Increase in the resistance to the *Bacillus thuringiensis* supernatant effect in a *Drosophila melanogaster* wild type Oregon R line

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We report here the genetical and X chromosome rDNA molecular study of two *Drosophila melanogaster* Oregon R lines. These lines differ extensively in the degree of resistance of the females both to the lethal effect of an increased temperature and to that of *Bacillus thuringiensis* beta-exotoxin, which is an inhibitor of the nucleolar RNA polymerase. The 3B line, whose females are resistant, came from an Oregon R population subjected over several generations to increased temperature, 28°C or over, while the other line is derived from the initial stock. Twofold variation was observed in the total number of ribosomal genes between the two lines. This variation applied to most ribosomal units, including the active ones. Variations among X chromosome rDNA content in a wild type population have thus been revealed using tests of resistance to the *Bacillus thuringiensis* beta-exotoxin. Additive variations in specific unit types between the two lines indicate that modifications to the rDNA content are not rare events.

Keywords: *Bacillus thuringiensis*, beta-exotoxin, *Drosophila melanogaster*, evolution, high temperature lethality, rDNA.

Introduction

The ribosomal DNA, located at the nucleolar organizer sites of the chromosomes, is composed of the genes for the 28S, 18S and 5.8S type RNA, clustered as repeated transcriptional units. In the *Drosophila melanogaster* species, the nucleolar organizers are on the heterochromosomes (Cooper, 1959; Ritossa & Spiegelman, 1965; Spear, 1974). Many ribosomal units of the X chromosome are interrupted by an insertion into the 28S gene sequence (Long & Dawid, 1980). Two classes of non-homologous insertions have been identified named type I, largely represented, and type II (Roiha *et al.*, 1981). Only the uninterrupted units seem to be transcriptionally active (Long & Dawid, 1979; Long *et al.*, 1980; Kidd & Glover, 1981; Terracol & Prud'homme, 1986). Compared to the X chromosome, interrupted units are in a smaller proportion on the Y chromosome where only type II insertions have been

identified (Tartof & Dawid, 1976; Wellauer *et al.*, 1978; Peacock *et al.*, 1981).

The supernatant of cultures of the *Bacillus thuringiensis* Berliner strain contains a thermostable toxin (McConnel & Richards, 1959): the beta-exotoxin or thuringiensin, which acts *in vivo* as a preferential inhibitor of nucleolar RNA polymerase (Mackedonski *et al.*, 1972; Beebe & Bond, 1973). Like the purified toxin, the supernatant has a lethal effect during the development stage if added to the nutritional medium (Paumard & Bourgin-Rosenberg, 1981). Using 'bobbed' mutants, we have previously shown that lethality increases when the ribosomal DNA amount decreases (Paumard & Bourgin-Rosenberg, 1981). In Oregon R populations, the males are more resistant than females to the lethal effect of the supernatant; this has been correlated with a greater number of uninterrupted units on the Y chromosome compared to the X chromosome (Rosenberg-Bourgin *et al.*, 1988b).

The amount of ribosomal DNA on the two chromosomes and the proportion of the different classes of uninterrupted and interrupted units can vary between different strains of *Drosophila melanogaster* and even

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between lines originated from the same strain (Endow & Glover, 1979; Dutton & Kridler, 1984; Rosenberg-Bourgin *et al.*, 1988a).

From an Oregon R stock bred for several generations at temperatures which often reach and sometimes exceed 28°C, we have spontaneously obtained a variant population in which the females are more resistant to the lethal effect of the supernatant than those of the initial stock. The initial and variant Oregon R populations do not exhibit any difference on a standard medium at 25°C. However, these two populations express differences when they are bred at 28°C: this temperature has a lethal effect during development and females of the variant population are more resistant than those of the initial population; we have shown that genotypes resistant to increased temperature were present in the initial stock and have been positively selected when bred at the high temperature (Paumard-Rigal & Rosenberg-Bourgin, 1990). We have postulated that variation in the degree of resistance to *Bacillus thuringiensis* supernatant could reveal variations in the amount of active rDNA units. By successive individual crosses, two lines, whose females differ extensively for resistance to beta-exotoxin, have been isolated: the 3B line from the variant population and the 1C line from the initial one (Paumard-Rigal & Rosenberg-Bourgin, 1990). We report here on the results of genetical and molecular studies of these two Oregon R lines.

Materials and methods

Drosophila melanogaster stocks

The 1C line and 3B line came from the Oregon R stock of the Centre de Génétique Moléculaire of Gif sur Yvette (France). The '94' strain originated from the collection of the Centre de Génétique Moléculaire of Gif sur Yvette (France). The females C1 (RM) have two attached X chromosomes and no Y chromosome (Lindsley & Grell, 1968).

Drosophila stocks were grown on cornmeal yeast extract agar medium. (75 g cornmeal, 75 g yeast-extract, 125 g agar-agar and 45 ml of a 10 g/100 ml solution of methyl-*p*-hydroxybenzoate in 90 per cent alcohol for 1 litre of medium).

Evaluation of the effect of the Bacillus thuringiensis thermostable supernatant

Drosophila were grown at 25°C. Supernatant from a culture of *Bacillus thuringiensis* Berliner strain harvested at the end of sporulation was autoclaved for 20 min at 120°C and added to the standard medium at

a final concentration of 1.5 or 2.5 per cent (vol./vol.). The *Bacillus thuringiensis* cultures were grown at 30°C using the conditions described in Lecadet & Dedonder (1971). The supernatant effect was assayed by counting the adults emerging after all their development had taken place on a supplemented supernatant medium, from eggs laid on this medium over 4 days by 12 females mated with 12 males. The same batch of bacterial culture supernatant was used for all experiments.

Extraction of brain DNA from third-instar larvae

Brains and imaginal discs of about 30 third-instar female larvae were dissected in *Drosophila* Ringer solution (Ephrussi & Beadle, 1936). After the addition of 7 µl of a 2 mg µl⁻¹ solution of self-digested pronase, they were incubated for 22–24 h at room temperature under slow agitation in an Eppendorf tube, in 250 µl of 50 mM Tris HCl pH 7.8, 100 mM EDTA and 0.5 per cent SDS. After incubation, 250 µl NaCl 2 M and 500 µl of chloroform-isoamyl alcohol (24:1) were added successively. The preparation was gently mixed for 3 min, kept for 10 min at 0°C and then centrifuged for 5 min at 12,000 g. Two microlitres of 10 mg ml⁻¹ RNase A were added to the supernatant and was incubated for 2 h at 37°C. After a second treatment with chloroform-isoamyl alcohol, the DNA was precipitated for 15 min at -70°C by the addition of 1 ml ethanol and was dissolved in 30 µl TE (10 mM Tris-HCl pH 8, 1 mM EDTA) after centrifugation and drying.

The pDmrY₁₂ plasmid

The pDmrY₁₂ plasmid, isolated by the method of Birnboim & Doly (1979), contains an 11.7 kb *EcoRI* insribosomal DmrY₁₂ fragment in the *EcoRI* site of *ColEI* (Wellauer *et al.*, 1978).

DNA electrophoresis, transfer and hybridization

Electrophoresis was performed in pH 8 borate buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8) with the addition of 1 µg ml⁻¹ of ethidium bromide. After electrophoresis, the agarose gels were treated successively, twice for 15 min with 0.25 M HCl, twice for 15 min with 0.5 M NaOH, 1.5 M NaCl, and twice for 30 min with 0.02 M NaOH, 1 M ammonium acetate. Transfer to a Schleicher and Schüll BA 85 nitrocellulose filter was carried out in 0.02 M NaOH, 1 M ammonium acetate for 10–12 h. DNA was fixed to the membrane by heating for 2 h at 80°C. Filters were prehybridized at 42°C for 2 h in the following mixture

(Wahl *et al.*, 1979): 50 per cent formamide–5×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate), 5×Denhardt solution (1×Denhardt is 0.02 per cent polyvinylpyrrolidone, 0.02 per cent ficoll, 0.02 per cent bovine serum albumin), 50 mM sodium phosphate buffer (pH 7.4), 1 per cent SDS. The filters were then hybridized at 42°C for 20–24 h in 50 per cent formamide 5×SSC, 1×Denhardt solution, 20 mM sodium phosphate buffer (pH 7.4), 6 per cent dextran sulfate 500, 1 per cent SDS, 100 µg ml⁻¹ of yeast tRNA, 2 ng ml⁻¹ ³²P-nick-translated pDmrY₁₂ or DmrY₁₂ (0.5–1×10⁸ cpm µg⁻¹) denatured for 10 min at 100°C. The filters were rinsed successively once at room temperature for 5 min in 2×SSC, three times at 42°C for 20 min in 2×SSC–1 per cent SDS, twice at 42°C for 20 min in 0.5×SSC–0.1 per cent SDS. Filters were exposed to Kodak X-Omat R films for different times (in general 1–72 h). Autoradiographs were scanned on a Shimadzu CS 930 densitometer. The areas were determined with several autoradiographs within the linear response range of the film.

Ribosomal DNA quantification

The number of ribosomal units per X chromosome was determined after loading of *Drosophila* DNA samples on a 1 per cent agarose minigel containing ethidium bromide. The quantity of total *Drosophila* DNA in each slot was first evaluated: known quantities of lambda DNA were loaded on the same minigel and, after a short migration, the fluorescence intensity, under ultraviolet light, of the different samples of *Drosophila* DNA was compared with that of the standards. The fluorescence intensity of each sample was determined precisely after scanning the photographic negatives. The number of haploid genomes (i.e. the same as the number of X chromosomes in the females) was calculated with the knowledge that the haploid genome contains 0.18 pg DNA.

The number of ribosomal units on each *Drosophila* DNA sample was determined by loading known quantities of pDmrY₁₂ added to 250 ng of calf thymus DNA in the slots where the lambda DNA had first been loaded. After a short electrophoresis, the DNA was transferred to nitrocellulose filters and hybridized with ³²P DmrY₁₂. The hybridization signals of the various *Drosophila* DNA samples were compared with those of the standards. The signal intensity was precisely determined after scanning the autoradiographs. The determination of the number of ribosomal units was realized by taking into account the fact that the *Drosophila* insert DmrY₁₂ (representing 67 per cent of the total pDmrY₁₂ plasmid and corresponding to a 11.7 kb uninterrupted unit) is equivalent to

1.52×10⁻⁵ pg of DNA. The method is illustrated in Rosenberg-Bourgin *et al.* (1988a).

The ratio between the number of ribosomal units and the number of haploid genomes was calculated for at least three independent DNA samples to determine the number of ribosomal units per X chromosome.

Distribution of the different ribosomal unit types

The extracted *Drosophila* DNA was diluted in a final volume of 45 µl in a buffer of the following composition: 5 mM MgCl₂, 100 mM Tris-HCl pH 7.5, 50 mM NaCl, 100 ng µl⁻¹ of bovine serum albumin. Lambda DNA (4.5 µg) was added in order to test the complete enzymatic digestion and to be used as a size marker in the autoradiographs. The DNA preparation was treated with *Eco*RI. Agarose gels (0.6 per cent) were loaded with 11 µl of the DNA digests obtained from 20 brains for the 3B line and 40 brains for the 1C line with the BRL size market '1 kb ladder', which gives rise to numerous fragments from 3 to 12 kb. After electrophoresis at 110 V for 15–20 h, transfer, and hybridization, the resulting autoradiographs were scanned to determine the percentage of the different size fragments.

Results

Genetical results

The characterization of the two Oregon R lines, 3B and 1C, in the presence of the 2.5 per cent supernatant is shown in Table 1. For the 1C line, the male/female sex ratio is increased in the presence of 2.5 per cent *Bacillus thuringiensis* supernatant: 1.1 without supernatant to 6.2 in the presence of the inhibitor. For the 3B line, there is no difference in the sex ratio value: 1.0 and 1.07. The difference in the sex ratio between these lines in the presence of 2.5 per cent supernatant is due to an increase in the resistance of 3B line females compared to the ones in line 1C.

We have compared the resistance of the homozygous 3B and 1C females to the resistance of the females heterozygous for the two genotypes, obtained in the reciprocal crosses between the 3B females and the 1C males or between 1C females and 3B males (Table 1). The male/female sex ratio is the same for the two crosses: respectively 2.04 and 2.0; this value lies between the value obtained for the 1C line and that obtained for the 3B line. The number of males is similar in each case, so females that have a heterozygous genotype 3B/1C have a similar resistance in both reciprocal crosses.

Table 2 shows the influence of the nature of the X chromosome and the nature of the autosomes on the resistance of X/O males generated from crosses between X.X females of the '94' strain and males of different genotypes. In these crosses, the X/O males receive the X chromosome from their father. There is no difference in the progeny of these different crosses in the standard medium and whatever may be the father's genotype, no X/O male exhibits a bobbed phenotype. In the presence of 2.5 per cent supernatant, X/O males generated by males with a heterozygous autosomal stock 3B/1C and the 3B X chromosome are very resistant (sex ratio 0.56) compared to X/O males generated by males with the same autosomal stock but with the 1C X chromosome (sex ratio 0.08). The resistance of X/O males was similar when a different autosomal stock was used, sex ratios of 0.67 and 0.56

for the males with the 3B X chromosome and 0.06 and 0.08 for the males with the 1C X chromosome.

Thus neither the nature of the autosomes nor any cytoplasmic heredity are involved in the difference in resistance between the two lines. This difference is only correlated with a difference between the X chromosomes of these lines.

Molecular results

The number of ribosomal units per X chromosome for the 3B and 1C Oregon R lines is 418 ± 10.6 and 195 ± 13.3 respectively, representing a twofold difference.

The *EcoRI* X chromosome ribosomal DNA restriction patterns of the two lines are presented in Fig. 1. The results of densitometric scanings are reported in

Table 1 Comparison of the effect of 2.5 per cent *Bacillus thuringiensis* supernatant at 25°C on the sex ratio, for the progeny of the two Oregon R lines 1C and 3B and for that of the reciprocal crosses, between these two lines

	X/Y males per flask		X/X females per flask		Male/female ratio	
	S+	S-	S+	S-	S+	S-
Parents						
1C* line	259	(232)	42	(211)	6.2	(1.1)
3B† line	275	(238)	257	(237)	1.07	(1)
3B females × 1C males	233	(233)	114	(225)	2.04	(1.04)
1C females × 3B males	241	(228)	120	(228)	2	(1)

S+ = with 2.5 per cent supernatant, S- = without supernatant.

S+ and S- values are means of at least five flasks.

*Line originated from the initial Oregon R population.

†Line originated from the variant Oregon R population.

Table 2 Comparison of the effect of 2.5 per cent *Bacillus thuringiensis* supernatant at 25°C on X/O genotype male progeny of crosses between X.X females and males of the 1C or 3B lines, or those obtained from reciprocal crosses between these two lines

Father's genotype		Descendents per flask					
		X/O males		X.X/Y females		Male/female ratio	
		S+	S-	S+	S-	S+	S-
Autosomes	X chromosome						
3B/3B	3B	114	(188)	170	(182)	0.67	(1.03)
1C/1C	1C	10	(188)	167	(174)	0.06	(1.08)
3B/1C	3B	100	(180)	179	(175)	0.56	(1.03)
3B/1C	1C	17	(187)	192	(183)	0.08	(1.02)

S+ = with 2.5 per cent supernatant, S- = without supernatant.

S+ values are means of at least five flasks; S- values are means of three flasks.

Fig. 1 *EcoRI* X ribosomal DNA restriction patterns in the 3B and in the 1C Oregon R lines. One-quarter of the *EcoRI* DNA digests from 20 female larvae brains and imaginal discs for the 3B line and one-quarter of the *EcoRI* DNA digests from 40 female larvae brains and imaginal discs for the 1C line were fractionated on 0.6 per cent agarose gel, blotted on to nitrocellulose and hybridized to the ^{32}P -labelled pDmrY₁₂ probe. 17, 12.4 and 11.7 kb are the size of the different fragments determined by reference to a DNA size marker: (the BRL '1 kb ladder' and *EcoRI* digested lambda DNA). Lane A = 3B line, Lane B = 1C line.

Table 3, which shows the distribution of the three major fragments (17, 12.4 and 11.7 kb) and of the two main smaller fragments detected for the two lines. This distribution is not significantly different between the two lines. The difference between the rDNA in the 3B and 1C Oregon R lines X chromosomes thus concerns most of the ribosomal units (both active and inactive) as a whole: the number in the 3B line is twice the number in the 1C line.

Some units nevertheless are represented differently in the two lines: the comparison of the two autoradio-

graphs (Fig. 1) shows an interruption of the signal in the area of the 14.5–15.4 kb fragments for the 1C line and not for the 3B line. In our experimental conditions, a similar intensity of the signal must be obtained on the autoradiographs for fragments that are twice as numerous in the 3B line compared to the 1C line. The difference between the two patterns shows that the amount of the 14.5–15.4 kb fragments in the 3B line is much more than twice the amount in the 1C line. A second difference concerns the 15–16.2 kb fragments: there is an interruption of the signal in the corresponding area for the 3B line and not for the 1C one. Thus, these fragments are less than half as abundant on the 3B X chromosome compared to the 1C X chromosome.

Discussion

Our genetic results show that the differences in resistance to the lethal effect of the *Bacillus thuringiensis* supernatant, observed between the females of the 3B Oregon R line derived from our variant population and the 1C Oregon R line derived from the initial stock, are related to differences in the properties of their X chromosomes. We have shown that twice as many ribosomal transcriptional units are present on the 3B line X chromosome compared to that of the 1C line; the amount of the rDNA on this 1C line X chromosome corresponds to that usually described for the Oregon strain (Tartof, 1971, 1973a; Ritossa, 1976). The better resistance of the 3B line females is thus correlated with an increase in the X chromosomal rDNA amount. This increase concerns the majority of the different unit types, including the uninterrupted units, which seem to be the only ones able to give an active product (Long & Dawid, 1979; Long *et al.*, 1980; Kidd & Glover, 1981; Terracol & Prud'homme, 1986; Hawley & Marcus, 1989). These units do correspond to the 11.7-kb *EcoRI* ribosomal fragments. The 17-kb fragments represent 5 kb type I insertion interrupted units while the 12.4-kb fragments correspond generally to 0.5–1 kb type I interrupted units or to uninterrupted units with a larger spacer. The fragments smaller than 10 kb are part of type II insertion interrupted units (Endow & Glover, 1979; Terracol & Prud'homme, 1986).

The two lines obtained from our Oregon R stock do not exhibit any difference in standard growth conditions. They both have a wild type phenotype. An increase in the X rDNA units content, particularly in the amount of active ribosomal units, has been revealed by the use of the *Bacillus thuringiensis* supernatant, which contains the beta-exotoxin nucleolar RNA polymerase inhibitor.

Table 3 Distribution of the different fragments detected on the *EcoRI* X ribosomal DNA restriction patterns in the two lines

Fragment size (kb)	3B line		1C line	
	Percentage among the total X ribosomal DNA	Number of fragments per X chromosome	Percentage of the total X ribosomal DNA	Number of fragments per X chromosome
17	23.9	100	26.8	52
12.4	16	67	16.2	32
11.7	28.4	119	26.8	52
Sum of the two main smaller fragments	6.2	26	8.2	16

The percentage of each fragment among the total X ribosomal DNA was determined by integration of the areas of the peaks obtained on densitometric scanning of the autoradiographs.

The number of fragments per X chromosome was calculated taking account of the average number of the X ribosomal units: 418 for the 3B line and 195 for the 1C line. The χ^2 calculated with these results shows that the ratios of the different fragments in the two lines is not significantly different.

Ribosomal DNA evolution has previously been studied with 'bobbed' mutants because of the possibility of following variations in the amount of functional rDNA by variation in the intensity of the 'bobbed' phenotype, particularly in the case of the magnification phenomenon (Ritossa, 1972; Locker & Prud'homme, 1973; Tartof, 1973b; Terracol & Prud'homme, 1986; Terracol, 1987). However this method needs particular crosses and genotypes with a very low level of active ribosomal units (Tartof, 1974; Hawley & Tartof, 1985; Marcus *et al.*, 1986) and cannot be used to follow variations in the rDNA amount in wild type flies. The method we describe here, however, could be used to follow such variations among a wild type population.

The 3B line was isolated from a variant Oregon R population selected at high temperature. We had previously observed a correlation between the sensitivity of different genotypes to the lethal effect of the increase in temperature and to that of the *Bacillus thuringiensis* supernatant (Paumard-Rigal & Rosenberg-Bourgin, 1990). The 3B line females have more active ribosomal transcriptional units compared to the 1C line females. Thus, the increase in the number of the active ribosomal transcriptional units could have given a selective advantage expressed when the developmental temperature had increased too much: the development time being shorter at 28°C than at 25°C; a higher rate of synthesis, including that of the rRNA, may be necessary at 28°C. In the 3B line compared to the 1C line; the different main unit types: interrupted and uninterrupted have been increased in the same proportion. This suggests that this increase could result from a single molecular event, for example, an unequal exchange which would have transferred the main part of the rDNA from one chromatid to another,

generating simultaneously a large deletion in the rDNA, similar to deletions identified in lethal 'bobbed' mutations (Ritossa *et al.*, 1966). Could such a mechanism: the simultaneous increase in interrupted and uninterrupted units, followed by positive selection of genotypes with an increased number of active units, occur in the natural environment? This could then have contributed to maintaining inserts in the rDNA, in addition to other processes including the possible transposition of the DNA insertions that have been identified as retrotransposable elements (Jakubczach *et al.*, 1990).

The mechanism postulated to explain the twofold increase in the number of most units on the 3B line X chromosome cannot, however, explain all the differences observed between the X rDNA of our two lines. The number of the 14.5–15.4 kb fragments in the 3B line is more than twice that in the 1C line. Two hypotheses could explain this observation: either the corresponding unit type had been amplified in the 3B line or these units had been eliminated from the 1C X chromosome. One of the mechanisms of this elimination could be unequal recombination. The same unit types are clustered in the rDNA locus (Appels & Hilliker, 1982; Sharp *et al.*, 1983; Salzano & Malva, 1984; Terracol & Prud'homme, 1986; Gillings *et al.*, 1987; Terracol, 1987; England *et al.*, 1988) and can be eliminated together during such an event. The observation is inverted for the 15–16.2 kb fragments, which are more represented on the 1C line X chromosome: they had been either eliminated from the 3B line X chromosome or amplified in the 1C line X chromosome. Thus, there is more than one modification of the rDNA between the two lines. Heterogeneity of our initial Oregon R population had been observed in

temperature-resistant tests on individual X/O genotypes (Paumard-Rigal & Rosenberg-Bourgin, 1990). This could be related to differences in the rDNA content and could thus reveal frequent variations in the rDNA locus. In non-selective conditions they do not lead to any modification of the global population characteristics and thus should be essentially reciprocal modifications probably resulting from unequal recombinations.

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