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Caenorhabditis elegans Mutants Resistant to Attachment of Yersinia Biofilms

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ABSTRACT

The detailed composition and structure of the *Caenorhabditis elegans* surface are unknown. Previous genetic studies used antibody or lectin binding to identify *srf* genes that play roles in surface determination. Infection by *Microbacterium nematophilum* identified *bus* (bacterially unswollen) genes that also affect surface characteristics. We report that biofilms produced by *Yersinia pestis* and *Y. pseudotuberculosis*, which bind the *C. elegans* surface predominantly on the head, can be used to identify additional surface-determining genes. A screen for *C. elegans* mutants with a biofilm absent on the head (Bah) phenotype identified three novel genes: *bah-1*, *bah-2*, and *bah-3*. The *bah-1* and *bah-2* mutants have slightly fragile cuticles but are neither Srf nor Bus, suggesting that they are specific for surface components involved in biofilm attachment. A *bah-3* mutant has normal cuticle integrity, but shows a stage-specific Srf phenotype. The screen produced alleles of five known surface genes: *srf-2*, *srf-3*, *bus-4*, *bus-12*, and *bus-17*. For the X-linked *bus-17*, a paternal effect was observed in biofilm assays.

THE nematode cuticle is a complex, multi-layered, dynamic extracellular matrix (Bird and Bird 1991). As a major site of interaction with the environment, cuticle is of interest physiologically, behaviorally, ecologically, and, for parasitic nematodes, immunologically. Complete determination of the composition and structure of cuticle has not been accomplished for any species. Cuticle can be dissected by hand from macroscopic parasitic nematodes, *e.g.*, *Ascaris lumbricoides* (Bird 1956, 1957), facilitating biochemical and structural studies, but robust genetic methods are not available for these animals. Conversely, the microscopic model nematode *Caenorhabditis elegans* is less convenient for biochemical and structural analysis, but is ideal for genetic studies.

As *C. elegans* develops through four larval stages into an adult, a new cuticle is synthesized at each molt by underlying hypodermal cells, and there are major differences in the composition and structure at different stages (Cox *et al.* 1981b). Ultrastructural studies show that the *C. elegans* adult cuticle is ~0.5 μm thick and comprises five distinct layers. Proximal to distal, these are the basal, medial, and cortical layers, the epicuticle, and the surface coat (Zuckerman *et al.* 1979; Cox *et al.* 1981a,b; Bird and Bird 1991). The first three are relatively thick and predominantly composed of collagens. The noncollagenous epicuticle is exceedingly thin, and electron microscopy reveals little of its structure. Lipid analog probes associate with the epicuticle of many nematodes, but the probes do not diffuse as they do in a cellular lipid bilayer, implying a different type of organization (Proudfoot *et al.* 1993). A biochemical study of the *C. elegans* surface suggested that the epicuticle contains a heterodimeric protein complex (Blaxter 1993).

The outermost nematode component, the surface coat, differs fundamentally from the underlying layers. While harsh treatments are required to separate highly crosslinked components of the collagenous layers and the epicuticle (Cox *et al.* 1981a), ethanol is sufficient to extract the surface coat (Page *et al.* 1992). The surface coat is therefore lost in standard transmission electron microscopy preparations that use ethanol dehydration, but alternative methods revealed this layer of *C. elegans* (Zuckerman *et al.* 1979) and *C. briggsae* (Himmelhoch and Zuckerman 1978).

Biochemical, immunological, and molecular biology approaches have produced descriptions of some parasitic nematode surface proteins at the primary amino acid level, but there are no comparable reports for *C. elegans*. The surface composition has been examined genetically using phenotypes of antibody binding (to unidentified epitopes) or lectin binding to whole animals. This identified three genes whose mutants appear to have primary defects in the surface: *srf-2*, *srf-3*, and *srf-5* (Politz *et al.* 1990; Link *et al.* 1992). Mutations in *srf-6* result in stage-specific defects in surface antigen display (Hemmer *et al.* 1991; Grenache *et al.* 1996).

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Additional surface-determining genes were identified using infection by the nematode-specific pathogen *Microbacterium nematophilum*, which causes anal swelling. Screening for a bacterially unswollen (Bus) phenotype identified 15 genes and also produced alleles of *srf-2*, *srf-3*, and *srf-5* (Gravato-Nobre *et al.* 2005).

Surface defects also affect adherence of the plague bacterium *Yersinia pestis* and the closely related *Y. pseudotuberculosis*. Yersinia make a biofilm—a polysaccharide-rich extracellular matrix in which the bacteria are embedded—that binds the worm, especially on the head (Darby *et al.* 2002; Tan and Darby 2004). The bacteria secrete the matrix when grown on agar in the absence of nematodes; when worms are then placed on the agar, their forward locomotion causes the tightly adhering material to accumulate on their heads (Tan and Darby 2004). *srf-2*, *srf-3*, and *srf-5* mutants are resistant to biofilm attachment (Joshua *et al.* 2003; Hoflich *et al.* 2004). We now describe a genetic screen using the Yersinia biofilm phenotype to identify additional surface-related genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions: *Y. pseudotuberculosis* YPIII is a standard laboratory strain (Gemski *et al.* 1980). Bacteria were grown in LB broth at 26° overnight; these cultures were used to inoculate NGM agar, and the plates were incubated overnight at room temperature to form lawns (Darby *et al.* 2005).

To score the Bus phenotype, mixed lawns of *M. nematophilum* CBX102 and *Escherichia coli* OP50 were prepared as described (Gravato-Nobre *et al.* 2005) except that CBX102 constituted 1% of the initial inoculum instead of 10%. *C. elegans* grown on these plates were scored as either deformed anal region (Dar, the wild-type phenotype) or Bus (bacterially unswollen).

***C. elegans* strains:** The N2 Bristol strain and its mutant derivatives were used, except for the experiment shown in Table 1. For linkage analysis and mapping, the mutations used, on chromosomes indicated by Roman numerals, were (I) *bli-3(e767)*, *unc-11(e47)*, *dpy-5(e61)*, *unc-13(e1091)*, *unc-29(e1072)*, *mom-5(or57)*, *lin-11(n566)*, *vab-10(e698)*, *unc-101(m1)*, *unc-54(e1092)*; (II) *lin-31(n310)*, *unc-4(e120)*, *unc-52(e444)*; (III) *dpy-1(e1)*, *unc-36(e251)*, *unc-25(e156)*; (IV) *dpy-9(e12)*, *unc-33(e204)*, *unc-5(e53)*, *dpy-4(e1166)*; (V) *unc-34(e566)*, *dpy-11(e224)*, *unc-51(e369)*; and (X) *lon-2(e678)*. Deficiencies used to map *bah-1* were *nDf24*, *qDf7*, *hDf17*, and *dxDf2*.

Biofilm assays: Biofilm susceptibility of *Caenorhabditis* sp. was determined by testing the ability to grow from hatching to L4 stage in 2 days (Darby *et al.* 2005). Adult hermaphrodites were placed on *Y. pseudotuberculosis* lawns, allowed to lay eggs for ~2 hr, and then removed. Developmental stage of the broods was scored after incubation at 20° for 2 days. In every experiment, development on *E. coli* OP50, the standard *C. elegans* food, was assayed in parallel.

When genetic analysis required genotyping individual worms, animals were grown to adult stage on *E. coli* and then incubated on *Y. pseudotuberculosis* lawns for 4–5 hr and the presence of biofilms was scored. Biofilm attachment to wild type is not 100% under these conditions, and therefore *bah* genotypes were confirmed by examining broods hatched and grown on *Y. pseudotuberculosis*. To score the Bah phenotype at particular growth stages, adults were placed on OP50-seeded plates, allowed to lay eggs for 1 hr, and then removed. The plates were incubated at 20° for 27, 40, and 47 hr to obtain stages L2, L3, and L4, respectively, and animals were then transferred to *Y. pseudotuberculosis* and scored after 4–5 hr.

To confirm the genotype of paternally rescued *bus-17* males, animals with biofilms were removed from *Y. pseudotuberculosis* lawns and placed in a drop of M9 buffer that had been raised to approximately pH 10 by the addition of sodium hydroxide, a condition that removes the matrix from worms (Tan and Darby 2004). Biofilms detached within a few minutes, after which the suspensions were restored to physiological pH by addition of excess M9, pH 6.5. The males were transferred to NGM mating plates, containing 30 μg/ml of kanamycin, that had been seeded with a kanamycin-resistant *E. coli* strain; this prevented growth of any *Y. pseudotuberculosis* carried over during the removal treatment. Each recovered male was separately mated to tester *dpy-5(e61); bus-17(br2)* hermaphrodites and the non-dumpy (Dpy) progeny were scored in biofilm assays.

Screen for Bah mutants: Strain N2 was mutagenized with *N*-ethyl-*N*-nitrosourea (ENU) as described (De Stasio and Dorman 2001). F_1 hermaphrodites were treated with alkaline hypochlorite (Wood 1988) to release their eggs, which were washed in water and deposited on lawns of *Y. pseudotuberculosis* grown on 10-cm NGM agar plates. It was necessary to plate the eggs at low density (<~1000/10-cm-diameter plate), so that bacterial exopolysaccharide was not limiting. Under these conditions, the feeding inhibition of the *Y. pseudotuberculosis* biofilm prevented almost all animals from developing to L4 stage after 2 days or to adult stage after 3 days. Plates were screened after 2 days for rare F_2 L4's that had no attached biofilm; some plates were rescreened a day later for biofilm-free adults. Candidate mutants were placed, one per plate, on new *Y. pseudotuberculosis* lawns for testing of their broods. To ensure that mutations were independent, only one strain was established from any mutagenized parent.

Cuticle fragility tests: A published protocol (Gravato-Nobre *et al.* 2005) was modified slightly, such that alkaline hypochlorite solution contained 5.4% NaOCl (instead of 40%) and 1 N NaOH. In other respects, the assay was unchanged. For each trial, 15 worms were collected on a wire pick and placed in a 10-μl drop of alkaline hypochlorite on an NGM plate. The animals were observed continuously with a stereomicroscope, and the time required for the last of them to stop spontaneous movement was noted. The time required for the first visible breach to appear in a cuticle, which almost always was later, was also recorded. For wild type, the thrash time was slightly longer than reported by Gravato-Nobre *et al.* (2005), presumably due to the lower chlorine concentration. The time required for cuticle breach was not appreciably different. Mutants were assigned scores of + to ++++ on the basis of differences in the means of multiple assays.

Lectin binding: Wheat germ agglutinin (WGA) conjugated to fluorescein isothiocyanate (FITC) (EY Laboratories, San Mateo, CA) was used at 20 μg/ml in a buffer of 0.01 M phosphate and 0.15 M NaCl, pH 7.3. Nematodes were stained at room temperature for 30 min, washed twice in buffer, and examined immediately by epifluorescence microscopy.

Antibody binding: A published protocol (Hemmer *et al.* 1991) was used with minor modifications. Nematodes were washed three times in phosphate-buffered saline (PBS) and then incubated for 2.5 hr with a 50-fold dilution of monoclonal antibody M37 in PBS. After three PBS washes, worms were incubated with goat anti-mouse-immunoglobulin M conjugated to FITC (Sigma, San Diego) for 1.5 hr and then washed six times. Because M37 sloughs off of *C. elegans* upon warming, all solutions were ice cold, and samples were not allowed to warm at any time during the preparation. After the final wash, worms were pipetted to chilled glass slides or spot plate wells and examined immediately by epifluorescence microscopy.

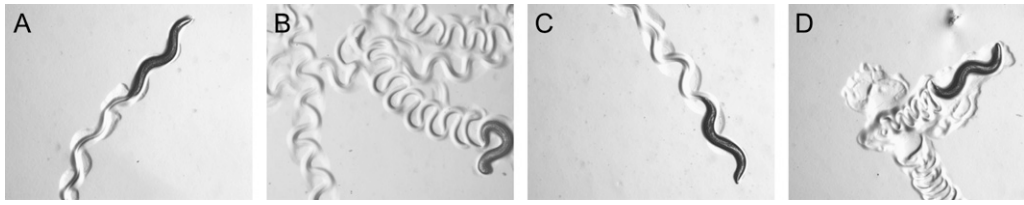

FIGURE 1.—Aberrant locomotion on bacterial lawns. (A) Normal locomotion by wild-type strain N2 on *E. coli* OP50, the standard laboratory food. (B) Skd phenotype of N2 on *Y. pseudotuberculosis*. (C) Normal locomotion by *bah-3(br9)* on *Y. pseudotuberculosis*. (D) Skd phenotype of *bus-17(br11)* on *E. coli*.

Strain constructions: Double mutants containing both *srf-6* and a *bah* allele were constructed by a procedure that obtained homozygous mutations sequentially. First, *bah*/+ males were mated to hermaphrodites carrying *dpy-10(e128)* and *unc-4(e120)*, markers that flank the *srf-6* locus on chromosome II. Male progeny of this first cross were mated to *srf-6(yj13)* animals, and hermaphrodite progeny of the second cross were placed on *Y. pseudotuberculosis* to lay eggs. Offspring with the Bah phenotype were allowed to self, one per plate, on fresh *Y. pseudotuberculosis* plates. From lines that were true breeding for Bah and segregated Dpy uncoordinated (Unc) animals, indicating a *srf-6/dpy-10 unc-4* genotype, multiple non-Dpy non-Unc animals were picked to individual plates. The double mutant was established from a plate on which neither Dpy nor Unc segregated.

RESULTS

Biofilm sensitivity of *C. elegans* laboratory strain and wild Caenorhabditis isolates: The *Y. pseudotuberculosis* biofilm binds close to all *C. elegans* hermaphrodites of the standard laboratory strain N2 (DARBY *et al.* 2002, 2005). Rarely, biofilms do not form on individual animals, but propagating these worms shows that they are not spontaneous biofilm-resistant mutants. Biofilm attachment to males is somewhat less penetrant: in parallel assays of hermaphrodites and males conducted on eight separate days, biofilms attached to a mean of 95.4% of hermaphrodites (SD = 9.8, $N = 274$) but to only 86.4% of males (SD = 8.8, $N = 278$). Biofilms are observed at all *C. elegans* growth stages except in the dauer larva, the alternative L3 stage that worms enter to survive harsh conditions. However, biofilm formation requires nematode locomotion through a bacterial lawn that contains the secreted biofilm matrix material (TAN and DARBY 2004), and dauers do not often move on Yersinia lawns, even when prodded. It is therefore not clear whether the absence of biofilms on dauers is due to the lack of locomotion or to alterations of the surface composition.

Although worms must move through the bacterial lawn to accumulate biofilm, the locomotion is aberrant. Normally the animals move by bending their bodies back and forth, which leaves sinusoidal tracks on *E. coli* lawns (Figure 1A). Animals placed on *Y. pseudotuberculosis* (Figure 1B) or *Y. pestis* (not shown) continue to bend back and forth, but they make less forward progress than on *E. coli*, leaving tracks that are compressed. Eventually, many worms on Yersinia are unable to translocate altogether, and they carve craters in the bacterial lawn as they continue bending back and forth. The appearance is that of slipping or skidding, and the aberration appears identical to the strong skiddy (Skd) phenotype observed for *bus-17* mutants (GRAVATO-NOBRE *et al.* 2005; YOOK and HODGKIN 2007).

Because the biofilm covers the mouth and blocks food intake, animals fail to develop normally on Yersinia lawns. Two days after eggs are laid on *Y. pseudotuberculosis*, few or no worms have reached the L4 larval stage, while essentially all grow to L4 in this period on *E. coli* OP50, the standard laboratory food (DARBY *et al.* 2002, 2005). To determine the extent of biofilm sensitivity, we tested 11 additional *C. elegans* wild isolates as well as several other *Caenorhabditis* sp. Of the 11 *C. elegans* strains, 10 were sensitive to biofilm formation (Table 1), while a Hawaiian isolate, CB4856, was resistant. In crosses with N2, the Hawaiian strain's resistance did not behave as a simple Mendelian trait and was not analyzed further.

TABLE 1

Biofilm sensitivity of Caenorhabditis isolates

Species and strain	Geographic origin	% L4
C. elegans		
N2	England	2.0 ± 1.7
CB4852	England	7.9 ± 10.0
CB4851	France	0 ± 0
CB4507	Western United States	0 ± 0
CB4555	Western United States	5.7 ± 3.2
CB4853	Western United States	1.5 ± 2.6
CB4854	Western United States	0 ± 0
CB4855	Western United States	0 ± 0
CB4857	Western United States	0 ± 0
CB4858	Western United States	0.8 ± 1.4
LSJ1	Western United States	0 ± 0
CB4856	Hawaii	100 ± 0
C. briggsae		
AF16	India	93.0 ± 2.2
ED3032	Taiwan	100 ± 0
ED3033	Taiwan	91.7 ± 11.8
VT847	Hawaii	94.7 ± 5.0
C. remanei		
EM464	Eastern United States	94.0 ± 4.3

Strains are described in BAIRD *et al.* (1992) or at http://www.wormbase.org. Growth was assayed on lawns of *Y. pseudotuberculosis* as described in MATERIALS AND METHODS. Data are mean and SD of three independent trials.

TABLE 2

Phenotypes of Bah mutants

Gene	Alleles	*M. nematophilum*[a]	Motion[b]	WGA[c]	M37[d]	Fragility[e]
Wild type	—	Dar	+	−	L1	+
bah-1	*br1*	Dar	+	−	L1	++
bah-2	*br7, br8*	Dar	+	−	L1	++
bah-3	*br9*	Dar	+	−	L1–L4	+
bus-4	*br4*	Bus	+	−	−	++
bus-12	*br5*	Bus	Sl Skd	−	−	++
bus-17	*br2, br11*	Bus	Skd	+	−	++++
srf-2	*br3, br10*	Bus	+	+	−	+
srf-3	*br6*	Bus	Sl Skd	+	−	+++

[a] Response to *M. nematophilum* exposure. Dar, deformed anal region; Bus, bacterially unswollen.
[b] Locomotion on *E. coli* lawns. +, normal; Skd, strong skiddy; Sl Skd, slightly skiddy.
[c] Cuticle staining of whole worms by wheat germ agglutinin. −, absence of staining except at vulva; +, ectopic staining.
[d] Binding of monoclonal antibody M37 to larval stages. −, no binding at any stage. The L1–L4 phenotype of *bah-3* is also known as Cld.
[e] + to ++++, increasing fragility on the basis of data in Table 3.

Four *C. briggsae* strains were all resistant, as was the single *C. remanei* strain tested.

Biofilm absent on head (Bah) phenotypes of *srf* and *bus* mutants: *srf-2*, *srf-3*, and *srf-5* mutants were previously reported to be biofilm resistant (Joshua *et al.* 2003), a phenotype we named Bah (biofilm absent on head). We confirmed this phenotype for *srf-2(yj262)*, *srf-3(yj10)*, and *srf-5(ct115)*, each of which is believed to have a surface-specific primary defect (Politz *et al.* 1990; Silverman *et al.* 1997). In contrast, biofilm attachment was normal for the pleiotropic *srf* mutations *srf-4(ct109)*, *srf-8(dv38)*, and *srf-9(dv4)* (Link *et al.* 1992).

Rectal and peri-anal *M. nematophilum* infection of wild type is accompanied by anal swelling, but *srf-2*, *srf-3*, and *srf-5* mutants are resistant (bacterially unswollen; Bus) (Hodgkin *et al.* 2000; Hoflich *et al.* 2004; Gravato-Nobre *et al.* 2005). Because these *srf* mutants are both Bah and Bus, we tested other Bus mutants (Gravato-Nobre *et al.* 2005) for the Bah phenotype. *bus-2(e2687)*, *bus-4(e2693)*, *bus-12(e2740)*, and multiple alleles of *bus-17* were Bah. The *bus-12(e2740)* phenotype was somewhat leaky, as further described below. No difference between mutant and wild-type biofilm accumulation was observed for *bus-1(e2678)*, *bus-3(e2696)*, *bus-5(e2688)*, *bus-6(e2691)*, *bus-8(e2698)*, *bus-10(e2702)*, *bus-13(e2710)*, *bus-14(e2779)*, *bus-15(e2709)*, *bus-16(e2802)*, *bus-18(e2795)*, and *sur-2(e2706)*.

Screen for Bah mutants: We mutagenized strain N2 with ENU and screened the F_2 for Bah. Because the *Y. pseudotuberculosis* biofilm inhibits feeding, and therefore growth, candidate Bah mutants were easily identified as normally growing worms in a background of stunted animals. Biofilm formation requires nematode locomotion, and severely Unc mutants do not accumulate the material for reasons irrelevant to surface studies (Tan and Darby 2004). Accordingly, strong Unc mutants were ignored, although three mutants were recovered that have the milder Skd locomotion phenotype, as discussed below.

From ~7000 mutagenized genomes, 11 independent mutations were obtained (Table 2), all recessive and fully penetrant. In every case, the absence of biofilm was complete, with no visible matrix on the head or elsewhere. There were no growth defects observed on the standard *E. coli* OP50 food, and all mutants grew at the same rate on *Y. pseudotuberculosis* or *Y. pestis* as they did when fed OP50.

Chromosomal linkages were established using standard methods, and complementation tests were performed between mutants that mapped to the same chromosome. The 11 mutations fell into eight complementation groups. When tested in *M. nematophilum* assays, members of five complementation groups were Bus, and complementation tests against known *srf* and *bus* mutants showed that all five represented previously identified genes: *bus-4*, *bus-12*, *bus-17*, *srf-2*, and *srf-3*.

***bah-1* and *bah-2* mutants lack Srf and Bus phenotypes:** Mutants in three complementation groups were not Bus. The mutants were also not Srf in lectin assays; *i.e.*, they did not ectopically bind WGA. We named these genes *bah-1*, *bah-2*, and *bah-3*. In tests for antibody binding, *bah-1* and *bah-2* worms showed the wild-type phenotype; *i.e.*, only L1 larvae were bound by monoclonal antibody M37 (Table 2). *bah-3* worms, however, showed the constitutive larval display (Cld) phenotype, *i.e.*, binding of all four larval stages, as further described below.

bah-1 and *bah-3* are each linked to *dpy-5* on chromosome I. The genetic deficiency *hDf17* fails to complement *bah-1*, which localizes the gene to a region of ~2 MU. Three-factor mapping showed that *bah-3* is to the right of (or close to) *mom-5* and to the left of *vab-10* (data not shown), an interval of ~5 MU. Numerous genetic deficiencies have been described in this region, but

repeated attempts to identify one that fails to complement *bah-3* were unsuccessful. *bah-2* is on the left arm of chromosome IV, linked to *dpy-9*, a cuticle collagen gene. *bah-2* animals are not Dpy, *dpy-9* animals are not Bah, and *bah-2(br7)/dpy-9(e12)* heterozygotes are neither Bah nor Dpy, indicating that *bah-2* is not the same gene as *dpy-9*.

Relationship of Skd locomotion phenotype to biofilms: As noted above and shown in Figure 1, Yersinia biofilms disrupt *C. elegans* locomotion, causing the worms to skid on the bacterial lawn. *bah-1*, *bah-2*, *bah-3*, *bus-4*, and *srf-2* mutants have normal locomotion on Yersinia (Figure 1C and data not shown), indicating that the wild-type locomotion defect is related to the biofilm on the worm's head, rather than to some property of Yersinia that affects all genotypes. Further evidence for this is the observation that wild-type worms do not skid on mutant Yersinia that are unable to make biofilms (data not shown).

bus-17 mutants have a strong Skd phenotype, and *srf-3* animals a weak one, when grown on normal *E. coli* food (Gravato-Nobre *et al.* 2005). In our mutant collection, we observed strong Skd for *bus-17* (Figure 1D) and weak Skd for *srf-3(br6)* and *bus-12(br5)*. All Skd mutants retain the aberrant locomotion on Yersinia lawns. In contrast to our results, *bus-12(e2740)* was not reported to be Skd (Gravato-Nobre *et al.* 2005). When *bus-12(e2740)* was tested in Yersinia assays, small biofilms formed on the side of the head of some animals, but they grew normally since the mouth was not covered and feeding was unimpaired. The absence of Skd and the leaky Bah phenotype suggest that *bus-12(e2740)* is not a null mutation.

Stage-specific phenotypes of *bah-3* and *srf-6* mutants: Antibody M37 binds to L1 larvae but not to other stages of wild-type *C. elegans* under normal growth conditions (Politz *et al.* 1990; Hemmer *et al.* 1991). Alone among the mutants recovered in the Bah screen, *bah-1* and *bah-2* had the wild-type antibody phenotype (Table 2). All of the *bus* and *srf* mutants obtained did not bind antibody at L1 or any other stage. *bah-3* mutants bound antibody at all four larval stages (Cld). There was no corresponding stage specificity to their Bah phenotype, however: no biofilm attached at any stage.

Cld was first described for mutations in *srf-6* (Hemmer *et al.* 1991; Grenache *et al.* 1996). We tested each stage of *srf-6* mutants by growing them synchronously on *E. coli* OP50 and then transferring them to *Y. pseudotuberculosis*. Biofilms appeared on stages L2–L4, but as adults the mutants were strongly Bah (Figure 2). L1 phenotypes were not quantified because the animals' small size made definitive scoring problematic, but qualitatively it was apparent that most wild-type and *srf-6* L1's acquire biofilms.

To begin analysis of the genetic pathways in which *srf-6* and *bah-3* participate, we constructed a *bah-3(br9); srf-6(yj13)* double mutant. These animals were Bah at all stages, not merely as adults, indicating that *bah-3* is epistatic to *srf-6* for Bah phenotypes. Double mutants with *bah-1* or *bah-2* combined with *srf-6* were constructed, and these animals also were Bah at all stages.

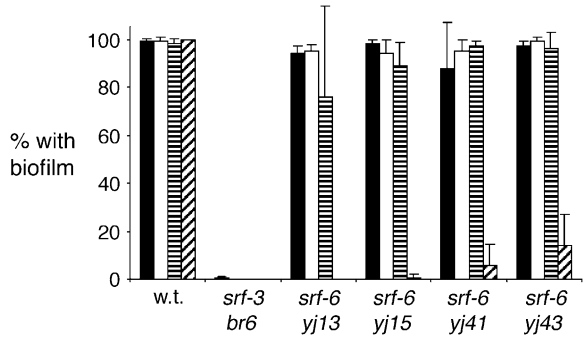

Figure 2.—Biofilm phenotype of *srf-6* mutants. Biofilm presence was scored 4 hr after transfer of animals to *Y. pseudotuberculosis* lawns. Solid bars, L2; open bars, L3; horizontal hatching, L4; diagonal hatching, adult. Data are mean and SD for at least three independent trials, with a minimum of 30 animals from each genotype and stage per trial. *srf-3(br6)* is a stage-independent Bah control.

Cuticle fragility of Bah mutants: Many Bus mutants have fragile cuticles, defined as increased sensitivity to alkaline hypochlorite treatment (Gravato-Nobre *et al.* 2005). We tested one allele of each gene found in the Bah screen, using a minor modification of the published protocol (materials and methods). In these experiments, 15 nematodes were immersed simultaneously in a drop of alkaline hypochlorite, and two times were recorded: the time required for the last of the worms to cease thrashing in the liquid and the time for the first of them to suffer a visible breach in its cuticle. *bah-3* and *srf-2* animals were indistinguishable from wild type (Table 3). *bah-1*, *bah-2*, *bus-4*, and *bus-12* mutants were slightly fragile, *srf-3* animals more so, and *bus-17* were by far the most sensitive. *bus-17* mutants ceased thrashing within the time required to release all worms from a wire pick, making exact measurement impossible. These results agree substantially with those reported for Bus mutants, the only discrepancy being with *bus-12*. We observed a mild fragility for *bus-12(br5)*, whereas *bus-12(e2740)* was reported to be similar to wild type (Gravato-Nobre *et al.* 2005). This is further evidence suggesting that *bus-12(e2740)* is not null.

Paternal effect of *bus-17* mutations: *C. elegans* has an XX/XO sex-determination system. Male progeny of crosses receive their single X chromosome from their hermaphrodite (functionally female) parent. Thus, male cross-progeny of a hermaphrodite with an X-linked mutation generally express the mutant phenotype. In initial crosses, we observed that some male progeny of *bus-17* hermaphrodites, expected to be Bah, were actually non-Bah if their fathers were wild type. This paternal effect was confirmed in crosses that allowed positive determination of progeny genotypes. Hermaphrodite

TABLE 3

Cuticle fragility of Bah mutants

Genotype	Mean longest thrash time (sec)	*N*	Mean time to first break (sec)	*N*	Fragility score
Wild type	48.7 ± 10.3	42	106.4 ± 43.2	54	+
bah-1(br1)	39.8 ± 8.6	23	83.3 ± 31.7	35	++
bah-2(br7)	41.3 ± 7.8	29	84.4 ± 31.8	39	++
bah-3(br9)	47.1 ± 10.1	26	103.3 ± 38.9	36	+
bus-4(br4)	39.5 ± 9.1	18	86.6 ± 23.4	18	++
bus-12(br5)	37.6 ± 4.9	21	81.9 ± 17.3	21	++
bus-17(br2)	<10	17	53.1 ± 10.7	18	++++
srf-2(br3)	44.1 ± 6.1	21	114.0 ± 56.1	21	+
srf-3(br6)	22.3 ± 5.7	22	73.7 ± 34.5	25	+++

Response to alkaline hypochlorite immersion as described in MATERIALS AND METHODS. Data are mean and SD for *N* trials that each contained 15 worms. Mutants with different fragility scores had significant differences (Student's *t*-test, $P < 0.05$) in pairwise comparisons for at least one assay, and in most cases for both.

parents carried the autosomal marker *dpy-5*, allowing cross-progeny to be identified by a non-Dpy phenotype. Because *bus-17* males mate with low efficiency, an ample supply of males was ensured by generating them with *him-5*, which produces a high frequency of self-progeny males by X chromosome nondisjunction in hermaphrodites. Crosses were done on standard *E. coli* plates using animals never exposed to Yersinia. Non-Dpy male progeny were transferred to *Y. pseudotuberculosis* as L4's or young adults and scored for biofilm attachment.

As shown in Table 4, male *bus-17* self-progeny of *him-5; bus-17* were invariably Bah. So, too, were male progeny of crosses in which both parents carried a mutant *bus-17* allele. However, numerous males with the identical genotypes, but whose fathers were *bus-17(+)*, were non-Bah, similar to the *him-5* control males. The paternal effect was also observed in crosses using wild-type male parents (data not shown), indicating that it is not related to the presence of *him-5*. There were no anomalies in the sex ratios of progeny, and in hermaphrodites, *bus-17* behaved as a conventional recessive mutation (data not shown).

Neither *bus-17* nor *dpy-5; bus-17* animals show the Him phenotype when they self-fertilize, so the aberrant males did not appear to be the result of X chromosome nondisjunction in the hermaphrodite parent and fertilization by an X-bearing paternal sperm. To confirm that the affected males were *bus-17* mutants, worms with biofilms were taken from Yersinia plates and briefly exposed to high pH to remove the biofilm (MATERIALS AND METHODS). Their genotypes were then determined by mating them individually to *dpy-5; bus-17(br2)*

TABLE 4

Paternal effect of *bus-17* mutations

Genotype of males tested	Paternal genotype	Maternal genotype	% with biofilm	*N*
him-5	None	*him-5*	70.8	511
him-5; bus-17(br2)	None	*him-5; bus-17(br2)*	0	384
him-5; bus-17(br11)	None	*him-5; bus-17(br11)*	0	494
him-5; bus-17(e2800)	None	*him-5; bus-17(e2800)*	0	399
dpy-5/+; him-5/+; bus-17(br2)/Ø	*him-5*	*dpy-5; bus-17(br2)*	48.5	241
	him-5; bus-17(br2)	*dpy-5; bus-17(br2)*	0	79
	him-5; bus-17(br11)	*dpy-5; bus-17(br2)*	0	92
	him-5; bus-17(e2800)	*dpy-5; bus-17(br2)*	0	74
dpy-5/+; him-5/+; bus-17(br11)/Ø	*him-5*	*dpy-5; bus-17(br11)*	33.5	176
	him-5; bus-17(br2)	*dpy-5; bus-17(br11)*	0	48
	him-5; bus-17(br11)	*dpy-5; bus-17(br11)*	0	32
	him-5; bus-17(e2800)	*dpy-5; bus-17(br11)*	0	90
dpy-5/+; him-5/+; bus-17(e2800)/Ø	*him-5*	*dpy-5; bus-17(e2800)*	29.7	118
	him-5; bus-17(br2)	*dpy-5; bus-17(2800)*	0	55
	him-5; bus-17(br11)	*dpy-5; bus-17(e2800)*	0	20
	him-5; bus-17(e2800)	*dpy-5; bus-17(e2800)*	0	53

"None" indicates male self-progeny of hermaphrodites carrying *him-5*, which confers a high frequency of spontaneous males. All *him-5* mutations are allele *e1490*; all *dpy-5* mutations are allele *e61*. Data are aggregates of assays conducted on at least three separate days.

hermaphrodites and assaying the non-Dpy cross-progeny on Yersinia plates. The recovery procedure was somewhat cumbersome, and the males mated poorly, so that results were obtained for only 11 worms. However, a total of 274 progeny were assayed and they were Bah without exception. Thus, the males on which biofilms appeared were in fact *bus-17* mutants, not worms that had obtained a paternal X chromosome by some reproductive aberration.

A potential explanation of the paternal effect is that during matings, *bus-17(+)* males secrete some molecule into the media, which then rescues their *bus-17* mutant progeny. To test this hypothesis, we placed *him-5*; *bus-17(+)* males on *E. coli*-seeded plates overnight, removed them, and then used the plates to cross *him-5; bus-17* males to *dpy-5; bus-17* hermaphrodites. None of the male progeny were rescued, suggesting that the paternal effect is related directly to mating rather than to a freely secreted molecule.

DISCUSSION

Intensive research on *C. elegans* has produced extraordinarily detailed pictures of many of the nematode's inner workings, but the same cannot be said of its outermost component. Despite the importance of the surface in the worm's interactions with the environment, it has been little characterized compared to much else about the animal. Although the collagenous inner layers of the *C. elegans* cuticle have been studied extensively, they do not contact the environment directly. The layers that do interact with the environment—the surface coat, and possibly the epicuticle beneath it—remain poorly understood. We have described a phenotype and genetic screen that allow identification of surface-determining genes that could not be found with previous assays.

The Bah phenotype identifies novel genes: A variety of methods have been used that identify *C. elegans* surface-related genes. The earliest studies used antibody binding and lectin binding to the surface of intact animals. *srf-1* was defined as an antigenic polymorphism between N2 and wild isolates of *C. elegans* (Politz *et al.* 1987). *srf-2* and *srf-3* were initially defined by ectopic binding of a polyclonal antibody that failed to bind wild-type adults (Politz *et al.* 1990). Subsequently, a different phenotype was observed in staining with monoclonal antibodies: these reagents recognized an epitope on the surface of wild-type L1 larvae that is absent from *srf-2* and *srf-3* animals (Hemmer *et al.* 1991). *srf-2* and *srf-3* worms also are bound ectopically by lectins that do not bind most of the wild-type surface, and this phenotype was used to identify an additional gene, *srf-5* (Link *et al.* 1992). Finally, *srf-6* was identified by the Cld phenotype, in which all larval stages display the M37 epitope that is normally L1 specific (Grenache *et al.* 1996).

Infection with *M. nematophilum* proved to be a highly productive tool for identifying additional surface-determining genes. Extensive screening for Bus mutants that are resistant to infection identified 15 new genes and also yielded alleles of *srf-2*, *srf-3*, and *srf-5* (Gravato-Nobre *et al.* 2005). This study also added to the methodological repertoire a simple assay for cuticle fragility, providing confirmation that cuticles are compromised in many of the mutants.

In this work, we used the Yersinia biofilm to obtain mutations in eight genes. In every case, the Bah phenotype was completely penetrant: no biofilm was observed on any worms. This could reflect a bias in screening because, under the conditions used, a reduction in biofilm, as opposed to complete absence, might have been difficult to observe. The screen was not saturated, as it produced only one allele each for five genes, and no alleles for two genes, *bus-2* and *srf-5*, that have Bah mutant phenotypes.

The Yersinia biofilm matrix binds the nematode surface, and there is no evidence that it interacts with underlying tissues (Darby *et al.* 2002; Tan and Darby 2004). Consistent with this, all five previously discovered genes that emerged in the screen (*srf-2*, *srf-3*, *bus-4*, *bus-12*, and *bus-17*) are known to have roles in surface determination. As discussed further below, four of these genes are in glycosylation pathways, indicating the importance of glycosylation for multiple surface phenotypes. (The fifth gene, *srf-2*, has not been cloned.)

The screen also identified three novel *bah* genes. *bah-1* and *bah-2* appear to be the most specific for biofilm attachment, since they lack other strong phenotypes. They could not have been found with previous screening methods, as the mutants have no phenotype in antibody, lectin, or *M. nematophilum* assays (Table 2). Although *bah-1* and *bah-2* mutants have somewhat fragile cuticles, the defects are in the mildest class observed. *bah-3* in principle could have been identified with antibody staining because of its Cld phenotype, but in fact was not. *bah-3* is unlike most other antibody-binding mutants (*bus-4*, *bus-12*, *bus-17*, and *srf-3*) in its absence of detectable cuticle fragility.

The Yersinia biofilm adheres primarily to the *C. elegans* head, although occasionally after prolonged incubations, matrix binds to more posterior surfaces. *M. nematophilum* exclusively colonizes the cuticle-lined rectum and the peri-anal cuticle. The difference between the pathogen binding sites is reflected in the mutants: 3 genes give Bah non-Bus mutants; 11 genes give Bus non-Bah. These findings imply that Yersinia and *M. nematophilum* adhere to *C. elegans* by way of distinct receptors and that the nematode expresses different molecules at different sites along the longitudinal axis.

Multiple genes with Bah mutant phenotypes are in glycosylation pathways: Glycosylation requires transporting nucleotide sugars from the cytoplasm, where

they are synthesized, to the Golgi or endoplasmic reticulum, where they are substrates of glycosyltransferases. Both nucleotide sugar transporters and glycosyltransferases are encoded by genes identified in the Bah screen. *srf-3* encodes a nucleotide sugar transporter with specificity for UDP-*N*-acetylglucosamine and UDP-galactose (Hoflich *et al.* 2004), while *bus-12* encodes a nucleotide sugar transporter of unknown specificity (J. Hodgkin, personal communication). Carbohydrate analysis confirmed that *srf-3* mutants have major reductions in galactosyl glycoconjugates (Cipollo *et al.* 2004). Both *bus-4* (J. Hodgkin, personal communication) and *bus-17* (Yook and Hodgkin 2007) encode glycosyltransferases.

Although *srf-3*, *bus-4*, *bus-12*, and *bus-17* are all in glycosylation pathways, the end-product glycoconjugates are not known, and therefore it is not clear how glycosylation defects lead to the Bah and Bus phenotypes. One straightforward possibility is that both Yersinia biofilms and *M. nematophilum* bind to glycan-containing receptors on the *C. elegans* surface and that these glycans are absent or aberrant in the mutants. However, it is also possible that the receptors themselves are not glycosylated, but require other glycan-containing molecules for their proper expression, localization, or function.

In the case of Srf, there is indirect evidence that the antigen recognized by monoclonal antibodies is an O-linked glycoprotein (Hemmer *et al.* 1991), and this is consistent with the finding of mucin-like glycoproteins in a variety of nematode species (Gems and Maizels 1996; Loukas *et al.* 2000; Theodoropoulos *et al.* 2001). Again, the simplest model is that glycosylation defects in *srf-3*, *bus-4*, *bus-12*, and *bus-17* mutants lead directly to loss or aberration of a glycan-containing epitope in the surface coat. However, surface coats are labile, and it is conceivable that this property permits environmental factors to reach the underlying epicuticle. Although we do not favor it, we cannot as yet exclude the possibility that either the biofilm receptor or the M37 epitope is in the epicuticle. Regardless of their locations, the biofilm receptor and the M37 epitope cannot be identical, since biofilms can attach to worms that do not display the epitope (wild-type L2–L4's and adults) and antibody can bind to Bah animals (*bah-1* and *bah-2* L1's and *bah-3* L1–L4's).

Evidence that *bah-3* is involved in surface regulation: *bah-3* animals are not Bus, have no detected cuticle fragility, and are not Srf in the lectin-binding assay. However, in the antibody assay, *bah-3* animals have the stage-specific Cld phenotype. Stage specificity does not extend to the biofilm phenotype: *bah-3* worms are Bah at every stage. Cld was first observed in *srf-6* mutants (Grenache *et al.* 1996), and we therefore tested these animals in biofilm assays. Here, too, *srf-6* mutations conferred a stage-dependent phenotype: the worms were wild type as larvae but Bah as adults (Figure 2). Curiously, the polarity of stage-specific defects is different for the two *srf-6* phenotypes. In the antibody assay, the worms show a mutant phenotype as L2–L4 larvae; in the biofilm assay, the mutant phenotype is only in adults. (No adult phenotypes can be discerned with M37, as the antibody has not been observed to bind adults of any genotype.)

The L1 specificity of M37 binding is dependent on environmental signals. Under standard *C. elegans* culture conditions, mutations are required to produce the Cld phenotype, but with certain additions to the media, wild-type L2–L4's display the epitope (inducible larval display, or Ild) (Grenache *et al.* 1996). Consistent with the involvement of environmental signals in determining the surface composition, it was recently shown that the Ild phenotype of wild type and the Cld phenotype of *srf-6* mutants require the chemotaxis genes *che-3*, *osm-3*, and *tax-4* (Olsen *et al.* 2007). Numerous chemotaxis genes are involved in formation of dauer larvae, and *srf-6* has been shown to interact with the dauer pathway (Grenache *et al.* 1996; Olsen *et al.* 2007). These results suggest that *srf-6* plays roles, likely regulatory, in the overlapping functions of chemotaxis, dauer formation, and surface determination. Because *bah-3* phenotypes overlap with those of *srf-6*, it seems probable that *bah-3* also participates in this complex regulatory network. As a first step toward determining its place, we performed epistasis analysis, which was possible because the mutations confer different Bah phenotypes. We found that *bah-3* (Bah at all stages) is epistatic to *srf-6* (Bah only as adult).

Paternal effect of *bus-17* mutations: *bus-17* mutants are unusual in several respects. Their cuticles are extremely fragile in the alkaline hypochlorite test (Gravato-Nobre *et al.* 2005) and their locomotion is markedly skiddy on an agar surface, even with normal *E. coli* food (Gravato-Nobre *et al.* 2005; Yook and Hodgkin 2007). A potential explanation of the Skd phenotype is that surface components that normally participate in locomotion, perhaps by making direct contact with the surface, are missing. Intriguingly, wild-type nematodes have the Skd phenotype on biofilm-producing lawns of Yersinia. A plausible hypothesis is that the Yersinia biofilm matrix binds to the locomotion components of wild-type worms, blocking their contact with agar and thus phenocopying *bus-17*. A similar phenomenon was reported for the plant-parasitic nematode *Meloidogyne javanica*, whose locomotion was altered by the binding of a surface-specific antibody (Sharon *et al.* 2002).

bus-17 mutations confer a paternal effect observable in male progeny. Only one paternal-effect gene, *spe-11*, has been described in *C. elegans*, but in that case a lethal phenotype was due to a direct requirement for sperm-produced protein in early embryogenesis (Hill *et al.* 1989; Browning and Strome 1996). In the case of *bus-17*, development is normal, and the only apparent defects are on the surface. The paternal rescue is incompletely

penetrant, but it persists to adulthood: a large fraction of adult *bus-17* males are non-Bah if their fathers were *bus-17(+)*. In contrast, Bah is completely penetrant for both sexes in true-breeding *bus-17* strains and in crosses in which both parents are *bus-17* mutants. It cannot be easily determined whether there is also a paternal effect for the Bus phenotype because of the difficulty of scoring Bus in males (J. Hodgkin, personal communication).

The paternal effect was not observed when mating plates were incubated with *bus-17(+)* males before mutant males were used in crosses, suggesting that the rescue is not the result of a freely secreted *bus-17*-dependent molecule. Rather, the effect appears to be related directly to mating. Since *C. elegans* males do not contribute an X chromosome to their male progeny, the effect cannot be genetic at the level of DNA. We hypothesize that a *bus-17*-dependent component of sperm or seminal fluid affects the phenotype of the male progeny.

Pathogenic microbes as specific probes for the *C. elegans* surface: Antibody staining, lectin staining, *M. nematophilum* infection, and Yersinia biofilms have identified more than two dozen genes with surface-determining functions. Even excluding those with pleiotropies that extend beyond cuticle and surface phenotypes (*srf-4*, *srf-8*, and *srf-9*), all of the genes identified by staining methods (*srf-2*, *srf-3*, *srf-5*, and *srf-6*) have multiple surface phenotypes. The mutants are all Bah (albeit restricted to adults for *srf-6*), and *srf-2*, *srf-3*, and *srf-5* are Bus. (*srf-6* has not been tested in *M. nematophilum* assays.) It therefore seems that antibody-staining phenotypes are biased toward mutations that cause far-reaching defects in the surface. Immunoblotting indicated that monoclonal antibodies recognized an epitope, probably a glycan, on more than one protein (Hemmer *et al.* 1991), a further indication that antibody phenotypes are unlikely to identify individual proteins of the surface coat.

Lectin-binding assays appear to have a similar limitation. WGA does not bind the wild-type surface (except the hermaphrodite vulva and male bursa), but ectopically binds the entire exterior of *srf-2*, *srf-3*, and *srf-5* worms. Fractionation experiments indicated that the WGA target is in the epicuticle, not on the surface coat (Silverman *et al.* 1997). The most parsimonious explanation is that the wild-type surface coat serves as a barrier that prevents lectins (and presumably other macromolecules) from reaching the epicuticle. Penetration of this barrier might occur only when mutations disrupt the surface coat extensively, *e.g.*, by affecting multiple components.

In contrast to these results with antibodies and lectins, screens using *M. nematophilum* and Yersinia have each recovered mutations with either no secondary phenotypes or only weak ones. Among those found using *M. nematophilum*, four—*bus-1*, *bus-3*, *bus-6*, and *bus-10*—have no phenotype in lectin binding or alkaline hypochlorite sensitivity (Gravato-Nobre *et al.* 2005), nor are they Bah. Of the three novel genes found with Yersinia biofilms, *bah-1* and *bah-2* do not have antibody, lectin, or Bus phenotypes, and they are only mildly sensitive to alkaline hypochlorite.

The two microbial infections also discriminate between positions on the anterior–posterior axis. Yersinia adheres copiously to the head but scantly or not at all elsewhere, while *M. nematophilum* attaches only to the cuticle-lined rectum and peri-anal cuticle. The screens for Bus and Bah each found mutations affecting only one of these infections, indicating that microbes are specific, high-resolution probes for genetic identification of nematode surface components.

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