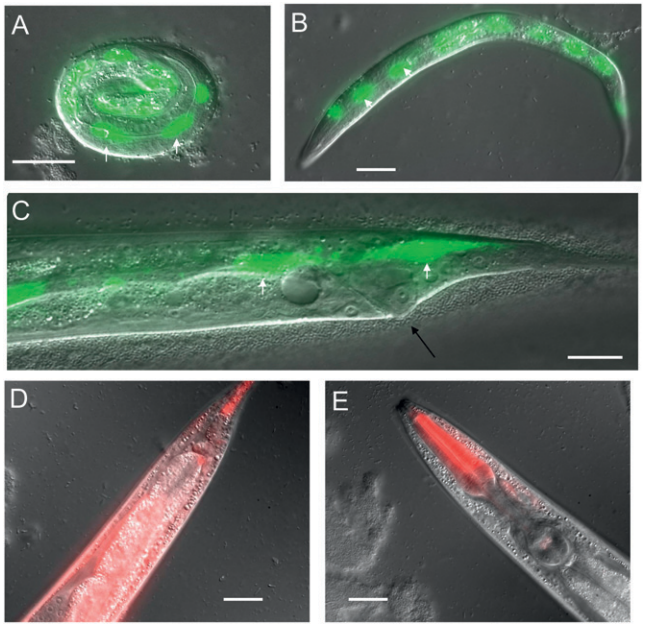

Figure 4.—Expression patterns for *bus-4*. (A–C) Expression from the rescuing operon construct *bus-4::GFP* in a *bus-4* background, strain CB6612; (D and E) Expression from the rescuing operon construct *bus-4::mCherry* in a *bus-4* background, strain CB6617. (A) Late embryo, illustrating strong seam-cell expression. (B) L2 larva. (C) L4 larva infected with *M. nematophilum*, showing tail swelling (black arrow) and seam-cell expression. (D) Tail region with intestinal expression. (E) Head region with anterior pharyngeal expression. Scale bar: ~25 μm.

phenotypes of *br5*, which are due only to loss of *bus-12*. Of the new alleles, five are nonsense mutations, and one of these (*e2977*, Gln123Amber) was used for most of the subsequent characterization of the gene.

On the basis of transcriptome analysis (WormBase), *JC8.12/bus-12* encodes two predicted isoforms of 315 and 304 amino acids. The shorter isoform appears to be generated by SL1 transplicing to the beginning of the second coding exon in the gene (Figure 5A) and would lack 11 N-terminal amino acids; it is not clear whether both isoforms are functional. The encoded proteins are predicted to be nucleotide-sugar transporters. The closest homolog of *bus-12* in the *C. elegans* genome is *sqv-7*, which has been shown to encode a protein capable of transporting UDP-glucuronic acid, UDP-*N*-acetylgalactosamine, and UDP-galactose, when expressed in yeast cells, and UDP-galactose when expressed in canine cells (Berninsone *et al.* 2001). Whether BUS-12 has similar substrates remains to be determined. Alignment between BUS-12 and SQV-7 is shown in Figure 5B. The *sqv-7* gene is expressed in seam cells, like *bus-12* (see below), and might therefore affect surface glycosylation, but *sqv-7* mutants were found to exhibit normal sensitivity to *M. nematophilum*. Possible redundancy between *bus-12* and *sqv-7* was nevertheless explored by constructing a double mutant *sqv-7(n2839); bus-12(br5)*. This strain did not exhibit any unexpected phenotypes, which argues against any redundancy in the function of the two genes.

Current gene models for *C. elegans* predict >15 different sugar transporters (Caffaro *et al.* 2007, 2008), of which three (*srf-3*, *sqv-7*, and *bus-12*) have now been associated with specific biological functions by means of mutations. Deletion alleles have been generated for five of the other predicted sugar transporter genes [*ZK896.9(ok3050)*, *K06H6.3(ok3012)*, *F44C8.7(ok2206)*, *T21B6.5(ok3220)*, and *C50F4.14(ok2860)*]. Strains homozygous for these alleles were examined for gross phenotype and resistance to *M. nematophilum*, but all appeared grossly normal. Seven other transporter genes (*C53B4.6*, *F15B10.1*, *ZK370.7/ugtp-1*, *ZC250.3*, *C03H5.2*, *B0212.4*, *F54E7.1/pst-2*) were tested by RNAi for altered sensitivity to *M. nematophilum* or other effects, using both *rrf-3* and *bus-12(br5)* mutant backgrounds, but no abnormalities or bacterial resistances were observed. Caffaro *et al.* (2007) have reported gonadal abnormalities associated with RNAi knockdown of *C03H5.2*, but only in a *srf-3* mutant background.

Expression patterns for *bus-12*: The expression pattern for *bus-12* was examined using the same methods as for *bus-2* and *bus-4*. A *bus-12p::GFP* reporter construct was expressed only weakly (data not shown), but a rescuing *bus-12* operon construct with dsRedII (materials and methods) exhibited strong expression in seam cells (Figure 6D), along with some gut expression mainly in the posterior intestine (Figure 6E) and in a few unidentified cells in the head (Figure 6F). Expression in the embryo or in the spermatheca was not detected. The major part of the expression pattern for *bus-12* therefore overlaps with that of *bus-2* and *bus-4*.

Growth comparisons between glycosylation gene mutants: Growth and fertility of hermaphrodites of reference mutants for *bus-2*, *bus-4*, and *bus-12* were compared with wild-type and reference mutants of the previously defined glycosylation genes *srf-3*, *bus-8*, and *bus-17*. No conspicuous differences were observed, with the exception of markedly slower larval growth in the *bus-17* mutant *e2800*. At 20°, generation time for most of the *bus* mutants was slightly longer than for wild type (3–5 hr difference) but ~15 hr longer for *bus-17(e2800)* mutants. An independent allele of *bus-17*, *e2693*, exhibited a similar growth delay as did *e2800/e2693* heterozygotes. Both mutants are notably smaller than wild type in mature adult body size. The viable *bus-8* allele used in this comparison, *e2698*, does not exhibit major growth retardation, but stronger viable alleles of *bus-8* exhibit severe growth retardation, small size, and some larval lethality, as previously described (Partridge *et al.* 2008).

All the mutants examined were both self-fertile (as hermaphrodites) and cross-fertile (as males). Strong gene expression in the hermaphrodite spermatheca was observed for *bus-2* and for *srf-3* (Höflich *et al.* 2004), but mutants of these genes failed to show any great

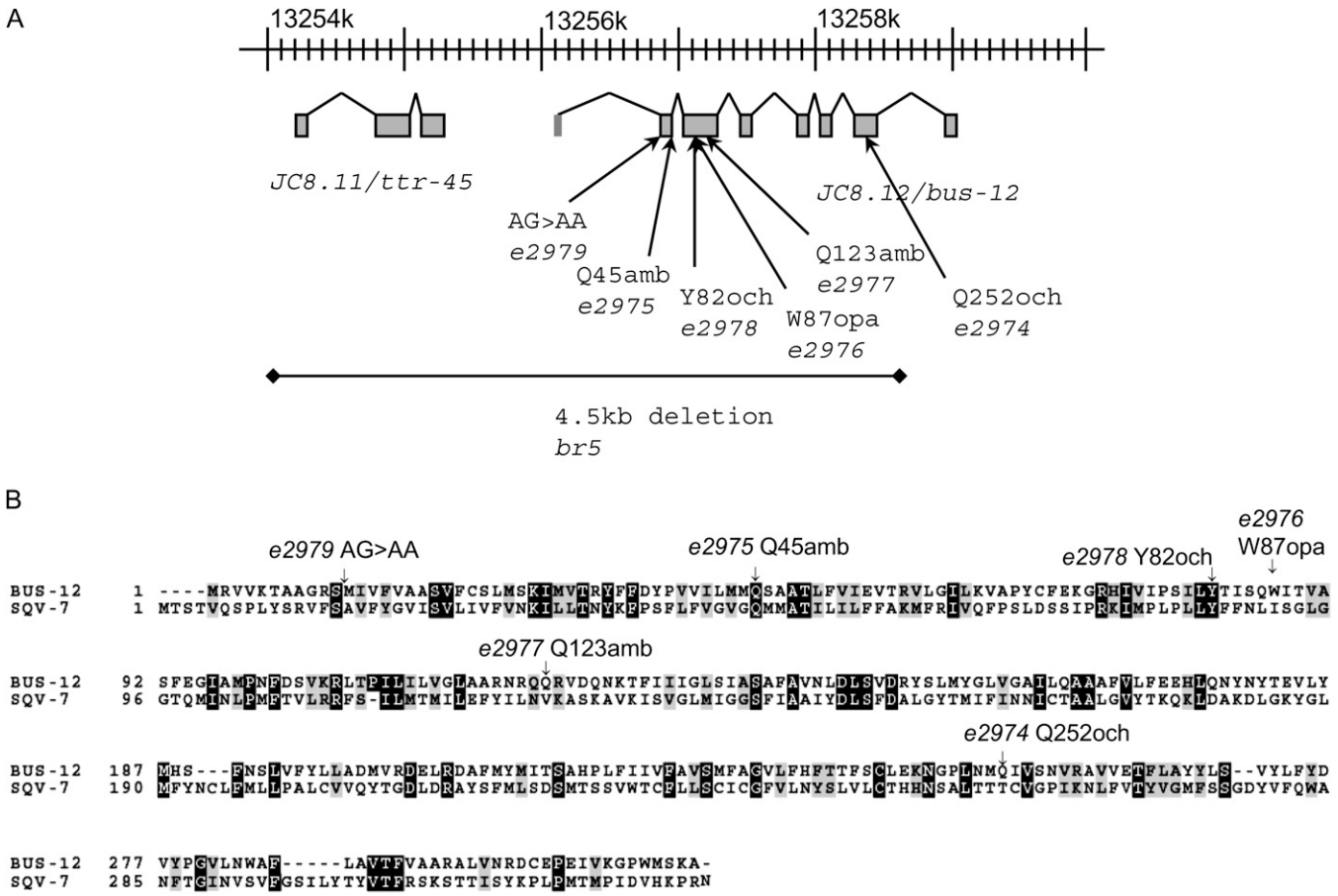

FIGURE 5.—Structure and mutations of *bus-12* and *ttr-45*. (A) Exon structure and localization of mutations, plotted against genomic coordinates of chromosome IV. (B) Sequence alignment of BUS-12 with SQV-7. Sequence alterations in *bus-12* mutants are indicated with arrows.

deficit in self-fertility, suggesting that spermathecal function is normal.

Effects on motility: interaction with *B. pumilus*: To make progress when crawling on a surface, nematodes need to exert a frictional force on that surface. For deformable, hydrophilic surfaces like those encountered on top of an agar gel, which is the usual place for examining *C. elegans* in a laboratory environment, it is not obvious how traction is exerted. As already noted in characterizing *bus-8*, *bus-17*, and *bus-18* mutants (YOOK and HODGKIN 2007; GRAVATO-NOBRE and HODGKIN 2008; PARTRIDGE *et al.* 2008), several of the surface-abnormal mutants identified by Bus phenotype also exhibit a "Skiddy" (Skd) locomotory mutant phenotype, in which the worm is able to generate a normal sinusoidal wave pattern but its body exhibits extensive slippage on agar and makes little forward progress. The three genes examined in detail in this work have only minor effects on this component of motility; *bus-12* and *bus-4* mutants exhibit a slight Skd phenotype, while *bus-2* mutants move almost normally under normal laboratory conditions.

A different aspect of motility, which is significant because it differentiates among the different surface glycosylation mutants, was discovered during a survey of interactions between *C. elegans* and plant-pathogenic pathovars of Pseudomonas (G. PRESTON and J. HODGKIN, unpublished results). One of the stocks examined was contaminated with a different, non-pseudomonad bacterial strain, CBX120, which had a striking effect on the motility of worms. Wild-type animals had great difficulty in moving through lawns of this contaminant and make progress only with a jerky or ratchet-like movement, as if trying to crawl through an extremely sticky medium. Sometimes worms eventually create small clearings in the lawn by eating the bacilli, but remain corralled within the clearing (Figure 7A). The aberrant locomotion has some similarities to the effect produced when wild-type nematodes are placed on lawns of *Yersinia* spp. (DARBY *et al.* 2007), which requires a bacterial exopolysaccharide (TAN and DARBY 2004). The contaminant was isolated and identified as a strain of the ubiquitous and important Gram-positive species *B. pumilus*, which has attracted detailed study as a common contaminant of spacecraft, among other reasons (KEMPF *et al.* 2005). A sample of one of the well-studied and fully sequenced strains of *B. pumilus*, SAFR-032, was obtained and found to lack the nematode stickiness

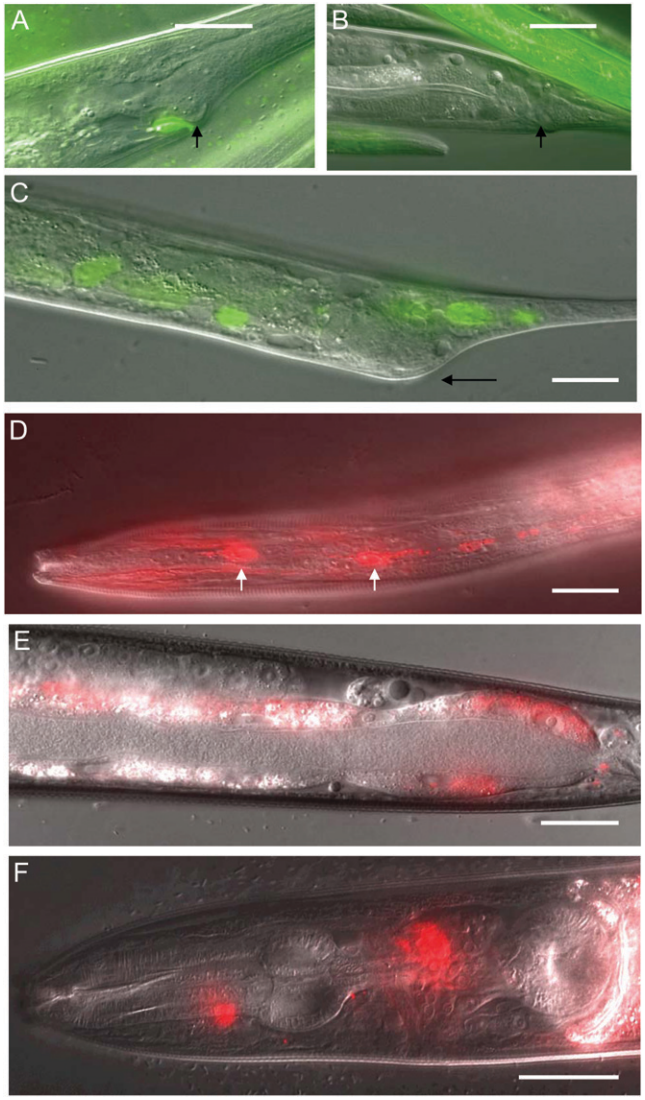

Figure 6.—Infection and expression patterns for *bus-12*. (A and B) *bus-12* mutants exposed to *M. nematophilum* and SYTO 13 stained to reveal bacteria; black arrowhead marks anus. (A) Allele *e2740*: rectal colonization, slight tail swelling. (B) Allele *br5*: no rectal staining, no tail swelling. (C) *bus-12(br5)* rescued with *bus-12(+); sur-5p::GFP* (strain CB6630) and exposed to *M. nematophilum*. Black arrow marks tail swelling. (D–F) *bus-12(e2977)* rescued with *bus-12::dsRedII*, strain CB6655; white arrowheads in D mark seam cells. (D) Head region, showing seam-cell expression. (E) Tail region, showing weak intestinal expression. (F) Head region, showing expression in a few head cells, of uncertain identity. Scale bar: ~25 μm.

observed in CBX120. Despite its extremely inhibitory effect on worm movement, pure lawns of CBX120 are little different from pure lawns of SAFR-032 in their ability to support good worm growth, and neither of these bacterial strains has much effect on fecundity or rate of maturation, as compared to *E. coli* OP50 (data not shown).

Microscopic examination of worms growing on CBX120 reveals that these bacteria do not stick tightly to the nematode surface, in contrast to the strong adhesion of infective *M. nematophilum* cells to the rectal and peri-anal cuticle. The impaired movement caused by this bacterial strain therefore seems to be due to extracellular secretions rather than to the bacteria themselves, but it is still likely to depend on specific interactions with the nematode surface. The movement of various surface mutants in CBX120 lawns was examined and found to be significantly different from wild type in a number of cases. Several mutant types were able to move much better than wild-type worms, with little jerkiness and immobility. In contrast, others appeared even more impaired in movement than wild type. To assess this interaction objectively, a "hurdles" assay was developed, in which worms were tested for the ability to crawl through three bands of CBX120 bacteria to reach a lawn of *E. coli* OP50, which they appear to prefer as an environment and food source (assay shown schematically in Figure 7C and described in more detail in materials and methods). As indicated in Figure 7D, wild-type worms perform poorly in this assay, with many worms remaining stuck at the first hurdle. In contrast, *bus-4* mutants are able to crawl efficiently across the hurdles.

Mutants of the six glycosylation genes discussed in this article were examined for movement on *B. pumilus* and found to exhibit a diversity of phenotypes: *srf-3*, *bus-4*, and *bus-17*, and to a lesser extent, *bus-8(weak)* mutants were able to move through CBX120 fairly efficiently, whereas *bus-2* and *bus-12* mutants were more impaired than wild type, with almost all worms remaining stuck at the first hurdle. For some of these genes, independent mutant alleles were tested and found to exhibit the same phenotypes as for the reference alleles (data not shown). The ability to move on CBX120 is not correlated with "skiddiness" during standard movement (Yook and Hodgkin 2007), nor with competence to support biofilm formation by *Yersinia* spp. (Darby *et al.* 2007). Moreover, mutants that appear very similar in other aspects of their phenotype, such as *bus-2* and *bus-4*, are strikingly different in this assay. *bus-4* mutants move well on CBX120, whereas *bus-2* mutants are impaired. The *bus-2*; *bus-4* double mutant resembles *bus-4* alone, not *bus-2*, indicating that *bus-2* mutants are not intrinsically inhibited in movement by CBX120.

These results reveal a further dimension to surface properties in *C. elegans* and demonstrate that some surface mutants can have a significant locomotory advantage over wild type in some environments. Sticky bacteria of many kinds are commonly seen as chance contaminants during the routine laboratory culture of *C. elegans*, so it seems very likely that colonies of such bacteria will encounter worms in their natural environments.

Effects on hermaphrodite surface recognition by males: A different kind of general alteration in surface properties in these glycosylation mutants was suggested by occasional difficulties encountered in crossing Srf

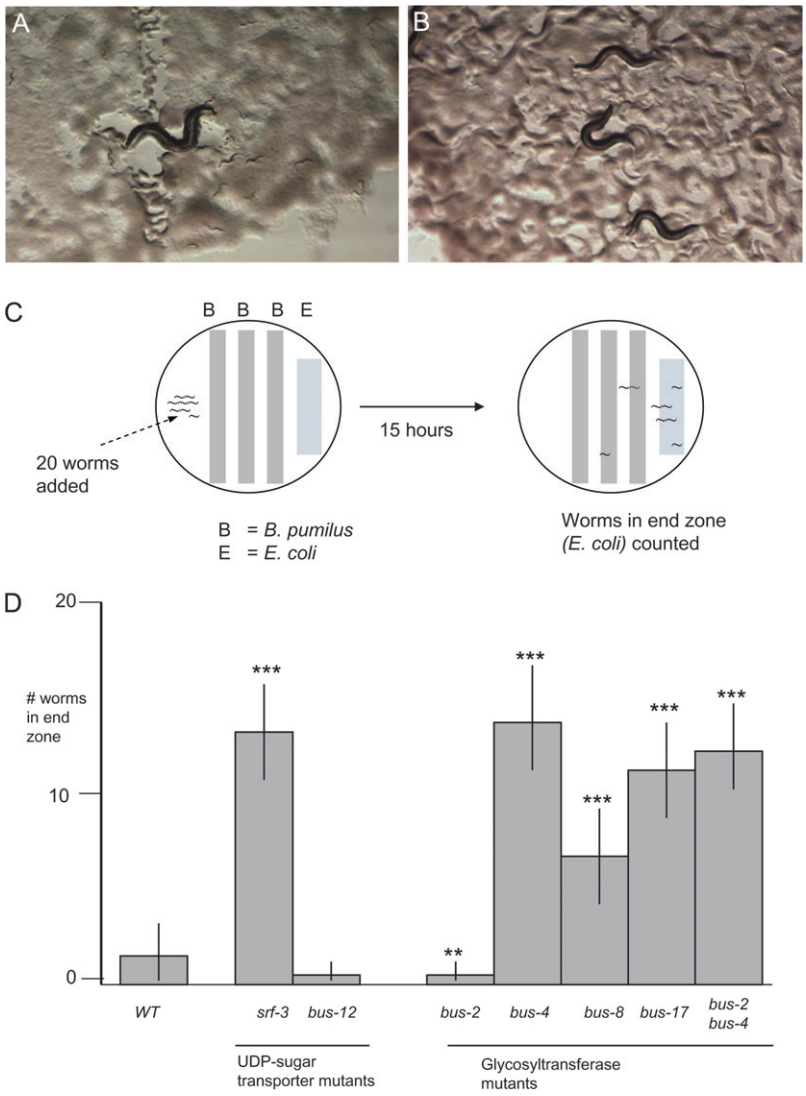

FIGURE 7.—Locomotory inhibition by *B. pumilus*. (A and B) Wild-type hermaphrodite (A) and *bus-4* hermaphrodites (B) on *B. pumilus* lawns. The wild-type animal is trapped in a cleared patch of the lawn, whereas the *bus-4* mutants can move well, leaving normal sinusoidal tracks. (C) Schematic of hurdles assay: worms attempt to swim across three bands of *B. pumilus* to reach a band of *E. coli*. (D) Numbers (maximum score 20) of animals accumulating in *E. coli* band after ~15 hr. Mean ± SD are shown for six to nine experiments with each genotype. Significant differences from wild type are indicated (two-tailed *t*-test).

or Bus hermaphrodites with phenotypically wild-type males. Cross-progeny were sometimes fewer than expected, or entirely absent, after crosses carried out under conditions in which mating is normally close to 100% successful. A possible explanation for this shortfall was that the males were less able to recognize the surface of the mutant hermaphrodites during mating and therefore less persistent in mating behavior. Male mating behavior (reviewed by BARR and GARCIA 2006) is a complex, multi-step process involving long-range pheromone-mediated detection of the presence of hermaphrodites, recognition of the hermaphrodite surface by direct contact between the hermaphrodite and the sensory rays of the male tail, tail-sliding and turning by the male, location of the hermaphrodite vulva by the male hook sensillum, and insertion of the male copulatory spicules, followed by ejaculation of sperm. The second of these steps, the recognition of the hermaphrodite surface, appears to involve some degree of specificity because *C. elegans* males will slide their tails over adults of other Caenorhabditis species, but they fail to slide over adults of other nematode genera and never remain in contact with them for long (BAIRD *et al.* 1992).

Time spent in contact between wild-type males and glycosylation-defective Bus/Srf hermaphrodites was therefore measured using a simple assay (see MATERIALS AND METHODS for details). Adult hermaphrodites to be tested were maintained on a small spot of bacteria, together with an excess number of adult wild-type males, and examined over time for mating contacts between males and hermaphrodites. Results of six independent tests are summarized in Table 1 and Figure S2. In all these tests, males remained in or very close to the bacterial spot with the hermaphrodites, indicating that the males continued to detect the presence of the hermaphrodites by diffusible chemical cues. Male leaving assays (LIPTON *et al.* 2004) confirmed this (data not shown). Under the conditions used, each wild-type hermaphrodite experienced one or more males sliding

TABLE 1

Mating contact assay

Genotype	% occupancy (mean ± SD)	% occupancy (range)	*P*-value relative to wild type
Wild type	44.33 ± 9.24	33–55	—
bus-2	13.50 ± 4.76	9–20	<0.001
bus-4	13.00 ± 6.87	4–21	<0.001
bus-8	25.83 ± 15.17	13–55	0.033
bus-12	13.00 ± 6.26	6–22	<0.001
bus-17	22.83 ± 11.54	6–39	0.006
srf-3	18.33 ± 5.75	10–28	<0.001

Occupancy indicates the percentage of time that an adult hermaphrodite spends in mating contact with a male under standard conditions (see MATERIALS AND METHODS). Data summarize six independent sets of tests. The statistical significance of each difference from wild type was calculated by a two-tailed *t*-test.

over its body for 40–50% of the time. In contrast, the various Bus/Srf hermaphrodites all experienced significantly reduced contact times, suggesting an impaired recognition by males. The defective recognition is distinct from a failure to detect the hermaphrodite vulva, the Lov (Location Of Vulva) phenotype, described by BARR and STERNBERG (1999), because it involves a prior sensory step and a different motor response (a "keep sliding" as opposed to a "stop sliding" response). Different sense organs may also be involved, as the hook sensillum appears to be the major vulva detector whereas the sensory rays may act as the main detectors in initial surface recognition.

None of the single mutants examined in these tests was completely unrecognizable by wild-type males, nor were any of the double-mutant combinations described below, because all eventually yielded cross-progeny when mated with males. This suggests that the detection of an appropriate hermaphrodite surface involves a complex set of chemical and/or physical cues that are recognized by the male tail sensilla, with significant but incomplete redundancy. Correct surface glycosylation evidently makes a substantial contribution to this contact recognition.

Tests for interaction between glycosylation genes: Multiple alleles of the three glycosylation genes that form the focus of this article (*bus-2*, *bus-4*, and *bus-12*) have been identified, some of which are likely to be null. The results presented above indicate that these mutants are altered in surface properties, in a variety of ways, but in other respects their gross properties and development appear fairly normal. The same is true for the previously analyzed *srf-3*, encoding a UDP-Gal transporter. In contrast, it has been shown that *bus-8* is an essential gene, required both for signaling during embryonic development and for successful molting in postembryonic life (PARTRIDGE *et al.* 2008). Also, the predicted galactosyltransferase gene *bus-17* is required for cuticle integrity, because severe *bus-17* mutants are viable, but exhibit significant cuticle fragility and drug sensitivity (GRAVATO-NOBRE *et al.* 2005), as well as the small size and slower larval growth rate reported here.

The relative mildness of the defects observed in the *bus-2*, *bus-4*, and *bus-12* mutants could be due to redundancy with other glycosylation genes. A precedent for such redundancy has been provided by CAFFARO *et al.* (2007), who found that RNAi knockdown of *C03H5.2* (encoding a nucleotide sugar transporter related to SRF-3) had no obvious effect in wild-type or RNAi hypersensitive strains, but resulted in gonad abnormality in a *srf-3* mutant background.

We therefore tested for possible redundancy or other interaction in the action of the three genes *bus-2*, *bus-4* and *bus-12*, both *inter se* and with the previously characterized *bus-8*, *bus-17*, and *srf-3*. This was done by constructing all 15 possible double mutants of the six genes under discussion. For the most part, probable null or extreme hypomorph alleles were used for these constructions (see MATERIALS AND METHODS), with the exception of *bus-8*, for which null alleles are inviable. The standard weak allele of *bus-8*, *e2698*, was therefore used instead for these constructions.

All double-mutant combinations proved to be viable, and no cases of unexpected phenotypes or mutual suppression (as tested by resistance to *M. nematophilum*) were encountered, apart from one anomalous and probably artifactual interaction, which is described below. In addition, the double mutant *bus-8(e2698) bus-17(e2800)* is more fragile, slower growing, and less healthy than either of its parents, but it is still viable. Since the *bus-8* allele used is non-null, this phenotypic enhancement is unremarkable.

The anomalous interaction was observed during the construction of double mutants of the two genes encoding the predicted transporters *srf-3* and *bus-12*. The double mutant *srf-3(e2689) bus-12(br5)* exhibited a severe uncoordinated (Unc) and egg-laying defective (Egl) phenotype, which is entirely different from that of either single parental mutant. However, alternative allelic combinations of these two genes (*e2689 e2977*, *yj10 br5*, and *yj10 e2977*) showed no sign of this severe

behavioral phenotype. As noted above, the *br5* allele is a deletion that removes both *bus-12* and the adjacent gene *ttr-45*, so it is probable that the synthetic phenotype is due to an interaction between *ttr-45* and a mutation linked to *srf-3(e2689)*, rather than to any allele-specific interaction between *srf-3* and *bus-12*. Very little is known about the function of the *ttr* gene family in *C. elegans*, with the exception of a recent report (Wang *et al.* 2010) demonstrating that *ttr-52* functions in the recognition of apoptotic cell corpses.

DISCUSSION

Previous work has implicated three *C. elegans* glycosylation genes in determining properties of the nematode surface, presumably by controlling the biochemistry of the glycocalyx, which is the outermost layer of the cuticle (Höflich *et al.* 2004; Yook and Hodgkin 2007; Partridge *et al.* 2008). The results presented here identify a three more genes (*bus-2* and *bus-4*, which encode predicted galactosyltransferases belonging to different families, and *bus-12*, which encodes a predicted UDP-sugar transporter protein) that have more subtle but still important roles in controlling the biological properties of the nematode surface. Characterization and cloning of all six genes were made easier by a shared feature, the fact that mutants of these genes are resistant to infection by the pathogen *M. nematophilum*, which induces a conspicuous and easily scored tail swelling (the Dar phenotype) in susceptible worms. In this work, we have further defined the mutant phenotypes of *bus-2*, *bus-4*, and *bus-12* and examined their expression patterns, as well as compared the properties of mutants of all six genes and defined new components of their phenotypes.

Definition of the exact biochemical alterations in *bus-2*, *bus-4*, and *bus-12* mutants, or *in vitro* examination of the enzymatic properties of the corresponding proteins, lies outside the scope of this article, not least because elucidation of the biochemistry of these mutants is unlikely to be easy. It is possible to examine substrate specificities for sugar transporters by expression in heterologous systems, as has been done for SRF-3, which was found to use UDP-galactose and UDP-*N*-acetylglucosamine as substrates (Höflich *et al.* 2004). A similar approach could be taken with BUS-12. However, such transporters probably contribute to multiple glycosylation pathways, and indeed, *srf-3* mutants were found to have reductions in both O-linked and N-linked glycoconjugates (Cipollo *et al.* 2004). A notable feature of the *C. elegans* sugar transporters, previously pointed out by Caffaro *et al.* (2007), is that the number of genes encoding putative transporters (18) appears more than sufficient to transport the seven different sugars in its glycoconjugates, especially as some of these transporters can deal with multiple substrates. Yet the two similar transporters SQV-7 and BUS-12, expressed in overlapping tissues such as the seam cells, have entirely different functions, as demonstrated previously for *sqv-7* mutants (Herman and Horvitz 1999) and here for *bus-12* mutants, We also find no evidence for redundant functions between the two in *sqv-7; bus-12* double mutants. It seems possible that these transporters, and other glycosylation factors, may act in separate subcellular components dedicated to the production of different extracellular carbohydrates.

Expression in heterologous systems could provide information about the possible enzymatic activities of BUS-2, BUS-4, BUS-8, and BUS-17, but it may be more immediately informative to examine glycomic changes in the mutant worms. Such investigations, using high-performance mass spectrometry, have been carried out in parallel work on mutants of *bus-2* (Palaima *et al.* 2010) and of *bus-4* (R. M. Mizanur, E. Jankowska, D. O'Rourke, D. Stroud, J. Hodgkin and J. F. Cipollo, unpublished results), revealing significant although complex alterations in glycans. Both of types of mutant were found to exhibit reductions, although not complete absence, of core-1 type O-glycans as well as reduced staining with ABA (*Agaricus bisporus*) lectin, especially in the tail region.

In contrast to the *sqv* and *bre* genes, which have been shown to affect chondroitin and glycosphingolipid biosynthesis, respectively, the substrate macromolecules for the *bus* glycosylation genes are not known. Enzymatic investigations *in vitro* may shed light on this question. A different approach may be provided by analysis of other *bus* and *srf* genes with mutant phenotypes comparable to the genes described here, because some of them may encode proteins that are core substrates for the relevant carbohydrate modifications. Recent advances in whole-genome sequencing (Sarin *et al.* 2008) have allowed us to identify a number of previously uncloned *bus* and *srf* genes, which may provide clues to possible substrates (D. O'Rourke, D. Stroud, M. J. Gravato-nobre and J. Hodgkin, unpublished results).

An important feature of this analysis is that each of the six genes exhibits a distinctive set of properties, as summarized in Table 2. This general result stands in contrast to two other realms of *C. elegans* biology in which glycosylation genes have been shown to have significant roles: vulval morphogenesis (affected by *sqv* genes) and susceptibility to *Bacillus thuringiensis* Cry toxins (affected by *bre* genes). As described in the Introduction, the five *sqv* genes all have similar mutant phenotypes (Herman and Horvitz 1999); likewise, the *bre* genes have similar mutant phenotypes (Marroquin *et al.* 2000). This is not surprising, if each set encode enzymes in a linear pathway for the production of a complex carbohydrate end-product. The situation with the six genes discussed in this article is different: null phenotypes for these genes range from subtle differ-

TABLE 2

Comparison of surface glycosylation genes

Gene	*WT*	*bus-2*	*bus-4*	*bus-8*	*bus-12*	*bus-17*	*srf-3*
Predicted activity	NA	Glycosyltransferase	Glycosyltransferase	Glycosyltransferase	UDP-sugar transporter	Glycosyltransferase	UDP-sugar transporter
Function	NA	Surface	Surface	Surface, embryo, molting	Surface	Surface	Surface
Growth on *E. coli*	Normal	Normal	Normal	Slow	Normal	Slow	Normal
Growth on *M. nematophilum*	Dar	Bus	Bus	Bus	Bus	Bus	Bus
Growth on Yersinia	Non-Bah	Bah	Bah	Non-Bah	Bah	Bah	Bah
Movement on *E. coli*	Normal	Normal	Weak Skd	Weak Skd	Weak Skd	Skd	Normal
Movement on *B. pumilus*	Poor	Very poor	Very good	Good	Very poor	Very good	Very good
Hermaphrodite/male recognition	Good	Poor	Poor	Defective	Poor	Defective	Defective
Cuticle fragility	Normal	Slight	Slight	Severe	Normal	Very severe	Severe

Phenotypes are those shown by reference alleles of the respective genes, which are putative nulls or severe hypomorphs, with the exception of *bus-8*, for which data refer to the weak viable allele *e2688*. The last row summarizes data on cuticle fragility as assayed by bleach sensitivity, previously published in Gravato-Nobre *et al.* (2005). Dar, Deformed Anal Region (*M. nematophilum* sensitive); Bus, Bacterially Un-Swollen (*M. nematophilum* resistant), Bah (Biofilm Absent on Head); Skd, Skiddy. NA, not applicable.

ences from wild type, detectable only by pathogen resistance or mating tests (as with *bus-2*), to complete lethality (as with *bus-8*). Moreover, assays such as mobility on *B. pumilus* reveal opposite alterations in some cases (increased mobility for *bus-4*, decreased mobility for *bus-12*), and these do not correlate with other kinds of mobility alteration such as "skiddiness" (maximal in *bus-8* and *bus-17* mutants). Both *bus-8* and *bus-17* affect cuticle integrity, and mutants exhibit increased drug sensitivity, but there are no obvious changes in cuticle integrity or permeability for mutants of the other four genes. Further diversity is revealed by surface lectin and antibody staining (Politz *et al.* 1990; Link *et al.* 1992; Gravato-Nobre *et al.* 2005).

An additional phenotype—defective recognition of hermaphrodite surfaces by males—was revealed in this work. Hermaphrodites of all six mutant types exhibited a significant difference from wild type in tactile attractiveness to mating males. This was measured by the time spent in direct contact between tested hermaphrodites and wild-type males under standard conditions. The males remain in the vicinity of mutant hermaphrodites, indicating that the hermaphrodites continue to emit pheromonal cues, but contact time between the sexes is reduced and consequently successful matings are rarer. We suggest that the mutants are deficient in surface features that males can detect only by direct contact, most probably by the sensory rays in the male tail. This detection might be achieved by short-range chemoreceptive "tasting" of some chemical feature on the hermaphrodite surface, or possibly by mechanoreceptive recognition of a physical surface property. Since none of the single or double mutants was completely unrecognizable by males, it seems likely that multiple cues are involved. Mate recognition is not essential for reproduction of a hermaphroditic species such as *C. elegans*, but is much more important for obligate male/female species such as most of the other species in this genus.

Tests for redundant function among these genes did not reveal any unexpected phenotypes in the double mutants examined. Redundancy is still possible, however. Indeed, the biochemical alterations in glycans so far detected by analysis of *srf-3* mutants (Cipollo *et al.* 2004), *bus-2* mutants (Palaima *et al.* 2010), and *bus-4* mutants (R. M. Mizanur, E. Jankowska, D. O'Rourke, D. Stroud, J. Hodgkin and J. F. Cipollo, unpublished results) appear to be quantitative rather than qualitative, in contrast to the more readily interpretable changes seen for *sqv* and *bre* mutants. Biochemical phenotypes may also be affected by compensatory changes in mutants if different modification enzymes are able to compete for the same substrate, and indeed, the biochemical analyses indicate increases in some some complex glycan types along with reductions in others.

A common feature of the three genes primarily discussed in this work (*bus-2*, *bus-4*, and *bus-12*) is that they all exhibit strong expression in seam cells, at least as inferred from the expression patterns of operonic rescuing constructs. Strong seam-cell expression has also been observed for *srf-3* (Höflich *et al.* 2004), *bus-8*

(Partridge *et al.* 2008), and *bus-17* (K. J. Yook and C. Darby, unpublished observations), as well as for *glf-1* (Novelli *et al.* 2009). These observations are in accord with the belief that the seam cells, rather than the hypodermis, are responsible for secreting the surface coat. The bulk of the cuticle consists of collagens secreted directly by the hypodermal cells, which envelop most of the worm, but the seam cells occupy only a small part of the worm's surface. Nevertheless, surface coat extends over the entire exterior of the worm, and the mutants described in this work affect both general surface properties and more local features, such as the rectal surface, that are anatomically remote from seam cells. Presumably, seam cells [which are known to be highly active in secretion during the molting process, as described by Singh and Sulston (1978)] produce precursor material that can spread in the space between old and new cuticles during molting, and thereby coat the whole surface, but exactly how this works, how the glycocalyx bonds to the underlying collagens and other cuticle proteins, and whether material can be added or turned over during the intermolt and adult stages of the life cycle, are all unresolved questions. Contributions from the intestinal secretions must also be considered in view of the significant gut-cell expression observed for *bus-2*, *bus-4*, and *bus-12*. On the other hand, the excretory system, pharyngeal gland cells, amphids, and phasmids do not seem to be involved, although they have been proposed as sources of surface coat material both in *C. elegans* and in other nematodes (Nelson *et al.* 1983; Bird *et al.* 1988; Jones and Baillie 1995).

This work, together with previous genetic and biochemical analyses, demonstrates that nematode surface glycosylation is complex in terms of biochemistry and biological function, both in the normal life of the worm (affecting locomotion, cuticle permeability, and mate recognition) and as a major factor in determining susceptibility to pathogens. The nematode cuticle is one of the most important defining features of the phylum Nematoda, which includes a very large number of important plant and animal pathogens. A surface coat is found on the surface of all nematodes, as far as is known, and is the largest interface between parasite and host in parasitic nematodes. Genomic data indicate that most or all of the *bus* and *srf* genes discussed in this article have orthologs or homologs in both animal and plant parasites, so the investigation of these surface-modification genes in *C. elegans* offers excellent opportunities for investigating a crucial interface both between an animal and its environment and between parasites and their hosts.

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Glycosylation Genes Expressed in Seam Cells Determine Complex Surface Properties and Bacterial Adhesion to the Cuticle of *Caenorhabditis elegans*

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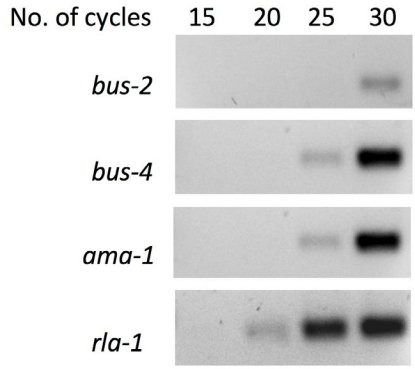

FIGURE S1.–Total RNA was isolated from N2 mixed stages using TRIzol (Invitrogen); 1 μg of total DNase-treated RNAs were reverse transcribed using the Superscript VILO cDNA Synthesis Kit (Invitrogen), according to the manufacturer's instructions. Semi-quantitative PCR was carried out using a Techne TC512 thermal cycler. Each reaction contained: 0.2 μM of forward and reverse primers and 1 μl of cDNA (1:20 RNA dilution) in a total volume of 50 μl. Gene specific primers were as follows:

*bus-2*_forw: 5′ GTCGAAATGGCTTCAAAACGAC 3′

*bus-2*_rev: 5′ GATCCCACCACCTGCATAGAAC 3′,

*bus-4*_forw: 5 ′GGGAGGAAGTGGCTATGTGATG 3′,

*bus-4*_rev; 5′ TCTGCCTACCCTTCTCATCTCG 3′, :

*ama-1*_forw: 5′ CCTACGATGTATCGAGGCAAA 3′,

*ama-1*_rev: 5′ CCTCCCTCCGGTGTAATAATG 3′

*rla-1*_forw: 5′ GAAGATCGCTACCCTTCTCAAG 3′

*rla-1*_rev 5′ CAGAAGTGATGAGGTTCTTCAC 3′.

Thermocycling was performed under conditions consisting of an initial denaturation step (95°C for 5 min), followed by 15/20/25 or 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. 30 μl of each transcript were analyzed on a 2% agarose gel and *bus-2* and *bus-4* levels were determined by comparison to the endogenous control genes *ama-1* and *rla-1* for the 4 cycle times tested. Detected levels were consistent in the 3 biological replicates analyzed. Similar results were obtained using a different pair of primers for *bus-2*.

Quantitation of relative RNA levels for these genes, and also for *bus-12*, was independently obtained from RNASeq data obtained by deep-sequencing RNA from intestinal cells (Simon Haenni and André Furger, personal communication). Read numbers for these genes in this dataset were as follows:

bus-2:	26
bus-4:	96
bus-12:	299
ama-1:	2537
rla-1:	4100

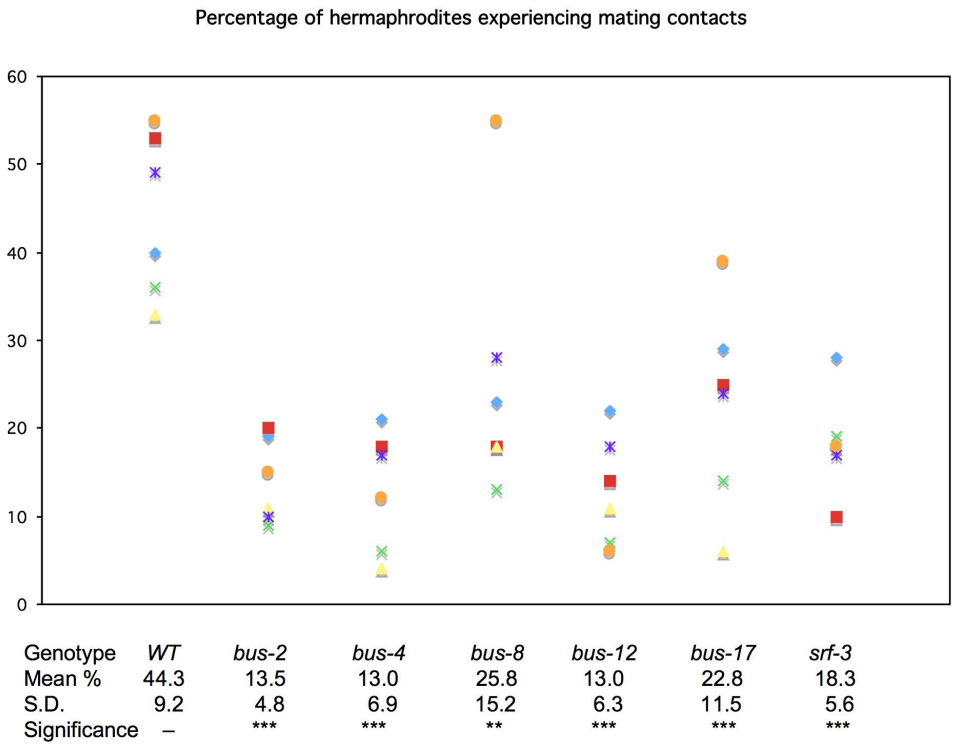

FIGURE S2.—Impaired recognition of Bus and Srf mutants by wildtype males. Each point is a measure of the fraction of time that wildtype or mutant hermaphrodites experienced sliding and mating contact with wildtype males, during a 50 minute observation period. Six separate experiments were carried out, assaying the seven genotypes in parallel in each experiment. Mean and standard deviation values are given below, together with significant differences from wildtype for each mutant (2-tailed T-test).