X chromosome variations of *Bacillus thuringiensis* supernatant resistance and ribosomal DNA content in *Drosophila melanogaster* wild-type Oregon R lines

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We have used the supernatant of *Bacillus thuringiensis* cultures to follow variations of the ribosomal DNA content in a wild-type Oregon R line of *Drosophila melanogaster*. These variations are revealed by differences in the degree of resistance to the lethal effect of the supernatant added in the culture medium. Different X chromosomes, all originated from the same X chromosome, confer different degrees of resistance. Increases and reductions in the number of the X ribosomal DNA transcriptional units have been found and are correlated with variations in the degree of supernatant resistance. However, no significant variations in X rDNA content were observed between the initial line and this line tested 20 generations later. These results show that the X rDNA is subjected to frequent modifications that must be essentially complementary and thus originate from reciprocal molecular events. Variations of resistance have been observed in the absence of recombination between homologous chromosomes; this is in agreement with the hypothesis that unequal recombinations between sister chromatids are largely involved in the variations of the X ribosomal DNA in Oregon R wild-type strains.

Keywords: *Bacillus thuringiensis*, *Drosophila melanogaster*, beta exotoxin resistance, ribosomal DNA.

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 *Drosophila melanogaster*, 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; Hawley & Marcus, 1989). 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 *D. melanogaster* and even

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

Ribosomal DNA evolution in *Drosophila* has been essentially studied with 'bobbed' mutants because of the possibility of following variations in the amount of functional rDNA by variation of the intensity of the 'bobbed' phenotype, particularly in the case of the magnification phenomenon (Ritossa, 1972; Locker & Prud'homme, 1973; Tartof, 1973; Terracol & Prud'homme, 1986; Terracol, 1987). 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.

We have developed a method based on the resistance to *Bacillus thuringiensis* supernatant that reveals modifications in the rDNA content of wild-type strains (Rosenberg-Bourgin *et al.*, 1988b; Paumard-Rigal & Rosenberg-Bourgin, 1993). The supernatant of cultures of *B. thuringiensis* Berliner strain contains a thermostable toxin (McConnel & Richards, 1959), the

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beta exotoxin or thuringiensin, that acts *in vivo* as a preferential inhibitor of the nucleolar RNA polymerase (Mackedonski *et al.*, 1972; Beebe & Bond, 1973). We have previously shown that the supernatant, like the purified toxin, has a lethal effect during the development stage when added to the nutritional medium of *Drosophila*; this lethal effect increases when the number of active ribosomal DNA units decreases (Paumard & Bourgin-Rosenberg, 1981; Rosenberg-Bourgin *et al.*, 1988b; Paumard-Rigal & Rosenberg-Bourgin, 1992).

We have shown (Paumard-Rigal & Rosenberg-Bourgin, 1992) that the addition of *B. thuringiensis* supernatant to *D. melanogaster* culture medium makes possible the detection of modifications in X ribosomal DNA content between two wild-type Oregon R lines, one isolated from a variant Oregon R population selected at high temperature and the other isolated from the original population (Paumard-Rigal & Rosenberg-Bourgin, 1990). The variations observed between the two lines have led us to hypothesize that modifications in the X rDNA content are not rare events.

Here we use *B. thuringiensis* supernatant to follow variations of the ribosomal DNA content of one Oregon R wild-type line X chromosome to determine how the modifications arise in the X rDNA.

Materials and methods

D. melanogaster stocks

All the *Drosophila* strains came from the stock of the Centre de Génétique Moléculaire of Gif sur Yvette (France).

The 1C line came from the Oregon R stock (Paumard-Rigal & Rosenberg-Bourgin, 1990).

The '94' strain has C1(RM) females with two attached X chromosomes (Lindsley & Grell, 1968).

The Müller 5 strain is (In(1) sc⁸IL sc⁸R + S, sc⁸ sc⁸ w^a B). This double inversion on the X chromosome includes the nucleolar organizer and makes the recovery of recombinants from X+ /X Mu₅ females very unlikely.

Drosophila stocks were grown on cornmeal yeast extract agar medium (75 g cornmeal, 75 g yeast extract, 12.5 g agar-agar and 45 ml of a 10 g/100 ml solution of methyl-para-hydroxy-benzoate in 90 per cent alcohol for 1 litre of medium).

Evaluation of the effect of *B. thuringiensis* thermostable supernatant

Drosophila were grown at 25°C. Supernatant from a culture of *B. 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 per cent and 1.5 per cent (v/v). *B. thuringiensis* cultures were grown at 30°C under the conditions described in Lecadet & Dedonder (1971). The supernatant effect was assayed by counting the adults emerging after all their development had taken place in 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.

Construction of the lines

From the 1C line previously described (Paumard-Rigal & Rosenberg-Bourgin, 1992), several lines, each isolated from one single X chromosome, were constructed by successive crosses involving the Müller 5 X chromosome introduced in the 1C autosomal context ((Müller 5)1C strain).

The 1C₁₁ line, which gives the same results in *B. thuringiensis*-supplemented supernatant medium as the initial 1C line, was selected for the construction of further lines.

At 20 generations later, new lines, each isolated from one single X chromosome of the 1C₁₁ line, were constructed using the same method.

Study of variations of supernatant resistance in the absence of recombination between homologous X chromosomes

From four of the new lines mentioned above, one male chosen at random from each population was crossed with (Müller 5)1C females and then one F₁ female was crossed with (Müller 5)1C males. The (B+) male descendants were individually crossed with ten females of the 94 strain on supplemented supernatant medium.

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 addition of 7 µl of a 2 mg/µl solution of self-digested pronase, they were incubated for 22–24 h at room temperature with slow agitation in Eppendorf tubes 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 2M 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. Then 2 µl of 10 mg ml⁻¹ RNase A was added to this supernatant and 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 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 pDmr Y₁₂ plasmid

The pDmr Y₁₂ plasmid, isolated by the method of Birnboim & Doly (1979), contains an 11.7 kb *Eco*RI ins⁻ ribosomal Dmr Y₁₂ fragment in the *Eco*RI 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 addition of 1 $\mu\text{g ml}^{-1}$ 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 Schleicher et 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, $5 \times \text{SSC}$ ($1 \times \text{SSC}$ is 150 mM NaCl, 15 mM sodium citrate), $5 \times$ Denhardt solution ($1 \times$ 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, $5 \times \text{SSC}$, $1 \times$ Denhardt solution, 20 mM sodium phosphate buffer (pH 7.4), 6 per cent dextranulphate 500, 1 per cent SDS, 100 $\mu\text{g ml}^{-1}$ of yeast tRNA, 2 ng ml^{-1} ^{32}P nicktranslated pDmrY₁₂ or DmrY₁₂ ($0.5\text{--}1.10^8$ c.p.m./ μg) denatured for 10 min at 100°C . The filters were rinsed successively, once at room temperature for 5 min in $2 \times \text{SSC}$, three times at 42°C for 20 min in $2 \times \text{SSC}$ –1 per cent SDS, twice at 42°C for 20 min in $0.5 \times \text{SSC}$ –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 *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 U.V. light of the different samples of *Drosophila* DNA was compared with that of the standards. Fluorescence intensity of each sample was determined precisely after scanning the photographic negatives. The number of haploid genomes (that is the same as the number of X chromosomes in the females), was calculated knowing that the haploid genome contains 0.18 pg DNA.

The number of ribosomal units in each *Drosophila* DNA sample was determined by loading known quantities of pDmr Y₁₂ with 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 ^{32}P DmrY₁₂. The hybridization signals of the various *Drosophila* DNA samples were compared with those of the standards.

The intensity of the signal was precisely determined by scanning the autoradiographs. The determination of the number of ribosomal units was realized by considering the *Drosophila* insert DmrY₁₂ (representing 67 per cent of the total pDmrY₁₂ plasmid and corresponding to a 11.7 kb uninterrupted unit) as 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, calculated for at least three independent DNA samples, allows the determination of the number of ribosomal units per X chromosome.

Results

X chromosome variations in supernatant resistance and in rDNA content between lines each isolated from one single X chromosome of the 1C₁₁ line

The results obtained in the presence of 1 per cent *B. thuringiensis* supernatant with the 1C₁₁ line isolated from the 1C Oregon R line (Paumard-Rigal and Rosenberg-Bourgin, 1990) and with 29 lines each isolated from one single X chromosome of this line, 20 generations after its establishment, are reported in Table 1.

The sex ratio is a measure of the supernatant resistance conferred by the X chromosomes of the different lines. Significant variations are observed between the lines; the sex ratio values are distributed on both sides of the 1C₁₁ value (0.43–1.03). For 11 lines, the X/0 males are significantly less resistant than those of the 1C₁₁ line; for six lines, they are significantly more resistant.

Table 1 Resistance to 1 per cent *B. thuringiensis* supernatant of the X/0 males obtained from crosses between X.X 94 females and males of the 1C₁₁ line or of the different lines derived from this line

Line number	Males/females X/0/X.X/Y	Ratio	Line number	Males/females X/0/X.X/Y	Ratio	Line number	Males/females X/0/X.X/Y	Ratio
9	78/183	0.43	26	91/143	0.64	28	125/157	0.80
18	79/179	0.44	12	110/168	0.65	32	121/149	0.81
10	80/165	0.48	24	107/165	0.65	27	151/178	0.85
23	87/170	0.51	14	145/159	0.66	8	155/181	0.86
2	64/130	0.53	5	117/174	0.67	31	140/158	0.89
6	85/156	0.54	4	101/150	0.68	21	145/141	1.03
11	90/161	0.55	1C ₁₁	111/163	0.68			
15	87/154	0.56	34	68/98	0.69			
7	97/171	0.57	20	119/168	0.70			
13	116/200	0.58	17	135/189	0.71			
22	108/186	0.58	1	140/185	0.75			
			30	115/153	0.75			
			25	136/176	0.77			

Male and female values are means of five flasks.

Table 2 Number of X ribosomal units of the different lines derived from the 1C₁₁ line

Line number	Number of X ribosomal units	S.E.M.	Difference with initial 1C ₁₁ line
Initial 1C ₁₁ line (DNA sampled at origin)	194	11	
1C ₁₁ line (DNA sampled 20 generations later)	201	10	+7
21	245	10	+44
31	248	10	+47
32	235	6	+34
6	166	7	-35
18	158	7	-43
9	156	8	-45

S.E.M. = standard error of the mean (three independent DNA samples for each line).

Six lines (numbered 9, 18, 31, 21, 6 and 32) were chosen for further biochemical studies. Lines 9 and 18 on one hand, lines 31 and 21 on the other, were selected because they present the most extreme variations of X/0 male resistance in a supernatant-supplemented medium compared with the 1C₁₁ line. The two other lines were chosen at random from lines with an intermediate X/0 male resistance (lower resistance for line 6, higher resistance for line 32).

The amount of X ribosomal DNA in each of these lines and in the 1C₁₁ line is reported in Table 2.

There is only a small variation between the number of X ribosomal DNA units in the 1C₁₁ line at origin and the number of X ribosomal DNA units of this line 20 generations later (DNA sampled at the same time as the other lines): 194 and 201, respectively.

On the contrary, the number of X ribosomal units varies between the 1C₁₁ line and the different lines as well as between the different lines themselves (from 156 to 245).

The variations are in agreement with the results obtained with the *B. thuringiensis* supernatant. The

Table 3 Resistance to 1.5 per cent *B. thuringiensis* supernatant of the X/0 males obtained from each individual cross between 94 females and the 20 (B+) males issued from one single X1C_{11n}/X Mu₅ female for the lines 18, 6, 32 and 21

(a) line 18		(b) line 6	
X/0 males/X.X/Y females		X/0 males/X.X/Y females	
34/112 = 0.28	55/133 = 0.41	40/129 = 0.31	72/139 = 0.52
43/139 = 0.31	71/168 = 0.42	31/95 = 0.33	77/137 = 0.56
42/134 = 0.31	64/151 = 0.42	45/126 = 0.36	59/100 = 0.59
49/145 = 0.34	56/131 = 0.43	50/129 = 0.39	104/177 = 0.59
44/124 = 0.35	64/146 = 0.44	53/131 = 0.40	57/94 = 0.61
40/170 = 0.37	58/124 = 0.47	77/191 = 0.40	72/117 = 0.61
47/122 = 0.38	42/104 = 0.50	68/169 = 0.40	69/110 = 0.63
51/127 = 0.40	87/162 = 0.54	63/157 = 0.40	73/116 = 0.63
80/198 = 0.40	57/104 = 0.55	53/128 = 0.41	59/89 = 0.66
46/112 = 0.41	112/131 = 0.85	69/162 = 0.42	81/116 = 0.70
		50/116 = 0.43	99/138 = 0.72
		38/84 = 0.45	69/96 = 0.72
		81/173 = 0.47	100/138 = 0.73
		72/148 = 0.49	93/125 = 0.74
		83/167 = 0.50	86/113 = 0.76
		40/77 = 0.52	82/91 = 0.90
(c) line 32		(d) line 21	
X/0 males/X.X/Y females		X/0 males/X.X/Y females	
32/122 = 0.26	106/152 = 0.70	41/103 = 0.40	65/89 = 0.70
53/101 = 0.52	96/133 = 0.72	49/112 = 0.44	66/89 = 0.74
70/131 = 0.53	77/105 = 0.73	98/210 = 0.47	81/107 = 0.76
65/118 = 0.55	83/111 = 0.75	69/143 = 0.48	105/134 = 0.78
74/121 = 0.61	89/116 = 0.78	56/114 = 0.49	105/127 = 0.83
72/117 = 0.61	79/101 = 0.78	55/96 = 0.57	122/147 = 0.83
57/89 = 0.64	67/82 = 0.82	86/144 = 0.60	77/92 = 0.84
97/135 = 0.64	109/130 = 0.84	96/158 = 0.61	112/134 = 0.84
64/98 = 0.65	151/150 = 1.0	71/114 = 0.62	75/88 = 0.85
80/120 = 0.67	105/103 = 1.02	80/129 = 0.62	132/146 = 0.90
		70/106 = 0.66	118/128 = 0.92
		101/153 = 0.66	150/161 = 0.93
		67/101 = 0.67	137/139 = 0.98
		93/138 = 0.67	92/90 = 1.03
		65/92 = 0.70	141/137 = 1.03
		87/122 = 0.71	127/116 = 1.09
		128/168 = 0.73	130/114 = 1.14

lines presenting a higher X resistance than the X 1C₁₁ (i.e. 21, 31 and 32) have a higher number of X ribosomal units. Conversely, the lines with lower X resistance (i.e. 9, 18 and 6) have a lower number of X ribosomal units.

It can be pointed out that the difference between the number of X ribosomal units of the two intermediate lines (32 and 6) and the number of X ribosomal units of the 1C₁₁ line is about 18 per cent, while this difference is more than 22 per cent for the most extreme lines.

Variations of supernatant resistance in the absence of recombination between homologous X chromosomes for lines 18, 6, 32 and 21

The results obtained with 1.5 per cent *B. thuringiensis* supernatant in crosses between 94 C1(RM) females and each of the (B+) males generated by one single X1C_{11n}/X Mu₅ female are reported in Table 3 (a: line 18, b: line 6, c: line 32, d: line 21). Significant variations in male/female ratios can be observed for each of the

four lines. They reveal variations in the degree of supernatant resistance conferred by different X chromosomes obtained from one single $X1C_{11n}/X Mu_5$ female.

The average male/female ratios calculated from the values presented in the four tables (respectively 0.43 for line 18, 0.53 for line 6, 0.69 for line 32 and 0.74 for line 21) are statistically different. Comparison of these ratios leads to the same classification of the four lines as previously reported in Table 1.

Discussion

We have shown that different X chromosomes, all originated from the $1C_{11}$ line which has been constructed from one single X chromosome, confer different degrees of resistance to the lethal effect of *B. thuringiensis* supernatant. Compared with the resistance conferred by X chromosomes of the $1C_{11}$ line, both an increase and a reduction in the degree of resistance was observed. For the six lines studied at a molecular level, we have observed a variation in the X rDNA content, compared with the $1C_{11}$ line and, for each line, a correlation between the variation in the degree of resistance and the variation at the X nucleolar organizer locus. No significant variations in X rDNA content have been observed between the initial $1C_{11}$ line and this line tested 20 generations later.

In a previous paper (Paumard-Rigal & Rosenberg-Bourgin, 1992), we had suggested that the modifications at the X rDNA locus were essentially reciprocal modifications. This hypothesis is corroborated by the results obtained here: we have observed, between the six lines studied at a molecular level, variations of X rDNA content in both directions while no variation in the X rDNA content of the $1C_{11}$ population from which the lines were isolated has been found. The X rDNA is thus subjected to frequent modifications that must be essentially complementary and thus originate from reciprocal molecular events.

The variations of resistance observed between the different X chromosomes obtained from single X $1C_{11}/X Mu_5$ females reveal variations of the X rDNA content in the absence of recombination between homologous chromosomes. Only unequal recombinations between sister chromatids can explain such results.

Unequal recombinations between sister chromatids had been considered by several authors as being the mechanism involved in the reversion of the 'bobbed' mutations during the magnification process (Tartof, 1973, 1974; Endow *et al.*, 1984; Hawley & Tartof, 1985).

Our results are in agreement with the hypothesis that these recombinations are extensively involved in the

variations of the X ribosomal DNA in wild-type strains.

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