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Characterization of Form Variants of *Xenorhabdus luminescens*

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From *Xenorhabdus luminescens* XE-87.3 four variants were isolated. One, which produced a red pigment and antibiotics, was luminescent, and could take up dye from culture media, was considered the primary form (XE-red). A pink-pigmented variant (XE-pink) differed from the primary form only in pigmentation and uptake of dye. Of the two other variants, one produced a yellow pigment and fewer antibiotics (XE-yellow), while the other did not produce a pigment or antibiotics (XE-white). Both were less luminescent, did not take up dye, and had small cell and colony sizes. These two variants were very unstable and shifted to the primary form after 3 to 5 days. It was not possible to separate the primary form and the white variant completely; subcultures of one colony always contained a few colonies of the other variant. The white variant was also found in several other *X. luminescens* strains. DNA fingerprints showed that all four variants are genetically identical and are therefore derivatives of the same parent. Protein patterns revealed a few differences among the four variants. None of the variants could be considered the secondary form. The pathogenicity of the variants decreased in the following order: XE-red, XE-pink, XE-yellow, and XE-white. The mechanism and function of this variability are discussed.

Xenorhabdus spp. are entomopathogenic bacteria symbiotically associated with nematodes of the genera *Steinernema* and *Heterorhabditis* (15, 20). The infective dauerlarvae of the nematode carry the bacterial symbiont in the intestine. The nematode penetrates an insect host, moves into the hemocoel, and releases the bacterium. The bacterium starts multiplying and kills the host, helped by excretion products of the nematode that repress the immune system of the insect. In addition, *Xenorhabdus* spp. produce antibiotics to inhibit the growth of other microorganisms in the insect cadaver and provide nutrients utilized by the nematodes (2, 20). Each *Steinernema* species is associated with its own *Xenorhabdus* species (3). The symbiont for all *Heterorhabditis* spp. is *Xenorhabdus luminescens*. However, the species *X. luminescens* is composed of several DNA homology groups that may be considered separate species (19). These bacterial groups are associated with *Heterorhabditis* spp. and strains that can be separated in the same DNA homology groups (19).

Xenorhabdus isolates tend to produce two colony forms, a primary form and a secondary form (1, 4, 6). The unstable primary form is preferentially taken up by the infective dauerlarvae and is often converted into the secondary form when cultured in vitro. The primary form has the ability to produce antibiotics and a pigment, can absorb dye from agar media and, in the case of *X. luminescens*, is luminescent. The secondary form has lost these abilities and does not support the growth of the nematodes as well as the primary form (1, 4, 7). Reversion from the secondary to the primary form has not yet been detected in any *X. luminescens* strain (4).

The nematode-bacterium complex shows great potential as a biological control agent for noxious insects in cryptic environments (13). A prerequisite for expanding the application of this agent is a low-cost in vitro production system for the nematodes. In both in vitro production systems, the Bedding method (5) and the liquid fermentation system (8), form variants of *Xenorhabdus* spp. are a major cause of failure, leading to a lower yield and a suboptimal quality of the nematodes (1, 9). Consequently, a better understanding of the variability of the bacterium is essential. In this paper, form variants of *X. luminescens* XE-87.3, the symbiont of Dutch *Heterorhabditis* strain NLH-E87.3, are described.

MATERIALS AND METHODS

Isolation and subculturing of bacteria. Table 1 lists the sources of the nematodes from which the bacteria were isolated. Bacteria were isolated directly from infective-stage nematodes. The nematodes were surface sterilized in 0.1% Merthiolate for 2 h, washed in sterile Ringer's solution (Oxoid), and macerated with a glass rod (1). Samples of the macerated material were spread on nutrient agar (0.8% Lab Lemco Broth [Oxoid]; 1.5% agar) and incubated in the dark at 25°C. The bacterium of *Heterorhabditis* strain NLH-E87.3 was also obtained by isolation from the hemocoel of waxmoth larvae (*Galleria mellonella*) infected with the nematodes. Red or orange luminescent colonies were selected. These colonies were subcultured every 3 or 4 days on nutrient agar. White and yellow variants were subcultured from single colonies without any red sectors. Shake cultures of the bacteria were made by inoculating single colonies into 50 ml of nutrient broth (0.8% Lab Lemco Broth [Oxoid]), shaken at 120 rpm and 25°C in the dark.

Isolation of form variants. Distinct colonies were selected from subcultures of the primary form and cultured separately.

General methods. Luminescence was determined by observation of cultures on nutrient agar in a dark room for 10 to 15 min. The production of antimicrobial substances by *X. luminescens* strains was determined as described by Akhurst (2). The test organism was *Micrococcus luteus*. The uptake of neutral red and bromothymol blue was tested on MacConkey agar and nutrient agar with 0.004% (wt/vol) triphenyltetrazolium chloride and 0.025% (wt/vol) bromothymol blue (NBTA), respectively.

DNA and protein analyses. Restriction endonuclease anal-

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TABLE 1. Origins of *X. luminescens* strains used in this study

Bacterial strain	Associated nematode strain or species	Homology group[a]	Original place of isolation
XE-87.3	NLH-E87.3	1	Eindhoven, The Netherlands
XL-81	NLH-L81	1	Limburg, The Netherlands
XF-85	NLH-F85	1	Flevoland, The Netherlands
XFR-86	NLH-FR86	1	Friesland, The Netherlands
XNH1-87	NLH-NH187	1	North Holland, The Netherlands
XW-79	NLH-W79	1	Wageningen, The Netherlands
XNB-87	NLH-NB87	1	North Brabant, The Netherlands
XB-87.1	NLH-B87.1	1	Bergeyk, The Netherlands
XSH-2	DH-SH-2	1	Kiel, Germany
XSie	PLH-Sie	1	Siedlce, Poland
XM145	IRLH-M145	2	Ireland
XMol	GISH-Mol	3	Moldavia
Xbac	*Heterorhabditis bacteriophora*	3	Brecon, South Australia

[a] *Heterorhabditis* DNA homology groups: 1, Northwest European group; 2, Irish group; 3, *H. bacteriophora* group. Data are from reference 19.

ysis, purification of DNA, agarose gel electrophoresis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described by Sambrook et al. (17). Restriction fragment length polymorphism analysis was performed with total DNA of the bacterium digested with the restriction enzyme *Msp*I or *Eco*RI. The fragments were separated on a 1% agarose gel. Protein patterns of whole-cell extracts of *X. luminescens* were made on a 12% SDS-polyacrylamide gel. The cells used for the DNA fingerprint and total protein analyses were collected from 3- to 4-day-old shake cultures. The cultures were checked for their color and cell size. Some cultures of the (unstable) yellow and white variants turned red. These cultures were not used.

Injection. Waxmoth larvae were injected with 3 μl of a suspension of bacteria in Ringer's solution. A 5-μl syringe was used to inject 2,000, 500, 50, 10, or 0 (control) bacterial cells per larva. The larvae were incubated in petri dishes at 25°C. Each treatment involved 15 larvae and was done in three replicates. The larvae were checked for mortality every day for 7 days. The results were statistically analyzed by analysis of variance. Logit transformation was performed to stabilize variance. Pairwise comparisons were made by the least-significant-difference test.

RESULTS

Form variants of XE-87.3. Four form variants of XE-87.3 were found (Table 2). A red variant (XE-red) and a white variant (XE-white) were isolated directly from both nematodes and infected waxmoth larvae. Yellow and pink variants (XE-yellow and XE-pink, respectively) were isolated, respectively, after 2 and 3 months of subculturing of XE-red on nutrient agar; neither occurred as frequently as XE-white. XE-red had all the characteristics of the primary form, as described by Akhurst (1): antibiotic and pigment production, inclusion bodies, luminescence, and uptake of dye. When a single red colony was subcultured, some small white colonies always developed in between the red colonies (Fig. 1A). Older red colonies, when subcultured, developed more white colonies than did younger ones. Occasionally, red colony plates developed no white colonies, but subcultures always developed them. In most small white colonies, red sectors started to appear after 3 to 5 days (Fig. 1B). White colonies and white parts of segmented colonies were composed of small cells, but the red sectors were composed of large cells, like XE-red cells. Young white colonies contained very few large cells, but after 48 h the number of large cells increased. When a single white colony without red sectors was subcultured, a few red colonies always developed in between the white colonies. When a red sector of a white colony was subcultured, more red than white colonies developed. Therefore, it was possible to select for more white colonies than red and vice versa, but is was not possible to separate them completely. The pink variant was first noticed on a MacConkey agar plate. This variant was very similar to the red variant, except for its pigmentation and uptake of dye. The yellow variant was very similar to the white variant. Like the white variant, it had small cells, was very unstable, and developed red sectors with large cells. Some of the yellow variant cells were slightly larger (2 to 3 μm) and carried inclusion bodies. The characteristics of the

TABLE 2. Characteristics of the four form variants of *X. luminescens* XE-87-3[a]

Variant	Pigmentation on nutrient agar	Colony form	Cell size (μm)	Luminescence	Inclusion granules	Antibiotic production	Uptake of:		Colony stability
							Neutral red	BTB	
XE-red	Red	Large, uneven margin	Large (4–8)	++	+	++	++	+	Stable[b]
XE-pink	Pink	Large, uneven margin or round	Large (4–8)	++	+	++	−	−	Stable[b]
XE-yellow	Yellow	Medium, round, smooth margin	Small (1–3)	+	±	+		−	Unstable; shifted to XE-red after 3 days
XE-white	None	Small, round, smooth margin	Small (1–2)	±	−	−		−	Unstable; shifted to XE-red after 3 days

[a] ++, positive response; +, moderate response; ±, weak response; −, negative response.
[b] Although small cells were produced in older colonies, the colonies stayed the original color.

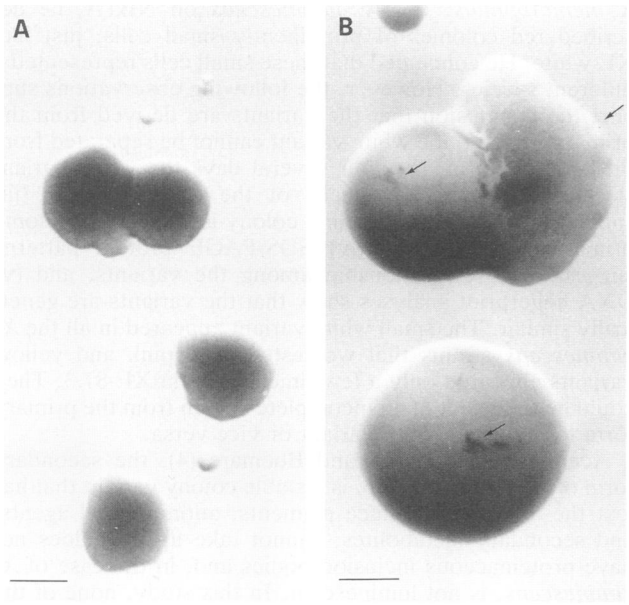

FIG. 1. Colony variants of *X. luminescens* XE-87.3. (A) XE-red colonies with some small white colonies (XE-white) in between. Bar, 0.8 mm. (B) A 4-day-old white colony (XE-white) with red sectors (arrows). Bar, 0.25 mm.

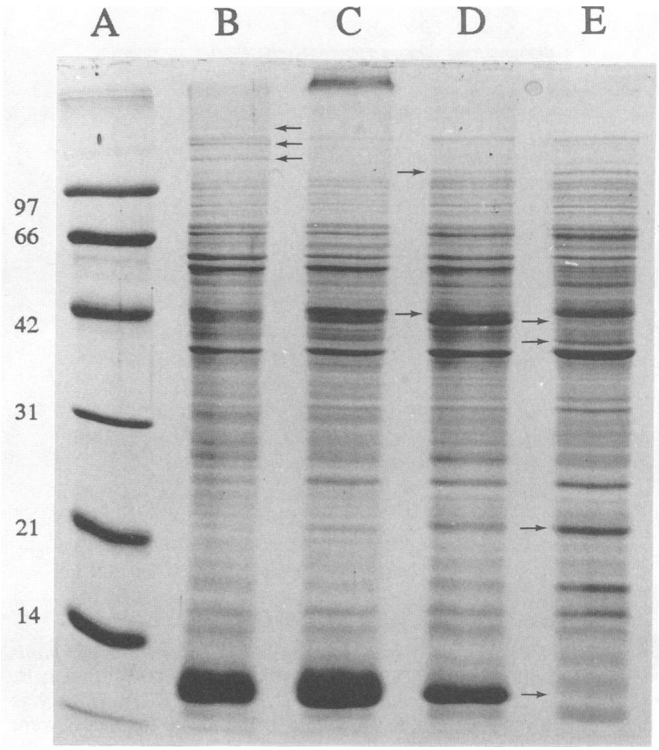

FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel (12%) of whole-cell extracts of *X. luminescens* variants. Arrows mark the positions of differences in protein bands among the variants. Lanes: A, molecular mass standards (in kilodaltons); B, XE-red; C, XE-pink; D, XE-yellow; E, XE-white.

four variants are listed in Table 2. On NBTA plates XE-pink, XE-yellow, and XE-white produced red colonies. The middle of the colonies was dark red, and the color faded toward the margins. XE-red produced greenish brown colonies on NBTA plates. When grown in shake cultures, the four variants raised the pH of nutrient broth (pH 6) to pH 8.5. When the pH was lowered to pH 2 with a few drops of 10 M HCl, XE-red and XE-pink turned yellow, while XE-white and XE-yellow did not change color. This result suggests that XE-white and XE-yellow do not produce the pH-sensitive anthraquinone pigment described by Richardson et al. (16).

Variants of other *X. luminescens* strains. From each of the other nematode strains listed in Table 1, including nematodes from two other DNA homology groups, large red and small white *X. luminescens* forms were isolated. In all cases, the red (sometimes orange) bacterium had all the characteristics of XE-red and the small white bacterium had all the characteristics of XE-white (Table 2). These strains were only subcultured twice, and no other colony forms were found.

Protein analyses. The SDS-PAGE protein patterns of the four form variants were compared to study differences in their metabolites. Figure 2 shows the SDS-PAGE protein patterns of the four XE-87.3 variants. The differences among the variants are marked with arrows. Although the patterns were not identical, they were very similar. There were a few differences in proteins of 40 to 42 kDa. XE-pink lacked some large proteins (>97 kDa). Some proteins (e.g., 21 kDa) were produced in smaller amounts in one or more variants. Characteristic was the large protein band of approximately 10 kDa. This band was absent in XE-white and smaller in XE-yellow. The similarity among the patterns of the four XE-87.3 variants was much greater than the similarity between the XE-87.3 patterns and the pattern of the closely related *X. luminescens* strain Xbac (unpublished results).

DNA fingerprint analyses. To make certain that the four variants were all *X. luminescens* XE-87.3 forms and not contaminants, we carried out DNA fingerprint analyses. The *Msp*I digestion patterns of the variants (Fig. 3) were identical. The *Eco*RI digestion patterns (not shown) were also identical for the four variants. Since the restriction enzyme digestion pattern of the genomic DNA of each bacterial species is unique, the four variants were all *X. luminescens* species and most likely derivatives of the same parent.

Injection experiment. Figure 4 shows the results of the injection experiment. None of the control larvae, injected with Ringer's solution, died. There was a significant difference in pathogenicity among the variants ($\alpha = 0.05$). The pathogenicity decreased in the following order: XE-red, XE-pink, XE-yellow, and XE-white; however, the difference in pathogenicity between XE-red and XE-pink was not significant ($\alpha = 0.01$; least significant difference test). XE-red not only killed the larvae at a lower dose (Fig. 4) but also killed the larvae more quickly than the other variants; at a concentration of 200 cells per larva, the mortality was 100% after 2 days with XE-red, while XE-pink, XE-yellow, and XE-white reached their maximum mortality after 4, 7, and 7 days, respectively. Dead waxmoth larvae were all red, independent of the color of the bacterium that was injected. The same color of the bacterium that was injected could be isolated from the dead larvae again. However, from larvae injected with XE-red or XE-pink, XE-white was also isolated, and from larvae injected with XE-yellow or XE-white, XE-red was also isolated.

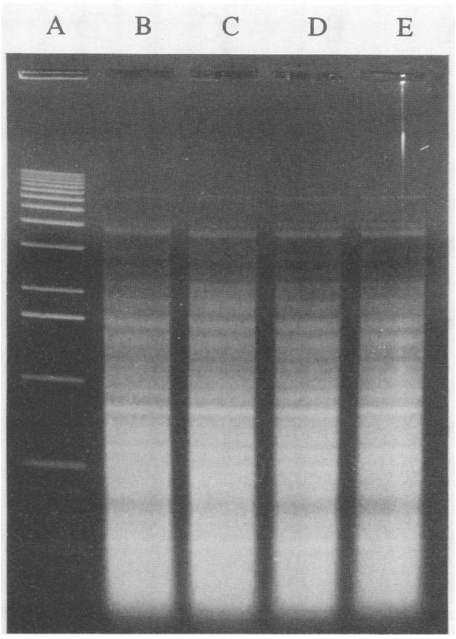

FIG. 3. Fingerprint of total DNAs isolated from four *X. luminescens* variants. Lanes: A, 1-kb DNA marker (GIBCO/BRL); B, XE-red; C, XE-pink; D, XE-yellow; E, XE-white. The DNA was digested with the restriction enzyme *Msp*I, and the fragments were separated on a 1% agarose gel.

DISCUSSION

X. luminescens XE-87.3 shows more morphological variation than the usual primary and secondary forms described for most *Xenorhabdus* strains (4). The red variant can be seen as the primary form of XE-87.3, but the other variants described here differ from the primary and secondary forms in several morphological and physiological characters. The DNA fingerprint and SDS-PAGE protein pattern analyses proved that all three variants are derived from the primary form and are not contaminants, especially the white variant, with its small colonies and cells, which differs considerably from the primary form. Wouts (21) found small cells in the primary form of *X. luminescens*, *X. poinari*, *X. bovieni*, and *X. nematophilus*. For *X. luminescens* on NBTA, he described red colonies of prominently small cells, just like XE-white. He concluded that these small cells represented a different species. However, the following observations support the conclusion that the variants are derived from the primary form: (i) the white variant cannot be separated from the primary form, (ii) after several days the white variant starts to produce large cells of the primary form, (iii) subcultures of one small white colony always contain some primary-form colonies, (iv) SDS-PAGE protein patterns suggest a close relationship among the variants, and (v) DNA fingerprint analyses show that the variants are genetically similar. The small white variant appeared in all the *X. luminescens* strains that we tested. The pink and yellow variants appeared only a few times in strain XE-87.3. They could be the result of an incomplete switch from the primary form to the small white variant or vice versa.

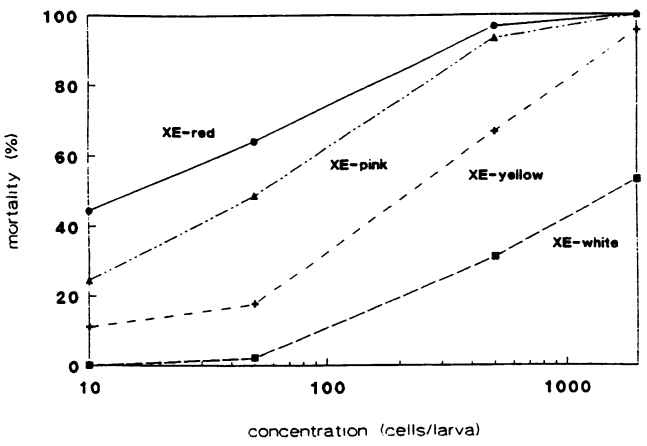

FIG. 4. Mortality of waxmoth larvae 7 days after injection with cells of the four form variants of *X. luminescens* XE-87.3, XE-red, XE-pink, XE-yellow, and XE-white.

According to Akhurst and Boemare (4), the secondary form of *Xenorhabdus* spp. is a stable colony variant that has lost the ability to produce pigments, antimicrobial agents, and secondary metabolites, cannot take up dye, does not have proteinaceous inclusion bodies and, in the case of *X. luminescens*, is not luminescent. In this study, none of the variants fits this description of a secondary form. The pink and yellow variants still produced antibiotics and were luminescent. The white variant had most of the abilities of a secondary form, except for the small cell and colony sizes and the instability. Therefore, it is most likely not a secondary form. Hurlbert et al. (12) described several form variants of *X. luminescens* HP88. One of these, a small-colony form, resembles the white variant described here. It also has small cells, is very unstable, and produces red sectors in the colonies. Hurlbert et al. (12) and Wouts (21) found their small variants in *Xenorhabdus* strains which also have a secondary form, indicating that the white variant should not be considered the secondary form of XE-87.3. Bleakley and Nealson (6) found a secondary form of *X. luminescens* Hm after several months in a shake-culture or after anaerobic growth. It is possible that a secondary form of XE-87.3 could be isolated this way. Hurlbert et al. (12) also observed their small variant in six other *X. luminescens* strains. We found a small variant in 13 *X. luminescens* strains, and Wouts (21) found small cells in the primary form of *X. luminescens*, *X. poinari*, *X. bovieni*, and *X. nematophilus*. These results indicate that this small-colony variant, consisting of small cells, is typical for *Xenorhabdus* spp.

The primary and secondary forms of most *Xenorhabdus* strains are equally pathogenic (1, 4). The variants of XE-87.3, on the other hand, differ significantly in their pathogenicity (Fig. 4). This variation may be of importance for the pathogenicity of the nematode. When an infective nematode carries a less pathogenic bacterium in the intestine, the combination also will be less pathogenic. The pathogenicity of XE-white is very low. It is possible that the small cells of XE-white are not pathogenic at all and that only the few large cells, actually XE-red cells, kill the insect. These cells may be present at injection of the bacterium or may develop after injection. Since it is not possible to separate the red and white variants completely, it is not clear which cells kill the insect. The loss of pathogenicity of XE-white can be caused by the lack of toxin production. Ensign et al. (10) purified the toxin of *X. luminescens* NC-19. In SDS-PAGE the toxin molecule had a major band of 40 kDa. Figure 2 shows at approximately 40 to 42 kDa two bands for XE-red and XE-pink. XE-yellow has a small upper band, while the lower band is wider. XE-white has lost the lower band and has only

a small upper band. It is possible that these bands, of 40 to 42 kDa, represent the toxin molecule of *X. luminescens* XE-87.3. The loss of this toxin would explain the loss of pathogenicity of the variants. The large protein band of 10 kDa might represent the protein of the inclusion bodies. This protein can constitute 60% of the total cell protein (10). XE-white does not have inclusion bodies and also lacks the 10-kDa protein band, while XE-yellow has only a few inclusion bodies and has a less wide band. In protein patterns of variants of *X. luminescens* XL-81, XF-85, XSH-2, XSie, and XMol, the 10-kDa protein band is also lacking in the white variant (unpublished results).

The significance of the polymorphism of *Xenorhabdus* spp. to the bacteria themselves is unknown at the present time. The small variant could have a survival advantage. It does not produce secondary metabolites, so it can use more energy for reproduction. Should the nematode prefer the primary form over the small variant, the latter could prevent the nematode from eating all the bacterial cells before they could multiply. This hypothesis might explain the shift from the primary form to the white variant and vice versa. After the small cells have multiplied for a while, there is a shift to large cells. The nematode can use these cells as a food source for reproduction and can take up the large (more pathogenic) cells to invade and kill a new insect host.

The mechanism by which these variants arise is still unknown. Several genes coding for different characteristics are switched on and off spontaneously at the same time. Frackman et al. (11) compared the genes coding for luminescence (*lux* genes) in primary and secondary forms of some *X. luminescens* strains by DNA hybridization. The in vivo luminescence of the secondary form was about 300 times lower than that of the primary form (14), but no difference was seen between the DNA structures of the *lux* genes of the two forms (11). This result strongly suggests that the difference between the primary and secondary forms is not caused by DNA deletion, insertion, or inversion within genes. Hurlbert et al. (12) have suggested that the variants arise by a mechanism similar to that of *Thiobacillus ferrooxidans* (18). The variation in *T. ferrooxidans* is caused by transposons or insertion sequences. Since these transposons or insertion sequences are not found in the *lux* genes of a secondary form, the conversion from the primary form to the secondary form is, at least for the change in luminescence, not regulated by this mechanism (11). The switch from the primary form to the secondary form may be caused by a mechanism different from that for the switch from the primary form to the small white variant. When conversion from one form to another is not regulated by deletion, inversion, or insertion within genes, it may be regulated by other genes that can at the same time switch off several genes coding for different properties.

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