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Multiple Genes Affect Sensitivity of *Caenorhabditis elegans* to the Bacterial Pathogen *Microbacterium nematophilum*

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ABSTRACT

Interactions with bacteria play a major role in immune responses, ecology, and evolution of all animals, but they have been neglected until recently in the case of *C. elegans*. We report a genetic investigation of the interaction of *C. elegans* with the nematode-specific pathogen *Microbacterium nematophilum*, which colonizes the rectum and causes distinctive tail swelling in its host. A total of 121 mutants with altered response to infection were isolated from selections or screens for a *b*acterially *u*n*s*wollen (Bus) phenotype, using both chemical and transposon mutagenesis. Some of these correspond to known genes, affecting either bacterial adhesion or colonization (*srf-2*, *srf-3*, *srf-5*) or host swelling response (*sur-2*, *egl-5*). Most mutants define 15 new genes (*bus-1*–*bus-6*, *bus-8*, *bus-10*, *bus-12*–*bus-18*). The majority of these mutants exhibit little or no rectal infection when challenged with the pathogen and are probably altered in surface properties such that the bacteria can no longer infect worms. A number have corresponding alterations in lectin staining and cuticle fragility. Most of the uninfectable mutants grow better than wild type in the presence of the pathogen, but the *sur-2* mutant is hypersensitive, indicating that the tail-swelling response is associated with a specific defense mechanism against this pathogen.

ALL multicellular organisms have to deal with bacterial pathogens of one kind or another and have evolved efficient defenses to survive in a world dominated, at least numerically, by potentially hostile prokaryotes. These defenses can be physical, providing mechanical barriers against invasion, or biological, in the form of antimicrobial compounds and dedicated phagocytic cells. Vertebrates have evolved sophisticated protection in the form of adaptive immunity, but most animal and plant species rely only on innate immune mechanisms, which are increasingly recognized as important and universal. Innate immune defenses can be studied effectively in model organisms such as Drosophila and *Caenorhabditis elegans* (Pradel and Ewbank 2004). For example, research on the Toll receptor in Drosophila was important in revealing the apparently conserved role of this receptor molecule in activating innate immunity. Recently, *C. elegans* has also become increasingly studied from the perspective of innate immunity (for recent reviews, see Millet and Ewbank 2004; Schulenburg *et al.* 2004; Gravato-Nobre and Hodgkin 2005; Sifri *et al.* 2005).

C. elegans is a species with a global distribution that lives by eating bacteria, so it is likely to encounter a very wide variety of bacteria in its diet and daily life. Few of these different bacterial species seem to have any deleterious effect on the worm, suggesting that it has effective means of protection. Recent work has identified a variety of different defense responses in the worm (Darby *et al.* 1999; Kim *et al.* 2002, 2004; Huffman *et al.* 2004; Nicholas and Hodgkin 2004). Thus far, most of the bacteria that have been examined with respect to pathogenic or toxic effects on *C. elegans* have been microbes known to have damaging effects on a variety of different metazoan organisms, such as *Bacillus thuringiensis*, *Pseudomonas aeruginosa, Salmonella enterica*, *Serratia marcescens*, and *Staphylococcus aureus* (Mahajan-Miklos *et al.* 1999; Aballay *et al.* 2000; Labrousse *et al.* 2000; Marroquin *et al.* 2000; Couillault and Ewbank 2002; Mallo *et al.* 2002; Sifri *et al.* 2003). Such broad-spectrum pathogens are likely to elicit relatively nonspecific defense mechanisms, for example, generalized responses to infection and stress. In contrast, bacteria with a narrow host range, which have evolved specialized mechanisms to infect *C. elegans*, may elicit separate kinds of response, with more dedicated roles in protecting the host from damage. Moreover, pathogens targeted to a restricted number of species may be more frequently associated with host-parasite co-evolution, with concomitant specialization and diversity in mechanisms of attack and defense. Schulenburg and Ewbank (2004) have provided evidence that coevolution can also occur

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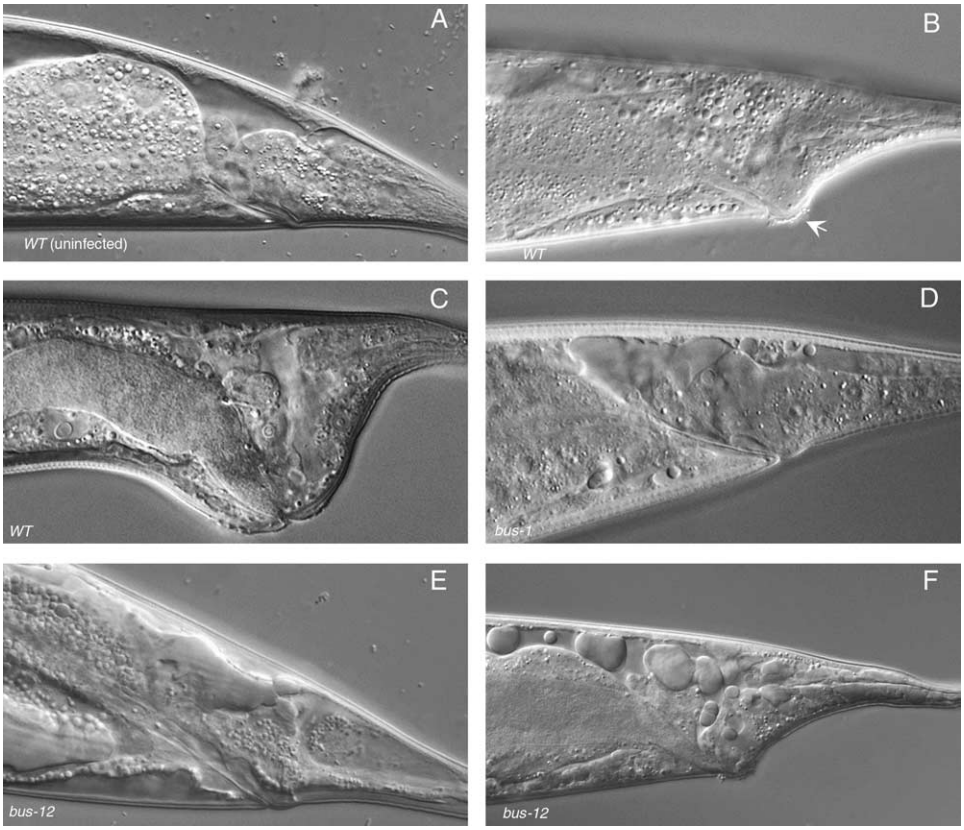

Figure 1.—Infection of wild-type and resistant worms. A–F show the tail region of adult hermaphrodites. (A) Uninfected wild type. (B) Infected wild type with weak Dar phenotype. Adherent bacteria are visible (arrow). (C) Infected wild type with strong Dar phenotype. (D) Infected *bus-1*, Bus phenotype. (E) Infected *bus-12*, Bus phenotype. (F) Infected *bus-12* with weak Dar phenotype, seen in a minority of animals.

between *C. elegans* and a broad-spectrum pathogen, *Serratia marcescens*. In general, however, it seems likely that detailed examination of interactions with specialist pathogens will reveal phenomena different from those involved in responses to generalist pathogens.

One pathogen of *C. elegans* with an apparently narrow host range among nematodes, and also a very unusual mode of infection and pathology, is the recently discovered coryneform species *Microbacterium nematophilum* (Hodgkin *et al.* 2000). This pathogen has been isolated on at least four independent occasions as a chance contaminant of laboratory cultures of nematodes, as a result of the striking morphological deformation that it induces in the tail of infected worms (Hodgkin *et al.* 2000, T. Akimkina, K. J. Yook, S. Curnock and J. Hodgkin, unpublished results). The bacterium is slow growing, so its presence in the lawns of *Escherichia coli* used for *C. elegans* culture is not easily noted. Nevertheless, it is able to infect *C. elegans* and establish a colony in the rectum of the animal, with cells adhering tightly to the rectal and post-anal cuticle. In response to the infection, the anal region of the worm becomes greatly enlarged, creating a distorted morphology that is easily scored by dissecting microscope (the deformed anal region, or Dar phenotype, illustrated in Figure 1). Worms become somewhat constipated as a result of the infection, and grow more slowly, but they do not generally experience more deleterious effects.

In the initial investigation of this phenomenon, it was not obvious whether the tail-swelling represents part of a defense reaction of the worm or is a deformation induced by the pathogen for its own benefit. Further analysis (Nicholas and Hodgkin 2004) has shown that the tail-swelling results from local activation of an ERK MAP kinase cascade and is associated with protection against the pathogen. When elements of this signaling cascade are inhibited, either by mutation or chemically, the pathogen is able to colonize the rectum but no swelling ensues. Mutants with defects in ERK MAP kinase signaling are hypersensitive to the pathogen, experiencing extreme constipation together with drastically reduced viability and fertility after infection. It therefore appears that the worm has evolved a specialized defense against attack by *M. nematophilum*. Different MAP kinase pathways have recently been implicated in protecting *C. elegans* against other kinds of bacterial infection or toxins (Kim *et al.* 2002, 2004; Huffman *et al.* 2004), but the ERK MAP kinase pathway appears to be specific for rectal infection.

Many questions remain to be answered about this host-parasite interaction, such as: How is the pathogen able to colonize the worm? What mechanisms are involved in its adherence to host cuticle? And with respect to the host side of the interaction: How does the host detect the presence of the pathogen? What cellular machinery is involved in the swelling reaction and other aspects of the defense response? What other factors limit the extent and consequences of infection?

To address these questions, and to explore the host-pathogen interaction further, we set out to isolate and

investigate *C. elegans* mutants with resistance or other alterations in response to infection by *M. nematophilum*. We found that resistant mutants can be efficiently selected, and we have isolated and studied a large number. This article provides a report on the initial characterization of these mutants, which represent a substantial number of both known and previously uncharacterized loci. In initial work on the infection (Hodgkin *et al.* 2000), it was observed that mutations in three known genes affecting surface antigenicity (*srf-2*, *-3*, *-5*) could confer resistance to *M. nematophilum*, so it was not surprising that new mutations in all three of these genes were recovered, but the majority of mutants represent genes with no previously reported mutant phenotype. Analysis of the biotic interactions between host and pathogen may reveal functions for many host genes for which knockout or knockdown has no obvious consequence in the absence of challenge by disease or other stress.

MATERIALS AND METHODS

Culture conditions: Procedures for growth and manipulation of *C. elegans* were as published (Brenner 1974; Sulston and Hodgkin 1988). Experiments were carried out at 20°–22° unless otherwise noted. Mutagenesis with 0.05 m ethyl methanesulfonate (EMS) was carried out as described by Brenner (1974).

Genetic markers: The following standard mutations were used in this study and are listed in Hodgkin (1997) and on WormBase (http://www.wormbase.org/), unless otherwise indicated. Bristol N2 was used as wild type.

LGI: *dpy-5(e61)*, *unc-13(e51)*, *srf-2(yj262)*, *spe-9(hc52ts)*, *sur-2(ku9)*
LGII: *bli-1(e769)*
LGIII: *egl-5(n486)*, *unc-32(e189)*, *mut-7(pk204)*
LGIV: *dpy-9(e12)*, *egl-4(n478)*, *dpy-13(e184)*, *unc-5(e53)*, *unc-24 (e138)*, *him-8(e1489)*, *dpy-20(e1282)*, *him-6(e1423)*, *srf-3(yj10)*, *unc-30(e191)*, *dpy-4(e1166)*
LGV: *dpy-11(e224)*, *unc-42(e270)*, *him-5(e1490)*, *lon-3(e2175)*, *fog-2(q71)*, *unc-51(e369)*
LGX: *unc-2(e55)*, *lon-2(e678)*, *unc-97(su110)*, *dpy-8(e130)*, *srf-5(ct115)*, *unc-7(e5)*

The multiply marked strain DA438 [*bli-4(e937) I; rol-6(e187) II; daf-2(e1368*ts*) vab-7(e1562) III; unc-31(e928) IV; dpy-11(e224) V; lon-2(e678) X*], constructed by L. Avery, was used for some mapping and construction.

Assays: For convenience, we adopted a standard procedure for preparing infective lawns: both *E. coli* strain OP50 and *M. nematophilum* strain CBX102 were grown to stationary phase in Luria-Bertani broth and mixed at a volumetric ratio of 9:1. Infection also occurs at much lower proportions of the pathogen, but a 10% mixture was used to ensure that infection was rapid and reliable. The mixture was used to seed lawns on standard NGM agar media (Brenner 1974). These lawns are referred to as mixed bacterial lawns (MBLs). The ratio of *E. coli* to *M. nematophilum* in the mixed lawns is unlikely to be constant with time for at least two reasons: initial doubling time is much lower for *M. nematophilum* than for *E. coli*, but *M. nematophilum* cells are able to survive better in stationary phase and continue to divide in the mixed lawns. Consequently, their proportion gradually rises. However, no great variability was observed in the response of worms to lawns of different ages. Pure lawns of *M. nematophilum* were not used in most of this work, because after a few days of incubation they become extremely sticky and barely edible even by resistant worms. Staining adherent bacteria with SYTO 13 is described in Nicholas and Hodgkin (2004).

Lectin staining: A total of 30–60 adult worms and larvae were picked and washed twice for 5 min in 1 ml of PBS-T (phosphate-buffered saline, 0.5% Triton X-100). Worms were incubated in 30 μl of 50 μg/ml fluorescein or rhodamine-conjugated lectin for 45 min at 25° and then washed three times for 10 min in 1 ml of PBS-T. Stained worms were anesthetized in 30 μl 0.5% phenoxypropanol; excess anesthetic was then removed and 10 μl of PBS-citifluor (Agar Scientific) was added to reduce fading before mounting on agarose pads and viewing with appropriate filters. Six fluorescent lectins (supplied by Sigma, St. Louis) with different specificities were used: wheat germ agglutinin (WGA), soy bean agglutinin (SBA), *Tetragonolobus purpureas* agglutinin (TPA), peanut lectin (PEA), concanavalin A (CON), and *Lycopersicon esculentum*, tomato lectin (LEA).

Testing bleach sensitivity: For each test, a 10-μl drop of freshly prepared alkaline hypochlorite solution [1 n NaOH, 40% NaOCl solution (>12% available chlorine)] was placed on NGM agar, and 15 adult hermaphrodites were immediately transferred together to the drop, using a platinum wire worm pick. Both the time taken before all worms stopped thrashing and the time taken for the first worms to begin to break up were noted. For wild-type worms, these times were usually 20–30 and 90–120 sec, respectively; for the most sensitive worms, <5 and <20 sec, respectively. Scores were assigned accordingly (+, wild type; ++++, most sensitive).

Analysis of mutants: All EMS-induced mutants were outcrossed at least once against wild type, and in most cases a high incidence of males (*him*) derivative strain was then constructed for convenience in further strain construction and mapping, using either *him-8 IV* or *him-5 V*. Difficulty in constructing either double mutant was an indication of linkage to either LGIV or LGV, which conveniently happens to be where most of the new genes identified in this study are located.

Mutants isolated from the *mut-7* strain NL917 (Ketting *et al.* 1999) were outcrossed more extensively to remove at least some of the extraneous transposon insertions that inevitably accumulate in this strain. The *mut-7* locus was explicitly removed by constructing double mutants with *unc-32* (tightly linked to *mut-7*) and then removing the *unc-32* marker by further crosses with wild type. This also served as a test for linkage to LGIII.

Mapping data for new genes: Linkage of all mutations was examined using the multiply marked strain DA438, permitting unambiguous assignment to a linkage group of all but a few *bus* mutations. Notably, *bus-14(e2779)* could not be easily located by this method and was therefore mapped using SNP markers, which demonstrated linkage to the right end of LGV. Three other mutations (*e2706*, *e2701*, *e2725*) gave initially misleading mapping results, for which reason they were at first assigned to distinct complementation groups (*bus-7*, *bus-9*, *bus-11*), but further characterization showed that *e2701* was a *bus-5* allele, and the other two were alleles of known genes [*sur-2(e2706)* (Nicholas and Hodgkin 2004) and *egl-5(e2725)* (H. R. Nichols and J. Hodgkin, unpublished results)]. Minimal data that define the approximate map positions of the 15 new genes identified in this study are given below. Where more than one allele was obtained for a given gene, most mapping results were obtained using only the reference allele for each gene; in a few cases confirmatory data were obtained with other alleles. These, plus more extensive additional map data, will be deposited in the public databases ACeDB and WormBase. Map positions are shown in Figure 2.

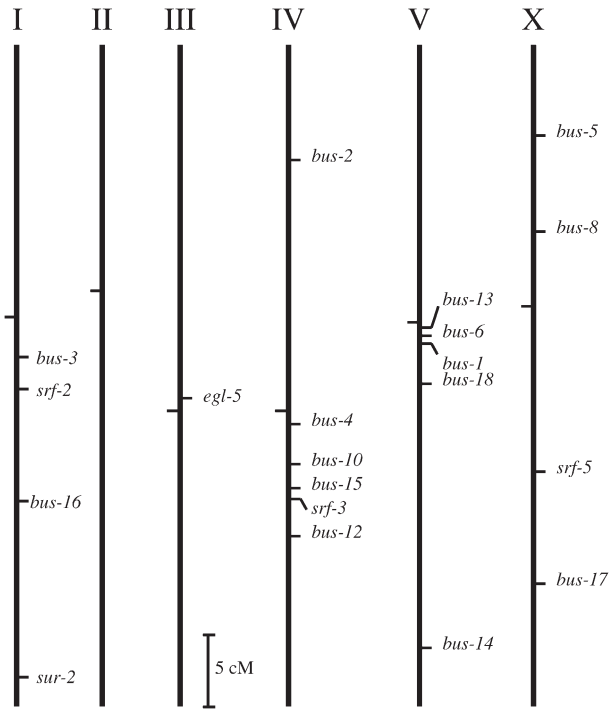

FIGURE 2.—Genetic map. Linkage groups (autosomes I–V and X chromosome) are shown in standard vertical format. The zero coordinate for each linkage group is shown as a bar to the left of each vertical line. Approximate positions of genes studied in this article (5 previously mapped, 15 new) are as shown.

bus-1: Dpy non-Unc and Unc non-Dpy recombinants picked from the progeny of *dpy-11* + *unc-42*/+ *bus-1(e2678)*+ gave the order *dpy-11* 4/6 *bus-1* 2/6 *unc-42*.

bus-2: Egl non-Dpy recombinants picked from the progeny of *dpy-9* + *egl-4*/+ *bus-2(e2687)*+ gave the order *dpy-9* 10/13 *bus-2* 3/13 *egl-4*.

bus-3: Dpy non-Unc and Unc non-Dpy recombinants picked from the progeny of *dpy-5 unc-13* +/+ + *bus-3* gave the order *dpy-5* 9/9 *unc-13* 0/9 *bus-3*.

bus-4: Dpy non-Unc and Unc non-Dpy recombinants picked from the progeny of *dpy-13 bus-4 unc-5*/+ + + gave the order *dpy-13* 37/57 *bus-4* 20/57 *unc-5*.

bus-5: Unc non-Dpy and Dpy non-Unc recombinants picked from the progeny of *unc-2* + *dpy-8*/+ *bus-5*+ gave the order *unc-2* 11/98 *bus-5* 87/98 *dpy-8*.

bus-6: Dpy non-Unc and Unc non-Dpy recombinants picked from the progeny of *dpy-11* + *unc-42*/+ *bus-6*+ gave the order *dpy-11* 7/16 *bus-6* 9/16 *unc-42*.

bus-8: Lon non-Unc recombinants picked from the progeny of *lon-2* + *unc-97*/+ *bus-8*+ gave the order *lon-2* 8/18 *bus-8* 10/18 *unc-97*.

bus-10: Unc non-Dpy and Dpy non-Unc recombinants picked from the progeny of *unc-24* + *dpy-20*/+ *bus-10*+ gave the order *unc-24* 7/8 *bus-10* 1/8 *dpy-20*.

bus-12: Unc non-Dpy recombinants picked from the progeny of *unc-30* + *dpy-4*/+ *bus-12*+ gave the order *unc-30* 1/8 *bus-12* 7/8 *dpy-4*.

bus-13: Dpy non-Unc and Unc non-Dpy recombinants picked from the progeny of *dpy-11* + *unc-42*/+ *bus-13*+ gave the order *dpy-11* 2/13 *bus-13* 11/13 *unc-42*.

*bus-14: B*acterially *u*n*s*wollen (Bus) non-Unc recombinants picked from the progeny of *bus-14 unc-51* +/+ + *fog-2* gave the order *bus-14* 7/7 *fog-2* 0/0 *unc-51*.

bus-15: Unc non-Dpy recombinants picked from the progeny of *dpy-20 him-6* + *unc-30*/+ + *bus-15*+ gave the order *dpy-20* 3/10 *him-6* 1/10 *bus-15* 6/10 *unc-30*.

bus-16: Bus non-Dpy recombinants picked from the progeny of *dpy-5* + *bus-16*/+ *spe-9*+ gave the order *dpy-5* 10/13 *spe-9* 3/13 *bus-16*.

bus-17: Lon non-Unc recombinants picked from the progeny of *lon-2* + *unc-7*/+ *bus-17*+ gave the order *lon-2* 11/12 *bus-17* 1/12 *unc-7*.

bus-18: Lon non-Unc recombinants picked from the progeny of *unc-42* + *lon-3*/+ *bus-18*+ gave the order *unc-42* 28/34 *bus-18* 6/34 *lon-3*.

RESULTS

Isolation of EMS-induced mutants resistant to infection by *M. nematophilum*: Wild-type worms of the standard N2 (Bristol) race of *C. elegans* can be infected by feeding on a mixed bacterial lawn containing *E. coli* OP50 and traces of *M. nematophilum*. They respond by developing a characteristic swollen tail, easily detected under the dissecting microscope, which we refer to as the Dar phenotype (Figure 1). Dar animals exhibit a slower growth rate and reduced fecundity (HODGKIN *et al.* 2000; HÖFLICH *et al.* 2004). In a survey of previously characterized *C. elegans* mutants, it was found that some of the *srf* mutants, originally isolated on the basis of altered surface antigenicity, were resistant to infection. Specifically, mutants of *srf-2*, *srf-3*, and *srf-5* did not develop swollen tails and exhibited little or no growth impairment in the presence of *M. nematophilum* (HODGKIN *et al.* 2000). We therefore sought additional mutants of this type by selecting for normal growth on mixed bacterial lawns and screening for absence of the Dar phenotype.

M. nematophilum does not normally kill its host, but the growth impairment it causes is sufficient to permit establishment of semiselective conditions for the isolation of resistant mutants. The following protocol was used for the isolation of most of the chemically induced mutants reported here: populations of *C. elegans* hermaphrodites were mutagenized with EMS and grown for two generations on *E. coli*. The population was then treated by bleaching with alkaline hypochlorite and the resulting eggs were allowed to hatch on a mixed lawn of *E. coli* and *M. nematophilum*. Under such conditions, hatched larvae normally all become infected by L2/L3 stage and develop a Dar (swollen-tail) phenotype. Rapidly growing worms were easily observed on these lawns after 2 days, and most of them did not have swollen tails. We refer to this as the Bus phenotype; normal tail anatomy is the most easily observed aspect of the resistance phenotype. Candidate resistant mutants were picked individually to mixed lawn plates to verify their phenotype. Further genetic characterization, summarized below, revealed that most of these mutants defined new genes, to which we have given the name *bus*.

A total of 37 independent resistant mutants were isolated from bulk screens of this nature. In addition,

TABLE 1

Isolation of mutants

Gene	EMS-induced alleles	*mut-7*-induced alleles	Totals
srf-2	*e2679*, *e2703*, *e2793*, *e2799*, *e2833*	*e2718*, *e2729*, *e2730*, *e2734*, *e2766*, *e2767*, *e2768*, *e2771*, *e2777*, *e2778*, *e2787*	5, 11
srf-3	*e2680*, *e2689*, *e2797*	*e2789*	3, 1
srf-5	*e2697*	—	1, 0
sur-2	*e2706*	—	1, 0
egl-5	—	*e2725*	0, 1
bus-1	*e2678*, *e2681*, *e2682*, *e2683*, *e2684*, *e2692*	*e2713*, *e2720*, *e2721*, *e2738*, *e2743*, *e2746*, *e2750*, *e2751*, *e2757*, *e2758*	6, 18
bus-2	*e2676*, *e2677*, *e2687* *e2705*	*e2776*, *e2780*, *e2781*	4, 3
bus-3	*e2696*	—	1, 0
bus-4	*e2693*, *e2700*, *e2803*	*e2752*, *e2788*	3, 2
bus-5	*e2685*, *e2686*, *e2688*, *e2699*, *e2701*, *e2704*, *e2794*, *e2801*	—	8, 0
bus-6	*e2690*, *e2691*	*e2728*, *e2756*, *e2759*, *e2772*, *e2790*	2, 5
bus-8	*e2698*		1, 0
bus-10	*e2694*, *e2702*	*e2714*, *e2715*, *e2716*, *e2717*, *e2719*, *e2722*, *e2723*, *e2724*, *e2731*, *e2732*, *e2733*, *e2736*, *e2737*, *e2742*, *e2745*, *e2753*	2, 31
bus-12	—	*e2740*	0, 1
bus-13	*e2710*	—	1, 0
bus-14	—	*e2779*	0, 1
bus-15	*e2709*	—	1, 0
bus-16	*e2802*	—	1, 0
bus-17	*e2695*, *e2800*	—	2, 0
bus-18	*e2795*	—	1, 0
		Total:	43, 74

Underlined alleles were chosen as representative for each locus and used in further characterization. Not all isolated alleles of *bus-1* and *bus-10* were retained and assigned allele numbers.

6 mutants were isolated from clonal screens in which individual worms from an F_2 population after mutagenesis were selfed for 1 day on *E. coli* and then transferred to mixed lawn plates. These screens were carried out with the intention of finding mutants with enhanced susceptibility to *M. nematophilum* (D. Whittington and J. Hodgkin, unpublished results), but the screens also yielded some apparently resistant mutants. Clonal screens of this type offer the opportunity of identifying mutants that have nonswollen tails in the presence of the pathogen, but are otherwise compromised in growth or resistance to other aspects of pathogenic attack. In contrast, the bulk screen is biased toward mutants with strong resistance and few pleiotropic defects. However, none of the 6 mutants isolated from the pilot clonal screens appeared initially to be very impaired in growth or movement.

Resistant mutants were identified at high frequency in these searches, suggesting that multiple different genes can be mutated to yield a Bus phenotype. On average, each mutagenized hermaphrodite yielded at least one resistant mutant in her F_2 progeny. In the bulk screens, often many *bus* mutants were recovered from the progeny of a given P_0, but, to ensure independence, only one was retained for each complementation group. The six independent mutants found in clonal screens were derived from 639 picked F_2 worms. Consistent with these high frequencies, it was found that the 43 mutants defined 17 different loci (Table 1).

Isolation of *mut-7*-induced resistant mutants: In addition to these searches for EMS-induced mutants, screens for resistant mutants were carried out using the mutator strain *mut-7*. This strain is deregulated for germline transposition of several different *C. elegans* transposons, so mutants isolated on this background are likely to result from transposon insertions in the relevant genes (Ketting *et al.* 1999). Transposon tagging of genes offers a potentially rapid route for their molecular identification, as opposed to conventional positional cloning (Collins *et al.* 1987). Mutation frequencies in *mut-7* populations are lower than those after EMS mutagenesis, and isolated mutants frequently do not breed true. Therefore, a different protocol was used for the isolation of resistant mutants from a *mut-7* strain. Individual hermaphrodites were picked from a *mut-7* population to separate plates spread with mixed lawns, and

their progeny were examined for the Dar phenotype. Almost all segregated only Dar animals in the first two generations, thereby establishing independent sensitive lines. These populations were then propagated for successive generations, passaging to fresh plates when the supply of bacterial food was exhausted. Plates were screened periodically for the appearance of many Bus animals. Populations grown in this way are under sustained selection for resistant mutants, because any Bus animal will have a growth advantage over its siblings, and its progeny will tend to take over the population. Sustained propagation of an unmutagenized wild-type population on mixed lawns resulted in the isolation of one spontaneous Bus mutant, demonstrating the effectiveness of long-term selection for resistance (this was found to be an allele of *bus-1*). Most *mut-7* populations eventually yielded a Bus mutant, with the exception of a few populations that died out in late passages, presumably as a result of accumulated extraneous mutations. The starting populations were all uniformly sensitive, so the different mutants represent independent events.

In the course of these screens, it became apparent that some loci appear to be hot spots (preferentially mutable loci) for *mut-7*-induced mutation to a Bus phenotype, in particular *bus-1* and *bus-10* (10 and 16 alleles, respectively, were isolated). Therefore, all stable mutants isolated from the *mut-7* screen were first complementation-tested against these two genes before further analysis. Eight additional *bus-1* strains and 15 additional *bus-10* strains were identified but not given allele numbers or retained for further analysis, as it seemed unlikely that they would provide further information.

The fact that two-thirds of the lines recovered from the *mut-7* screen carried alleles of either *bus-1* or *bus-10* suggests that these loci may be preferred targets for transposition. An alternative explanation is that these mutations represent frequent imprecise excisions from silent transposons already present in the two genes in the starting population of the mutator strain. However, this explanation can be excluded in the case of *bus-1*, because subsequent molecular analysis of this gene has shown that the 10 retained *mut-7* alleles all carry different insertion events of either Tc1 or Tc3 (M. J. Gravato-Nobre and J. Hodgkin, unpublished results). The *mut-7* line used in these experiments may also exhibit some preference for transposition into some chromosomal regions, because most (61/74) of the mutations recovered were on LGIV or LGV.

A total of 74 stable Bus strains were recovered from the mutator screen (Table 1). In addition, a number of lines exhibiting a weak or variable Bus phenotype were recovered, but these proved to be more difficult to analyze or map genetically and is not further discussed here.

Genetic characterization: Mutants were crossed at least once against wild type (more extensively in the case of *mut-7* alleles) and tested for chromosomal linkage. Almost all mutants were found to behave as simple Mendelian recessives. The only significant exception was the *sur-2* mutation *e2706*, which exhibited a marked maternal rescue effect: *e2706*/+ progeny of *e2706* homozygous mothers crossed with wild-type males exhibited a weak Bus phenotype.

Mutations were assigned to different loci by complementation testing with genes mapped to the same chromosome. Extensive complementation testing between alleles of the same gene and of different genes took place during the assignment of the 118 mutations to 20 loci, but almost no cases of intragenic complementation or, conversely, nonallelic noncomplementation, were observed. One case of anomalous noncomplementation was observed in that animals of genotype *bus-3(e2696)/+; bus-17(e2695)/+* are sometimes Bus, although the two loci concerned are unlinked.

For most of the nine genes with multiple independent mutations, all alleles appeared superficially similar in properties. Exceptions were a temperature-sensitive allele of *srf-3* (described in more detail by Höflich *et al.* 2004) and some alleles of *bus-5*, which exhibited distinctive differences in cuticle fragility, as described below; one *bus-5* allele was also weakly temperature-sensitive for the Bus phenotype.

Most loci (12/20) identified in this work are defined by a single mutation, and the number of mutations does not follow a Poisson distribution, even allowing for hot spots (preferentially mutable loci). It follows from this that these screens are far from saturated, so numerous further genes affecting sensitivity to *M. nematophilum* await discovery.

The spectrum of EMS mutants and *mut-7* mutants is substantially different: Both EMS and *mut-7* screens yielded multiple mutations in seven genes (*srf-2*, *srf-3*, *bus-1*, *bus-2*, *bus-4*, *bus-6*, *bus-10*) but most genes were identified in only one screen (Table 1). This indicates that the spectrum of mutants is significantly different between the two screens, even though neither has reached saturation. Such difference is scarcely surprising, given that transposon insertion is likely to produce either no phenotype (insertion into an intron) or an extreme loss-of-function phenotype. Weak viable mutations in essential genes are unlikely to be recovered after transposon mutagenesis and are more likely to be recovered after chemical mutagenesis. Genes such as *bus-5* (eight EMS alleles, no *mut-7* alleles) may fall into this class. The gene *bus-8*, defined by a single EMS allele, is demonstrably of this type, because a noncomplementation screen for further alleles yielded multiple additional mutations, some of which are lethal as homozygotes (J. Hodgkin, unpublished results).

Phenotypic characterization—growth in the absence of pathogen: Reference mutants for each of the 20 loci were grown on normal *E. coli* lawns and compared with wild type for differences in growth rate, viability, and fertility, as well as other phenotypic characteristics such as morphology and locomotion, with results

TABLE 2

Mutant characteristics and growth in the absence and presence of pathogen

Strain	Pure *E. coli*	Mixed lawn	Pure *M. nematophilum*
N2	Wild type	Dar	Dar
srf-2(yj262)	Srf	Bus	Bus
srf-3(yj10)	Srf, sl Skd	Bus	Bus
srf-5(ct115)	Srf, sl Gro	Bus	Bus
sur-2(e2706)	Egl-cs, Hin-cs	Bus (poor growth)	Bus (poor growth)
egl-5(e2725)	Egl, Unc	Bus	Bus
bus-1(e2678)	Wild type	Bus	Bus
bus-2(e2687)	Wild type	Bus	Bus
bus-3(e2696)	Sl Unc	Bus	Bus
bus-4(e2693)	Sl Sma	Bus	Bus
bus-5(e2701)	Sl Skd	Bus	Bus/sl Dar
bus-6(e2728)	Wild type	Bus	Bus
bus-8(e2698)	Sl Skd	Bus/v sl Dar	Bus/sl Dar
bus-10(e2702)	Wild type	Bus	Bus
bus-12(e2740)	Wild type	Bus/sl Dar	Bus/sl Dar
bus-13(e2710)	Rare Rup	Bus/v sl Dar	Bus/sl Dar
bus-14(e2779)	Wild type	Bus	Bus/sl Dar
bus-15(e2709)	Wild type	Bus	Bus/sl Dar
bus-16(e2802)	Skd, Rup	Bus	Bus
bus-17(e2800)	Skd	Bus	Bus
bus-18(e2795)	Sma, Skd, Gro, Rup	Bus/sl Dar	Bus/sl Dar

Skd, skiddy; Gro, slow growth; sl, slight; v sl, very slight.

summarized in Table 2. None showed obvious defects in growth rate or fertility, apart from *egl-5*, which showed a strong egg-laying defect and uncoordinated movement, like most other *egl-5* mutants. The *sur-2* mutant exhibited an egg-laying defect and some vulval induction abnormalities (a *h*yper*in*duced, or Hin, phenotype) at low temperatures, as reported elsewhere (Nicholas and Hodgkin 2004). One mutant, *bus-18*, exhibited slow growth and somewhat reduced body size. Slight and variable reduction in size was also noted in some of the other mutants (*bus-4*, *bus-13*, *bus-16*).

A number of mutants showed a distinctive movement defect, referred to as the "skiddy" phenotype, characterized by normal sinusoidal movement of the body but apparently poor traction on the agar surface. Consequently, animals make relatively little forward progress for each locomotory wave that passes down the body. The skiddy phenotype is conspicuous in *bus-16*, *bus-17*, and *bus-18* mutants and also detectable in *srf-3*, *bus-5*, and *bus-8*. It is correlated with cuticle abnormality, as described further below. Cuticular alae, which are believed to be important in providing traction, appear grossly normal in these mutants.

A more distinctive weak coiler uncoordinated phenotype was seen in *bus-3* animals. This phenotype cosegregated with the Bus phenotype during extensive crosses, so it is likely to be caused by the same mutation, but it is conceivable that it arises from a distinct tightly linked mutation. Comparison of multiple alleles would resolve this question, but only one *bus-3* allele has been identified thus far.

One mutant, *bus-16*, exhibited a phenotype of vulval rupture (Rup) at or soon after the last larval molt in ~40% of individuals. A similar phenotype was seen at lower penetrance (<10%) in some of the other mutants (*bus-13*, *bus-18*).

Male phenotypes: Male phenotypes were examined by constructing *him-5* or *him-8* strains for each of the 20 mutants. The *egl-5* mutant exhibited severely abnormal male tail development and was completely defective in male mating, consistent with its genotype. Other mutants did not show obvious alterations in male anatomy, and all were capable of mating, although in some cases less efficiently than wild type. The *bus-18* mutant males had very low mating efficiency, which can be explained by their relatively small size and poor movement.

Growth in the presence of pathogen: Strains were grown both on mixtures of *E. coli* and *M. nematophilum* (MBL plates) and on pure lawns of *M. nematophilum*, at 20° and 25°. As expected from the method of isolation, all mutants exhibited a complete or almost complete absence of tail swelling on mixed lawns, in contrast to the fully penetrant Dar phenotype of wild-type worms, and most mutants grew much more vigorously than wild type on such lawns, with the exception of *sur-2*. Occasional individuals of some strains, such as *bus-12*, exhibited a slight tail swelling (weak Dar phenotype), as indicated in Table 2 and illustrated in Figure 1, E and F.

Growth on pure lawns was slower than on mixed lawns in all cases. Most mutants grew more rapidly than wild type on pure lawns of the pathogen, even mutants with significant pleiotropic abnormalities such as *egl-5*, *bus-16*, and *bus-18*. A conspicuous exception, again, was the *sur-2* mutant, which exhibits very poor growth on pure *M. nematophilum*. In some, but not all, experiments, *bus-14* worms grew poorly relative to wild type. More mutants exhibited a leaky phenotype (variable weak tail swelling) on pure lawns than on mixed lawns, notably *bus-5*, *bus-8*, *bus-12*, *bus-13*, and *bus-15*.

Wild-type male worms are more severely affected than wild-type hermaphrodites by *M. nematophilum* (M. J. Gravato-Nobre and J. Hodgkin, unpublished results). The response of *bus* males to infection has not yet been systematically examined, but where tested, both sexes appear to be similarly resistant.

Tests for colonization by the pathogen: Worms exhibiting a Bus mutant phenotype might be unswollen because of a failure in the initial adherence of bacteria to the surface of the worm or because of an abnormality in the subsequent response to infection. To distinguish between these possibilities, wild-type and mutant worms were grown on mixed bacterial lawns, washed to remove

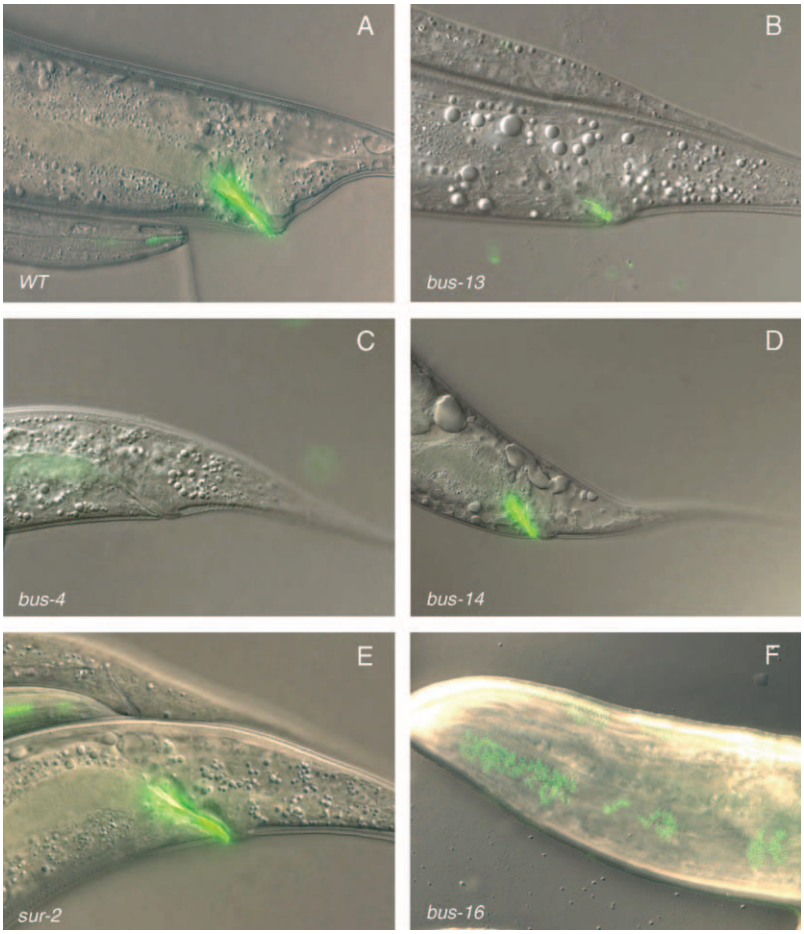

FIGURE 3.—Tests for colonization: SYTO 13 staining. A–E show the tail region of adult hermaphrodites after infection, extensive washing to remove *E. coli* cells, and brief staining with SYTO 13 to reveal infecting bacteria (green fluorescence). (A) Wild type: extensive rectal colonization and strong Dar phenotype. (B) *bus-13*: trace infection and very weak Dar response. (C) *bus-4*: no colonization and no Dar response. (D) *bus-14*: strong colonization and no Dar response. (E) *sur-2*: strong colonization and no Dar response. (F) Head region of adult *bus-16* hermaphrodite, showing patchy adherence of bacteria to the cuticle surface.

nonadhering *E. coli* and to complete digestion of ingested bacteria, and stained briefly with the fluorescent vital dye SYTO 13. This procedure allows visualization of adherent bacteria in the living worms. Wild-type worms infected with *M. nematophilum* exhibit strong rectal and post-anal fluorescence, reflecting extensive colonization of the rectum by the pathogen, whereas uninfected worms exhibit no fluorescence (Figure 3).

Representative alleles of 20 different mutants were examined by this procedure, with results summarized in Table 3 and Figure 3. The mutants fall roughly into four groups: 3 mutants (*egl-5*, *sur-2*, *bus-14*) showed strong rectal fluorescence, comparable to wild type, which suggests that these mutants are impaired in response to the pathogen, rather than to the initial infection. Two mutants (*bus-12*, *bus-15*) exhibited substantial rectal infection, but less than wild type, suggesting that both infection and response are reduced. Six mutants showed very low or variable rectal fluorescence (*srf-3*, *bus-2*, *bus-3*, *bus-5*, *bus-8*, *bus-13*), suggesting that some bacterial adherence can occur, but not enough to establish a full infection or elicit a strong response. The remaining 9 mutants exhibited essentially no rectal fluorescence, indicating that no infection had occurred. The most likely interpretation of the last two classes is that these mutants have alterations in rectal cuticle properties that prevent or impair the initial adherence by infecting bacteria. Additional and more general alterations in the cuticle properties of some of these mutants are also suggested by the observation that several mutants (*srf-3*, *srf-5*, *bus-3*, *bus-12*, *bus-13*, *bus-14*, *bus-16*, *bus-18*) exhibit occasional patches of bacterial adherence elsewhere on the outside of the worms, which are never seen in wild-type worms; an example is shown in Figure 3F (*bus-16*). One mutant, *bus-12*, sometimes exhibited an unusual pattern of strong ectopic bacterial attachment, but only in adult males.

Lectin staining: The data in the previous section suggest that the majority of mutants reported here are resistant to *M. nematophilum* because the bacteria are unable to attach to the rectal or anal cuticle and therefore cannot initiate an infection. This failure to adhere could be due to altered surface properties of the worm cuticle, either locally or over the whole body. Such alterations have already been demonstrated in the case of the three *srf* mutants (LINK *et al.* 1992), which show changes in the binding of both antibodies and labeled lectins to the surface of the worm. We therefore examined lectin-binding properties of the mutants isolated in this work, testing one or two alleles for each of the 20 loci identified as affecting susceptibility to *M. nematophilum*. Six lectins were tested, as described in more detail in MATERIALS AND METHODS.

TABLE 3

Tests for rectal colonization by vital dye staining for bacteria

Strain	Rectal staining	Other staining	Comment
N2	+++	0	
srf-2(yj262)	0	0	
srf-3(yj10)	+	++	Some general surface staining
srf-5(ct115)	0	+	Some alae staining
sur-2(e2706)	+++	0	
egl-5(e2725)	+++	0	
bus-1(e2678)	0	0	
bus-2(e2687)	+	0	
bus-3(e2606)	+	+	
bus-4(e2693)	0	0	
bus-5(e2701)	+	0	Rare rectal staining
bus-6(e2728)	0	0	Occasional weak staining
bus-8(e2698)	+	0	
bus-10(e2702)	0	0	
bus-12(e2740)	++	+	Abnormal male staining
bus-13(e2716)	+	+	Variable staining
bus-14(e2779)	+++	+	
bus-15(e2709)	++	0	
bus-16(e2802)	0	+	Patches on cuticle
bus-17(e2800)	0	0	
bus-18(e2795)	0	+	Pre- and post-anal patches

0, no bacterial staining; +, detectable staining; ++, some staining; +++, strong staining.

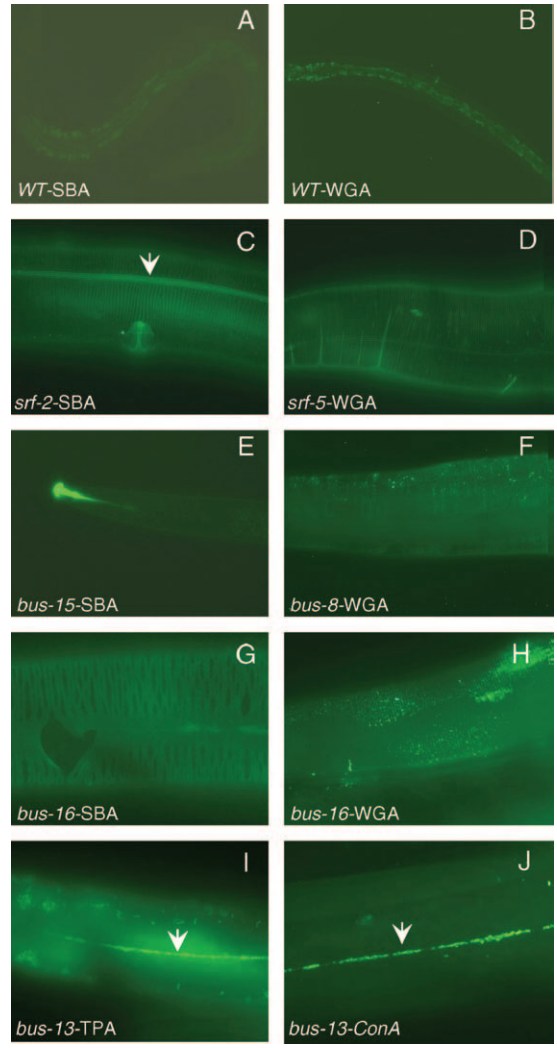

FIGURE 4.—Lectin staining. (A and B) Wild-type worms after staining with SBA and WGA, respectively; long exposure. No surface staining is seen, only faint background autofluorescence from the gut. (C) SBA-stained *srf-2*, vulval region: strong surface staining, especially of vulva and alae. (D) WGA-stained *srf-5*: strong staining. (E) SBA-stained *bus-15*: specific staining of nose. (F) WGA-stained *bus-8*: patchy surface staining. (G) SBA-stained *bus-16* showing disintegration of outer cuticle. (H) WGA-stained *bus-16*: strong patchy surface staining. (I) TPA-stained *bus-13*: alae staining. (J) CON-stained *bus-13*: alae staining. In C, I, and J, alae are indicated with an arrow.

As expected from previous observations (LINK *et al.* 1992), *srf-2* and *srf-5* adult mutants exhibited stronger binding of WGA and SBA, as compared to wild type. None of the other mutants shared exactly this phenotype, and none exhibited consistently strong staining with any of the other four lectins. However, distinctive patterns of staining were observed with a number of the remaining mutants. Several mutants exhibited increased but variable and patchy staining, most notably with SBA but sometimes also with other lectins. This phenotype is characteristic of *srf-3*, but a similar and often stronger phenotype was seen with *bus-8*, *bus-16*, *bus-17*, and *bus-18*. One of two tested alleles of *bus-5* showed this phenotype (*e2701*) but the other did not (*e2688*). This pattern of lectin staining is correlated with cuticle fragility, as described below. The fragility was sometimes also directly apparent in these staining experiments, because the outer layer of the adult cuticle tended to disintegrate during the process of mounting the worms for microscopy and was sometimes sloughed off (Figure 4G).

Three mutants (*bus-13*, *bus-14*, *bus-15*) had other patterns of abnormal lectin staining. The *bus-13* mutant exhibited increased but variable staining by most of the six lectins tested (all but WGA), with staining tending to be strongest along the alae (lateral ridges that run along the sides of the animal), as illustrated in Figure 4, I and J. It is possible that this effect results from nonspecific trapping of lectins on the cuticle surface, rather than from a more specific interaction, but it still indicates that the surface of *bus-13* worms is significantly different from wild type. Similarly, some *bus-14* mutant animals exhibited increased and patchy staining with SBA, PEA, and LEA lectins, but the penetrance of this phenotype was low. Finally, *bus-15* mutants could be labeled with SBA in the head, although the rest of the body was unstained (Figure 4E).

TABLE 4

Lectin staining

Strain	WGA	SBA	TPA	PEA	CON	LEA
N2	−	−	−	−	−	−
srf-2(yj262)	++	++	−	−	−	−
srf-3(yj10)	V	V	V	V	−	V
srf-5(ct115)	++	++	−	−	−	−
egl-5(e2725)	−	−	−	−	−	−
sur-2(e2706)	−	−	−	−	−	−
bus-1(e2678)	−	−	−	−	−	−
bus-2(e2687)	−	−	−	−	−	NE
bus-3(e2606)	−	−	−	−	−	−
bus-4(e2693)	−	−	−	−	−	−
bus-5(e2688)	−	−	−	−	−	−
bus-5(e2701)	V	−	−	−	−	V
bus-6(e2728)	−	−	−	−	−	−
bus-8(e2698)	V	V	−	V	V	V
bus-10(e2702)	−	−	−	−	−	−
bus-12(e2740)	−	−	−	−	V	−
bus-13(e2716)	−	A	A	A	A	A
bus-14(e2779)	−	V	−	V	−	V
bus-15(e2709)	H	H	−	−	−	NE
bus-16(e2802)	V	V	−	V	V	V
bus-17(e2800)	−	V	−	V	V	NE
bus-18(e2795)	−	V	−	V	V	−

The six lectins used are described in MATERIALS AND METHODS. −, no staining; ++, strong staining. V, variable, patchy staining; A, variable, mainly alae staining; H, variable, nose/head staining; NE, not examined.

TABLE 5

Cuticle fragility

Strain	Bleach sensitivity	Comment
N2	+	
srf-2(yj262)	+	
srf-3(yj10)	+++	Poor long-term survival
srf-5(ct115)	+	
egl-5(e2725)	+	
sur-2(e2706)	+	
bus-1(e2678)	+	
bus-2(e2687)	++	
bus-3(e2606)	+	
bus-4(e2693)	++	
bus-5(e2688)	++	
bus-5(e2701)	++++	Poor long-term survival
bus-6(e2728)	+	
bus-8(e2698)	+++	
bus-10(e2702)	+	
bus-12(e2740)	+	
bus-13(e2716)	++	
bus-14(e2779)	+	
bus-15(e2709)	+	
bus-16(e2802)	++++	Poor long-term survival
bus-17(e2800)	++++	Poor long-term survival
bus-18(e2795)	++++	Poor long-term survival

For scoring, see MATERIALS AND METHODS.

Larvae of these strains were also examined after staining with the same set of six lectins, and some further differences from wild-type staining were observed, most notably increased staining of *bus-12* larvae by WGA. Also, both WGA and SBA bind weakly to the cuticle of wild-type larvae, and some mutants (*bus-1*, *bus-4*, *bus-15*) showed a possible reduction of binding of these two lectins in larvae. Observations are summarized in Table 4.

Altered surface properties in some of these mutants may also explain the abnormal skiddy movement phenotype that they exhibit, described above. Skiddiness is seen to a greater or lesser degree in many of the mutants with lectin-staining phenotypes.

Cuticle fragility: Several other phenotypes, in addition to the lectin-staining data described above, indicated that some of these mutants are defective in cuticle integrity. It has been reported that dauer larvae of *srf-3* mutants are less resistant to detergent treatment. We found that some of the *bus* mutants exhibited notably poor survival on starved or desiccated plates. The vast majority of *C. elegans* mutants remain viable for weeks on standard NGM agar plates, long after bacterial food has been exhausted, and even when the plate has dried down to half or less of its original volume. Worms usually recover rapidly after "chunk transfer" to a fresh plate with a bacterial lawn (SULSTON and HODGKIN 1988). In contrast, some of the *bus* and *srf* mutants died out under desiccation conditions.

The reduced survival of these strains was correlated with increased sensitivity to alkaline hypochlorite treatment. The standard bleach sterilization procedure for cleaning contaminated worm stocks involves picking a few gravid hermaphrodites to a drop of alkaline hypochlorite (see MATERIALS AND METHODS). Worms rapidly stop moving and subsequently break up and dissolve, leaving behind only debris and surface-sterilized eggs. Bleach-sensitive worms exhibit a markedly weaker resistance to this treatment: on exposure to alkaline hypochlorite they stop moving almost instantly and start to disintegrate much faster than wild-type worms. The time in seconds to breakup provides a convenient measure of bleach sensitivity and allows the mutants to be grouped into four classes (Table 5). About half of the mutants showed little or no difference from wild type, but the remainder were distinctly more sensitive: mildly (*bus-2*, *bus-4*, *bus-13*), strongly (*srf-3*, *bus-8*), or very strongly (*bus-5*, *bus-16*, *bus-17*, *bus-18*).

Where tested, different alleles of a given gene usually exhibited similar sensitivity, but alleles of *bus-5* were strikingly heterogeneous: four alleles were hypersensitive (*e2685*, *e2701*, *e2704*, *e2801*) whereas four were only

slightly more sensitive than wild type (*e2686*, *e2688*, *e2699*, *e2794*). All eight showed similar resistance to *M. nematophilum* infection, however.

To explore the possibility of more rapid permeation of drugs into the animal, drug sensitivity of some mutants was also examined. Consistent with this, *bus-16* and *bus-18* mutants were found to be unable to grow on plates containing concentrations of emetine or colchicine (0.5–1 mM) that did not prevent growth of wild-type worms. However, no direct measurements of drug uptake or cuticle permeability have yet been made.

Further evidence of cuticle fragility was provided by making double mutants with *bli-1(e769)*. This and other *bli* mutants exhibit fluid-filled blisters on the surface of the cuticle at the adult stage, which has a double-layered cuticle and can therefore become blistered by fluid accumulation between the cuticle layers. Double mutants such as *bus-16; bli-1* were found to develop only small blisters, presumably because larger blisters rupture as a result of weakness in the outer cuticle layer. This phenotype correlates with the disintegration and sloughing of the cuticle seen in some mutants after lectin staining (*bus-8*, *bus-16*, *bus-17*, *bus-18*).

The Rup phenotype noted in some of the cuticle mutants, notably *bus-16*, may also result from cuticle fragility, but this phenotype seems to occur at high frequency only in *bus-16* and is not obviously correlated with the relative fragility of different mutants.

DISCUSSION

This article reports the results of an initial survey for mutants altered in response to infection by *M. nematophilum*. We have shown that it is easy to find such mutants and that a large number of genes are involved.

The majority of mutants were isolated by selection both for resistance to the debilitating effects of the pathogen and for absence of the distinctive swollen-tail phenotype caused by infection. Many of these mutants appear to be resistant because the bacterium is unable to adhere to the cuticle and initiate an infection. For nematodes, the cuticle represents the first and most general line of defense against bacterial attack, and its surface properties are certain to be critical in determining whether many different bacteria are able to act as pathogens or otherwise interact with a host species.

A few mutants that are able to permit the establishment of infection, but lack the swelling response, were obtained. However, it appears likely that most such mutants are also hypersensitive to the pathogen, as illustrated by the *sur-2* mutant (Nicholas and Hodgkin 2004; this study), and therefore are not directly selectable. The *egl-5* mutant is anomalous in this regard in that its growth is relatively unimpaired by the pathogen despite rectal infection, but this may be a consequence of the multiple alterations caused by defects in this gene, which encodes a Hox protein. Further mutants defective in response are being sought in clonal screens such as the one that yielded the *sur-2* mutant, rather than in direct selections.

Direct selections for resistant mutants do not appear to be saturated, as noted in the text, even though 15 new loci have been identified thus far. Different selection protocols and different mutagens will probably yield yet more classes of mutants; indeed, experiments using Mos transposon mutagenesis (Bessereau *et al.* 2001) have already defined further *bus* genes (K. J. Yook and J. Hodgkin, unpublished results). It is also likely that a large class of weakly resistant mutants exist, which exhibit a reduced or variable response to *M. nematophilum*, distinguishable both from the standard Dar phenotype exhibited by wild-type worms and from the robust Bus phenotype of the mutants described here. Some mutants of this type were recovered from the *mut-7* screen, but they have not yet been further investigated. Such weak mutants are necessarily harder to work with, but may be informative about other aspects of the biology of this host-pathogen interaction.

The mutants studied so far can be tentatively placed in different groups on the basis of the different properties set out in Tables 3–5. For example, mutations of *srf-3*, *bus-5*, *bus-8*, *bus-16*, *bus-17*, *bus-18* all result in rather similar phenotypes, with no rectal colonization, increased lectin staining, cuticle fragility, and skiddy movement. However, in the absence of further information about their properties, it would be premature to classify these six genes together. For most of them, the null phenotype is as yet uncertain, and it is already clear that mutations of *bus-8*, *bus-16*, and *bus-18* can have much more severe consequences than null mutations of *srf-3*. Proper classification must await cloning and molecular analysis of the relevant loci.

The majority of mutants exhibit little or no accumulation of bacteria in the rectum when exposed to *M. nematophilum* and also exhibit additional phenotypes that suggest that cuticle properties have been altered. The simplest explanation for resistance in these mutants is that the cuticle surface has been altered in such a way that *M. nematophilum* can no longer adhere to the cuticular lining of the rectum and is therefore unable to initiate an infection. Consistent with this idea, molecular characterization of *srf-3* has shown that it encodes a nucleotide sugar transporter and appears to be defective in surface glycosylation (Cipollo *et al.* 2004; Höflich *et al.* 2004).

However, some of the mutants do not have obviously altered cuticle. This may be because our assays for surface alteration do not detect abnormalities such as subtle changes in glycosylation or other surface properties. It is also possible that the cuticle is altered only in the region of infection, as opposed to the whole body surface. Nevertheless, other explanations for the failure to establish a rectal colony can be considered: for example, it may be that the pathogen needs to detect signals from

the host to activate the infection process and that these mutants are defective in producing such signals.

It is significant that the majority of the mutants that we have examined no longer permit rectal colonization by the pathogen and do not exhibit a Dar phenotype even when grown on a pure lawn of *M. nematophilum*. This suggests that the signal or signals that trigger the swelling response act only over a short range or are emitted only by bacteria or damaged tissues after successful infection. Formation of a rectal colony of a sufficient size (a quorum) could be an essential step in eliciting the response, because some of the mutants did exhibit significant adherence of bacteria to the rectum, but much less than that seen in wild-type or *sur-2* mutants.

Testing for a Bus phenotype is a convenient, simple, and robust assay, which can be used effectively in the analysis and cloning of genes that affect this response. For example, *M. nematophilum* and the new *srf-3* alleles isolated in this work were essential in the molecular cloning and characterization of *srf-3* (Höflich *et al.* 2004). These screens have also substantially increased the number of alleles of *srf-2* and *srf-5*, and the Bus phenotype is likely to be equally useful in the further investigation of these genes.

The fact that most of the mutants reported here affect adherence by *M. nematophilum* and are either probably or demonstrably altered in surface properties means that they may also be significantly altered in response to other pathogens that attack nematodes through an initial surface attachment step. It has already been reported that *srf-2*, *srf-3*, and *srf-5* mutants are increased in susceptibility to the nematopathogenic fungus *Duddingtonia flagrans* (Mendoza De Gives *et al.* 1999). Conversely, the same three mutants are resistant to formation of a surface biofilm on the head by Yersinia *spp.* (Darby *et al.* 2002; Tan and Darby 2004). Examination of some of the other mutants reported here has shown that a subset of the *bus* mutants are resistant to Yersinia biofilm attachment (C. Darby, personal communication). Other surface pathogens of *C. elegans* may also show changes in interaction with *bus* mutants.

Moreover, the isolation of these mutants substantially increases the number of genes known to affect the surface of *C. elegans*. Previous screens have utilized surface antigenicity and/or lectin staining to identify such genes, leading to the definition of nine *srf* genes, only one of which has been cloned to date (Politz *et al.* 1987; Link *et al.* 1992). At least 9 of the 15 *bus* genes reported here appear to affect the cuticle in one way or another, and their further investigation should provide new information on the development and properties of nematode cuticle, which is one of the defining features of the phylum and a major reason for its great evolutionary success. A number of the *bus* mutants are clearly altered in mechanical and structural properties of the cuticle, exhibiting abnormal skiddy movement, fragility, and increased sensitivity to chemicals and drugs. They provide a new means of looking at the interface between nematodes and their surrounding environment.

The majority of the mutants do not exhibit any obvious major alterations in phenotype other than in response to *M. nematophilum*. Many of them nevertheless probably result from complete loss of function in the gene concerned, and therefore they are members of the large set of *C. elegans* genes for which knockout has no obvious effect. Both classical and reverse genetic approaches (such as RNA interference) indicate that >70% of *C. elegans* genes fall into this set. As discussed elsewhere (Hodgkin 2001), it may be that the functions of many of these genes pertain to biotic interactions, such as that with *M. nematophilum*, and that to understand them it will be necessary to have a better understanding of the natural ecology of *C. elegans* and to identify more dedicated pathogens like *M. nematophilum*.

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