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Drosophila genes *cut* and *miniature* are associated with the susceptibility to infection by *Serratia marcescens*

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Summary

A mutant strain of *Drosophila melanogaster* with five markers on the *X*-chromosome was found to be more sensitive than the wild type when infected with an insect-pathogenic strain of *Serratia marcescens*. Two of the five mutations in this fly strain, *cut* and *miniature*, were found to be responsible for this sensitivity. A double-mutant, with both *cut* and *miniature*, was as sensitive to *Serratia* infection as was the original sensitive *Drosophila* strain with all five mutations. Recombinant flies with other alleles of *cut* and *miniature* were also sensitive. A revertant of *cut* was found to be less sensitive than the parental flies. Our insect pathogenic strain of *Serratia* produces several proteases and a chitinase. A bacterial mutant, lacking proteases and chitinase, was found to be less virulent than wild-type bacteria. When pupal shells from resistant and *cut-miniature* flies were incubated with a mixture of protease and chitinase there was a release of *N*-acetyl glucosamine, and 50% more material was liberated from pupal shells of sensitive flies. Sensitive flies reared on sucrose infected with *Serratia* showed bacteria in their hemolymph earlier than wild-type flies. We conclude that *Drosophila* genes for *cut* and *miniature* are associated with the sensitivity to *Serratia* infection, presumably because the gut peritrophic membrane is more susceptible to bacterial proteases and chitinase.

1. Introduction

Serratia marcescens is known to be a facultative insect pathogen (for a recent review see Lysenko, 1985). During a spontaneous infection of a *Drosophila* collection in a genetics department we isolated a non-pigmented strain of *S. marcesens* (strain Db10) from sick flies. A streptomycin-resistant mutant of this strain (Db11) was used for the fulfilment of Koch's postulates (Flyg, Kenne & Boman, 1980) and the organism was found to be pathogenic for adult *Drosophila* whether injected in low doses in the abdomen or if flies were fed infected sucrose. Further work showed that certain phage-resistant mutants had lost most of the virulence (Flyg *et al.* 1980) and that proteases alone were not a virulence factor (Flyg & Xanthopoulos, 1983).

During the outbreak of the original infection it was noticed that, while a number of fly cultures were infected with Db10, only one strain of *Drosophila* (\$153) was really sick. This strain had been constructed by Valentin (1970) with five markers on its *X*-chromosome, namely *yellow*, *white*, *cut*, *miniature*, and *forked* (*y w* ct^6 *m f*). We here demonstrate that \$153 is more susceptible to *Serratia* infections than wild-type strains and that this sensitivity can be traced to two of the markers, namely ct^6 and *m*.

The exoskeleton of insects (the cuticle) functions as an effective barrier against micro-organisms (Götz & Boman, 1985). This highly complex structure is composed mainly of proteins and chitin (Kramer & Koga, 1986; Fristrom, Doctor & Fristrom, 1986). Since *cut* and *miniature* affect the wings and certain parts of the cuticle, it was our working hypothesis that the chitin layer in the gut peritrophic membrane is also weakened by these two mutations. Experiments supporting this hypothesis are given.

2. Materials and methods

(i) *Mutants of* D. melanogaster, *and crosses*

Strain \$153 of *D. melanogaster* has the following genotype: *yellow* (*y*), *white* (*w*), *cut* (ct^6), *miniature* (*m*), and *forked* (*f*) (Valentin, 1970). All mutations are

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of spontaneous origin and located on the *X*-chromosome (Morgan, 1916, 1925; Lindsley & Grell, 1968). KAS60 and other wild-type strains were from Umeå Stock Collection Center, Department of Genetics, University of Umeå, S-901 87 Umeå, Sweden, Healthy \$153 were mated with KAS60 in reciprocal crosses to produce the whole set of genotypes in the F_2 offspring: wild-type and \$153 males; heterozygous females (two types) and homozygous females of wild type and \$153 males. The *cut* strain (*ct*; Morgan *et al.* 1925) was crossed from a compound X-strain, also from Umeå. The new, spontaneous *m*-strain (m_{SL}) was isolated from an inbred Oregon line (Lake & Cederberg, 1984). An X-ray-induced revertant of ct^6 and its parental strain ($y^2\ w^1\ ct^6\ f$) were obtained from M. Green. All *Drosophila* strains were reared and maintained on an autoclaved, ordinary *Drosophila* cornflower yeast agar food, at 25 °C as described earlier (Flyg *et al.* 1980). Adult flies less than 7 days old were used in all experiments.

(ii) *Strains and mutants of S. marcescens*

Our insect-pathogenic strain of *S. marcescens*, Db10, isolated from diseased *Drosophila* (Flyg *et al.* 1980), produces several proteases (Flyg & Xanthopolous, 1983) as well as a chitinase. From a streptomycin-resistant mutant (Db11) of Db10 we isolated a bacteriophage-resistant mutant, Db1101, which was found to be less virulent in *Drosophila* (Flyg *et al.* 1980). The chitinase and protease-deficient mutant Db1139 was obtained from Db11 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) according to Miller (1974). Db10 and Db11 produce as much chitinase as *S. marcescens* IMR-1E1, a chitinase-overproducing mutant (Reid & Ogrydziak, 1981).

(iii) *Production of culture filtrates rich in proteases or chitinase*

For protease production, Db11 was grown in LB-medium (Bertani, 1951). For the induction of chitinase Db11 was grown in a chitin medium described by Monreal & Reese (1969). In both cases cultures were grown at 30 °C on a rotatory shaker (100 rev/min) for 6 days. Sterile culture filtrates were concentrated with Amicon PM10 filters (Amicon Corp. Sci. Div. System, Mass., USA) which retain molecules larger than 10 kDa. In this way the culture filtrate from LB-grown bacteria was concentrated 100 times (= the crude protease). In the same way filtrate from bacteria grown on chitin medium was concentrated 50 times (= the crude chitinase). *Serratia* proteases are subject to autodegradation but the chitinase was unaffected by the proteases.

(iv) *Feeding of* Drosophila *flies on infected sucrose*

The test bacteria were grown exponentially in LB-medium at 37 °C, harvested, and diluted with sterile sucrose to give a final concentration of 10^8 viable bacteria/ml of 5% (w/v) sucrose. About 2.5 ml of this suspension was added to sterile pillows of cellulose sponge which were placed in the bottom of sterile plastic tubes (7.8 × 2.8 cm) with flat bottoms. Each tube contained 20–25 flies. Surviving flies were counted at least once a day.

(v) *Enzyme assays*

Proteases were measured with Hide Powder (Sigma Chem. Co.) according to Rinderknecht *et al.* (1968), as modified by Flyg & Xanthopoulos (1983). Chitinase was measured either as clear zones in chitin-agar plates (Monreal & Reese, 1969) or as liberated *N*-acetyl-glucosamine (NAG) according to Jeuniaux (1966). Empty pupal shells from strains *y w f* and ct^6 *m* (offspring types 6 and 7 in Table 1) were water-washed several times and stored desiccated. In each reaction mixture for Fig. 4 we used 8 mg of finely ground shells incubated on a rotatory shaker at 37 °C with 250 μl of crude chitinase and 250 μl of buffer or 250 μl of crude chitinase and 250 μl of crude protease. In addition, all samples contained 2·25 or 2·00 ml of 0·10 M citrate-phosphate buffer, pH 6·6, with 0·02% NaN_3. At the intervals indicated, 500 μl of the mixture were withdrawn and analysed for liberated NAG according to Jeuniaux (1966). The addition of azide does not affect the enzymes used. The average weight of a single pupal shell from the ct^6 *m* strain was 56 μg, the corresponding value for the *y w f* strain was 47 μg.

3. Results

We began by an experimental demonstration of our original observation, namely that flies of strain \$153 are more susceptible than wild-type flies to an infection. Fig. 1 shows survival of male and female flies feeding on sucrose infected with our phage-resistant mutant Db1101. This bacterial mutant was used because it gives slightly larger differences than the parental strain Db11. The figure shows that male flies of strain \$153 or homozygous females did not survive 8 days. However, flies with at least one wild-type *X* chromosome survived unaffected more than 10 days, which suggests that the wild-type alleles are dominant.

In order to investigate the role of each of the five mutations present in \$153, recombinant flies with only one of the markers were isolated after crossing to wild-type strain KAS60. We also selected recombinants homozygous for ct^6 *m* and for *y w f* respectively. These seven *Drosophila* strains (offspring types 1–7) were then compared to their parental strains in feeding experiments using *Serratia* Db11. Table 1 shows that ct^6 and *m* separately and in combination caused a significant reduction in the number of surviving flies. Flies with either one or all of the three other markers (*y*, *w*, *f*) were as resistant

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as the wild type. In order to estimate the experimental error and normal strain variations, we also compared six wild-type strains originating from different parts of the world (lower part of Table 1). These strains only varied within 10% and thus we are confident that the results with *cut* and *miniature* strains are significant.

The effects recorded with flies carrying ct^6 and *m* could be caused by unknown mutations linked to these markers. We therefore investigated two independent and spontaneous alleles for *cut* and *miniature* inserted into the background of KAS60. The other *cut* allele used was *ct* isolated by Morgan (1925); the *miniature* allele, called m_{SL}, was a new isolate (Lake & Cederberg, 1984). The survival found was 58% for *ct*, 57% for m_{SL} and for the double mutant *ct* m_{SL}, 44% (strain CF1 in Table 1). Moreover, we have compared a revertant of ct^6 and its parental strain for their survival on infected food. Fig. 2 shows that the revertant survived considerably better than the ct^6-mutant strain. Thus, the results obtained in Figs. 1 and 2 and Table 1 can be attributed to the *cut* and *miniature* loci.

If the sensitive strains have a more fragile cuticle, then virulent bacteria should more easily penetrate the body or the gut, enter the hemocoel and thus be detected in the hemolymph. Table 2 shows that some ct^6 *m* flies had bacteria in their hemolymph already at day 4, while KAS60 only rarely carried *Serratia* Db11 unless they were already dead.

Both *cut* and *miniature* flies show several defects in their cuticle. We hypothesized that these genes could somehow affect the biosynthesis of the chitin. If so we should expect the *Serratia* chitinase to be a virulence factor that would show up in feeding tests. This was also found to be the case when Db1139, a chitinase- and protease-deficient mutant, and Db11 were compared in feeding experiments (Fig. 3). It has not been possible to obtain a bacterial mutant deficient only in chitinase. However, since we have earlier shown that proteases alone do not significantly contribute to the

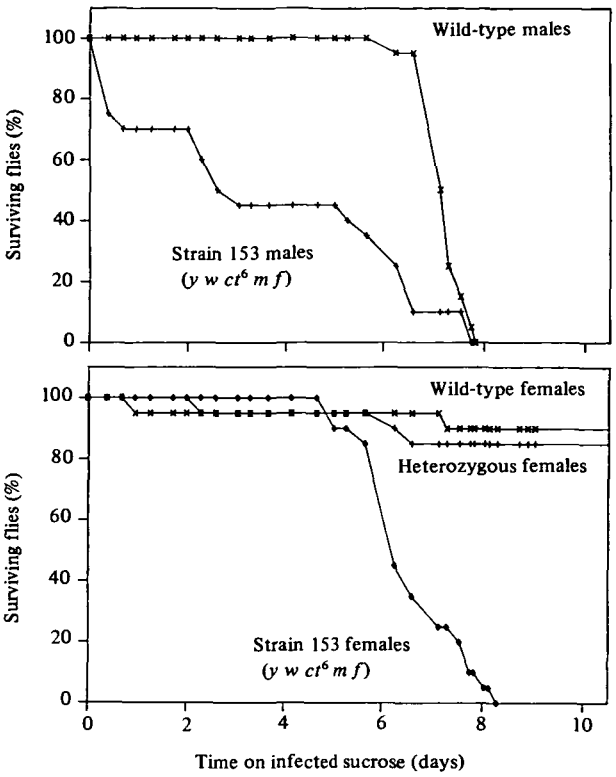

Fig. 1. Survival of different wild-type *Drosophila* and strain \$153 feeding on sucrose containing *Serratia* strain Db1101. The upper part shows male and the lower part female flies. In this experiment the total number of flies from each strain was 20. Larger numbers of flies were used for the experiments in Table 1.

Table 1. *Survival of parental and recombinant* Drosophila *infected with* Serratia

Strain	Genotype	Total number of flies tested	Surviving flies after 6 days (%)
\$153	*y w* ct^6 *m f*	255	31 ± 7·0
KAS60	Wild type	271	83 ± 8·8
Offspring 1	*y*	249	87 ± 4·6
Offspring 2	*w*	249	92 ± 4·5
Offspring 3	ct^6	230	55 ± 2·9
Offspring 4	*m*	250	58 ± 8·0
Offspring 5	*f*	246	78 ± 9·0
Offspring 6	*y w f*	250	78 ± 0·71
Offspring 7	ct^6 *m*	250	32 ± 5·4
CF1	*ct* m_{SL}	325	44 ± 8·7
Akaya	Wild type (Japan)	250	90 ± 9·6
Ashtarak	Wild type (USSR)	250	82 ± 9·6
Coffs Harbour	Wild type (Australia)	250	93 ± 3·8
Hodejice	Wild type (Czechoslovakia)	250	92 ± 6·3
Oregon R	Wild type (USA)	250	92 ± 6·9
Sandra	Wild type, isolated 1984 (Sweden)	250	86 ± 7·6

Male flies of the respective strains were fed on sucrose containing *Serratia* Db11 as described in Materials and Methods.

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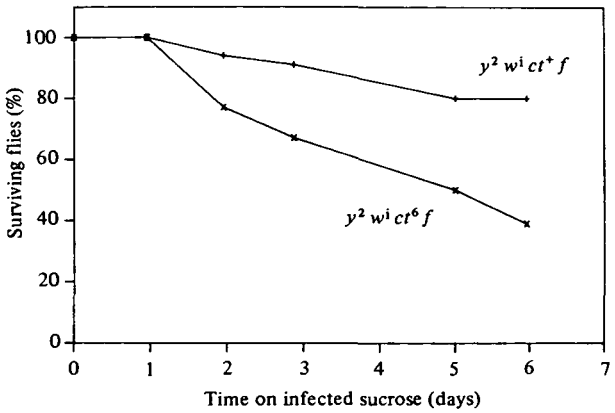

Fig. 2. Survival of adult male *Drosophila* containing an X-ray induced revertant of ct^6 and its parental strain, both feeding on sucrose infected with *Serratia* strain Db11. The markers in the *Drosophila* strains are the same as four of those in Fig. 1, but the alleles for *yellow* and *white* are different. The total number of flies from each strain was 110.

virulence (Flyg & Xanthopolous 1983), we can conclude that chitinase is of importance for the virulence.

The pupal shell of insects are also of cuticular origin. Therefore, as the next step we incubated empty pupal shells from ct^6 *m* and *y w f* flies with a concentrated culture filtrate containing chitinase and protease. Fig. 4 shows the release of *N*-acetyl glucosamine (NAG) after treatment with chitinase or chitinase and protease together. Both enzymes contribute to the release of NAG from pupal shells. From an equal number of shells there was a 50% increase in the NAG from ct^6 *m* compared to *y w f*. Thus, the chitin and/or the proteins in the cuticle of ct^6 *m* flies are affected in such a way that digestion with chitinase and protease is facilitated.

Our overall results indicate that *cut* and *miniature* affect in some way the formation of the chitin layer. Finally, to rule out that any of the markers involved

Table 2. *Mortality and appearance of* Serratia *DB11 in hemolymph samples from adult male wild type (KAS60) and* ct^6 m *flies*

Time on infected sucrose (days)	Number of dead flies		Flies with Db11 in hemolymph	
	KAS60	ct^6 *m*	KAS60	ct^6 *m*
4	1/50	20/50	0/5	2/5
5	3/50	24/50	0/5	1/5
6	5/50	29/50	1/5	1/5
7	7/50	34/50	0/5	4/5
8	50/50	50/50	5/5*	5/5*

For each *Drosophila* strain, 75 flies (25 per tube) were reared on sucrose infected with *Serratia*, strain Db11 (resistant to streptomycin and sensitive to bacteriophage ØJ, described by Flyg *et al.* 1980). Before sampling of the hemolymph the flies were surface-sterilized by washing with 70% ethanol. All bacterial colonies from infected flies were tested for the two markers that identify Db11. The number of dead flies was recorded from two of the tubes while the third was used for selecting visibly ill or dead flies for withdrawal of haemolymph. An asterisk indicates that the flies had died.

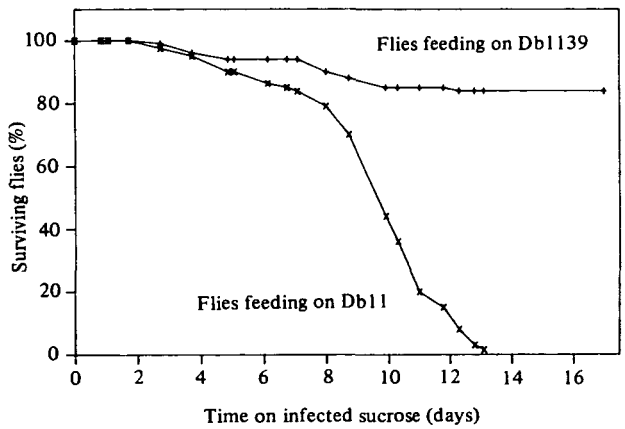

Fig. 3. Survival of adult male wild-type *Drosophila* (KAS60) feeding on sucrose containing either wild-type *Serratia* (Db11) or a bacterial mutant deficient in chitinase and protease (Db1139). The total number of flies used for each of the two bacteria was 80.

could affect the humoral immune system of the flies (Flyg *et al.* 1987), a control experiment was performed which showed that the inducible antibacterial activity was similar in immunized flies of strain \$153 and in wild-type KAS60.

4. Discussion

Both *miniature* and *cut* are classical wing markers first isolated by Morgan (1916, 1925). Relatively little has been written about *miniature*, and the last paper found was Dorn & Burdick (1962). Nothing is known about the gene product(s) of *miniature*. The *cut* locus was recently subject to an extensive investigation that included chromosome walking and restriction mapping (Jack, 1985). More than 40 different mutants have been mapped but also in this case nothing is known about the gene product(s). We have here

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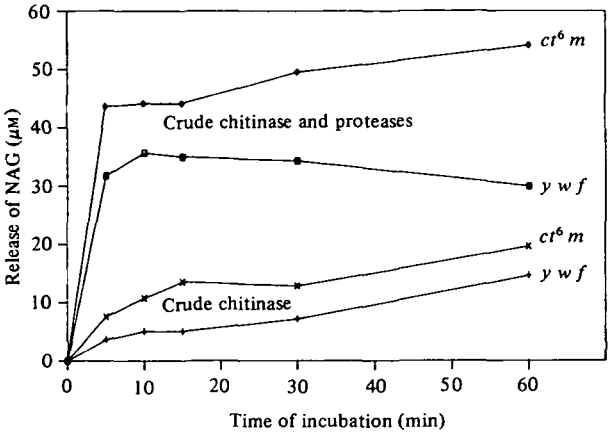

Fig. 4. Liberation of *N*-acetyl glucosamine from pupal shells of strains *y w f* and ct^6 *m* (offspring types 6 and 7 in Table 1) incubated with chitinase alone or in combination with proteases. Each incubation mixture contained 8 mg of finely ground pupal shells and crude enzymes from *Serratia* Db11 as indicated. Incubation was carried out at 37 °C on a rotatory shaker. Further details are given in Materials and Methods.

shown that S153 is more susceptible to *Serratia* infection than the wild type KAS60 (Fig. 1), and that the effects recorded with S153 can be traced to *cut* and *miniature*. Mutants in these two loci separately and in combination increase the susceptibility to infection (Table 1, Fig. 2).

Chitin is a main constituent of the insect cuticle and is also found in the trachaea and in the peritrophic membrane of the gut (Chapman, 1972). Chitinase is known to be highly toxic to insects (Lysenko, 1985) and in agreement we here found that a chitinase-deficient mutant Db1139 had lost its virulence (Fig. 3). Jeuniaux (1963) introduced the names 'free' and 'bound' for different types of chitin in cuticle. 'Free' chitin is the part of the total content that is accessible to chitinase. We have here shown that cuticle from *cut miniature* pupae is slightly more susceptible than cuticle from *yellow white forked* pupae to degradation by *Serratia* chitinase and proteases (Fig. 4). Thus, pupal shells from ct^6 *m* contain about 50% more 'free' chitin than shells from *y w f*. This difference is rather small but it has been obtained consistently in repeated experiments. The importance of an intact cuticle was also indicated by the fact that an *ebony* mutant (e^{11}, Lindsley & Grell, 1968), with a known cuticle deficiency (Jacobs, 1985), was susceptible to *Serratia* infection by feeding (results not shown).

The thin peritrophic membrane of the insect gut has been shown to be attacked by proteases and chitinases in *in vitro* experiments on the moth *Orgyia pseudotsugata* (Brandt, Adang & Spence, 1978). Histological studies have also indicated that *Serratia* can destroy the peritrophic membrane and break through to the haemocoel of moths (Goodwin, 1968; Podgwaite & Consenza, 1976). It has therefore been our working hypothesis that the peritrophic membrane in the gut is the part most susceptible during a feeding experiment with *Serratia*-infected sucrose. That this assumption is correct is supported by the experiment in which *Serratia* Db11 was isolated from the hemolymph of surface-sterilized infected flies (Table 2). We therefore believe that in our feeding experiments *Serratia* breaks through the gut and then quickly grows up in the hemolymph and kills the insects.

Susceptibility to infection is a new parameter in *Drosophila* genetics. The genes here studied, *cut* and *miniature*, are, however, probably not unique. We therefore predict that other genes affecting the cuticle as well as genes for cellular and humoral immunity will be found to affect the survival of flies to bacterial infections.

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