NOTES AND COMMENTS

ECOLOGICAL BACTERIOLOGY OF THE MEADOW BROWN BUTTERFLY

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1. INTRODUCTION

BACTERIA have contributed generously to our knowledge of formal, biochemical and molecular genetics, but their ecological genetics has not attracted so much attention. Yet the ecological approach here outlined suggests certain novel and economical methods of finding strains of bacteria which may have been selected for many years as symbionts, protecting their hosts by antagonising other, potentially pathogenic, micro-organisms.

2. BACKGROUND

We have studied the microflora of the larval stages of the Meadow Brown Butterfly, *Maniola jurtina*, L., an insect about whose ecological genetics many data are available (see Ford, 1964). Subsequent work by McWhirter (two papers in prep.) shows that spot-number is strongly heritable and suggests that in the Isles of Scilly and in the Channel Islands intensive endocylic selection pressures operate against larvae carrying low-spot genes at an early stage in the life cycle and on fifth instar larvae or pupae carrying high-spot genes in June and July. Either sex or both may be affected according to habitat. The later, anti-high-spot, phase of selection may eliminate about 80 per cent. of the high-spot specimens of a susceptible sex, yet the colonies studied are usually stable *qua* spotting from year to year.

Very little is known of the agents procuring this intensive endocyclic selection; laboratory data suggest that the early phase of elimination may be partly or wholly mediated by (*a*) pathogenic bacteria or (*b*) failure of larvae to establish beneficial or necessary elements in their microfloras. Working with Scillonian eggs in the seasons 1961-62 to 1964-65 one of us (K.McW.) found that larvae derived from them do not as a rule succumb to a bacterial syndrome " Black Death " (in which the posterior third of the body blackens and the larva dies in 2 or 3 days); those English and Cornish larvae which reached third instar almost invariably died of Black Death in the laboratory environment then used. One exceptional Scillonian family of 17 tested, did succumb to Black Death, losing 45 out of 47 larvae; members of all other families from these islands, although kept in open containers near dying English larvae seemed to be immune. There must be a presumption of genetic immunity in these circumstances. *Pseudomonas fluorescens*

is found in the cadavers (Dowdeswell, 1965); this might be the primary pathogen in Black Death. Polyhedral virus is excluded as a factor in this syndrome.

In 1964-65 two typical lethal syndromes were observed for the first time in presumptive high-spot and low-spot specimens from the north end of Herm, Channel Islands. These larvae were grown in a cool laboratory; no analyses of them have yet been made, but virus is excluded.

In both English and Scillonian cultures as many as 50-80 per cent. of laboratory-reared larvae may fail to come through first and second instar; in some high-spotted Scillonian families this early larval mortality may be mitigated if the temperature of the laboratory is raised to 22° C.

Dowdeswell (1961, 1962) found it possible to raise fifth instar larvae swept from the field near Winchester in a cool laboratory without undue loss. One explanation of this could be that some degree of immunity from a bacterial pathogen is acquired in the field. Since haemolymph from laboratory-reared Scillonian larvae did not inhibit *Pseudomonas aeruginosa in vitro* (Lowbury, personal communication), it began to seem more likely that the microflora of the insects, though it might well be controlled by the genotype of the larva, was mediating both genetic and acquired immunity. Pinnock, working on healthy third-instar Scillonian and Channel Island larvae, found the haemolymph to be in every case sterile (personal communication). Attention therefore centred on the microfloras present in the gut and frass.

3. METHODS

In June 1965 three larvae swept in May from each of four habitats in England and the Channel Islands, which had been kept in open containers in the laboratory on growing grass, were passaged three times in sterile boiling tubes; washed but non-sterile cut grass was supplied in the first two tubes. The last passage was made without grass, but no attempt was made to sterilise the surface of the insect. Frass was collected from the last tube and thenceforward kept in controlled conditions; the larvae were grown on to maturity.

An analysis of the frass was made by Mr W. K. Stevens of Twyford Laboratories Ltd., and revealed surprisingly few species of bacteria, though cultures were plated out on aerobic, anaerobic and cooked meat media. Only 10 bacterial species were found in the samples, several recurring in frass from more than one habitat. Three types of *Pseudomonas* and one species of *Xanthomonas* occurred, along with a few representatives of the order Eubacteriales, from the genera *Aerobacter*, *Escherichia*, *Paracolobactrum*, *Micrococcus*, and *Achromobacter*.

In January 1966 we swept larvae from two Scillonian habitats and these were analysed in a different, but comparable way. Some of the larvae were kept in open containers in the laboratory for up to two months, but this hardly increased the variety of the microfloras of the guts. Larvae were killed with chloroform and put into one per cent. mercuric chloride solution for five minutes. They were then carefully washed in saline solution and homogenised in sterile conditions. A small amount of the homogenate was put into cooked meat and nutrient broth bottles to be used one or two days afterwards as controls and a second part was directly inoculated on blood

agar, nutrient agar and MacConkey agar plates both aerobically and anaerobically. The fore-guts of the larger larvae were excised in order to reduce the number of contaminants from the grass and the laboratory environment; in healthy larvae these do not persist in the mid- and hind-gut.

4. RESULTS AND COMMENTS

The following general observations based on 22 analyses were made; detailed lists of bacteria will be published elsewhere.

(1) Representatives of only three orders of bacteria have been found so far, Pseudomonadales, Eubacteriales and Actinomycetales. The first is found only in English and Channel Island larvae and is represented by the genera *Pseudomonas* and *Xanthomonas*; the third, represented by *Nocardia* spp. is found only in Scillonian larvae. Any species within the Eubacteriales which is found in Scilly has not so far been found in English and Channel Island larvae, and *vice versa*. So far there appears to be a rule of mutual exclusiveness; Scillonian bacteria are mainly Gram-positive and the English and Channel Island ones mainly Gram-negative.

(2) The larvae from the two Scillonian habitats have different microfloras, though *Bacillus licheniformis*, *Nocardia* spp. *Streptococcus* and *Staphylococcus* (sub-groups II-VI, Baird-Parker (1965)) are common to both. Genera represented in swept larvae from only one of the Scillonian habitats are *Gaffkya*, *Sarcina*, *Kurthia*, *Achromobacter* and *Arthrobacter*.

(3) Many bacteria were aberrant in one respect or another, but they could generally be approximately allocated to a known species.

(4) No evidence of phage was seen in several hundreds of cultures.

(5) Samples of the microflora from the two Scillonian habitats were taken from the grass and soil. These contained, as would be expected, rich microfloras with many representatives of the order Pseudomonadales and of the family Enterobacteriaceae. None of these, however, has been found in the mid-gut of 10 larvae from either habitat.

(6) The fore-guts of healthy Scillonian larvae contain a number of *Bacillus* species, but only *B. licheniformis* persists in the mid-gut.

(7) *B. licheniformis* is known as a source of antibiotic substances and is present in all Scillonian larvae so far examined, whether these are survivors of eliminative processes in the field or in the laboratory; *Nocardia* spp. also sometimes produce antibiotics, but these bacteria were not found in third instar larvae early in February, though they were frequent in March. There is a chance that these species, or some of them, are laboratory contaminants; the patterning of the microfloras according to venue, which has otherwise occurred, renders it unlikely that the other bacteria were not derived from the original microfloras of the swept larvae.

5. DISCUSSION

The genotypes of English and Channel Island larvae should (to judge from imaginal appearances) be more akin than those of the English and Scillonian strains. The bacterial repertoires seem to reflect this situation; hence we may suggest that interaction between larval and bacterial genotypes occurs in the field as it clearly does in the laboratory. There is virtually

no gene-flow between the two Scillonian *jurtina* colonies. Each must have co-evolved with the local bacteria for many years. It is quite possible that a series of different strains of the same bacterial species has evolved specially in each *jurtina* isolate, since not only does the host's gene-pool differ, but also the microfloral environments within the surviving larvae are various.

Although a function of some of the bacteria present could be to assist in the digestion of grass or to provide vitamin-like micro-nutrients, the ecological pattern as a whole, the unexpected absence of the whole order Pseudomonadales and the family Enterobacteriaceae from both wild and laboratory-reared Scillonian larvae and the apparently obligate occurrence in them of one or more strains of *B. licheniformis* make it probable that evolution of antagonistic systems has occurred.

The next experimental step is obviously to raise bacteriologically sterile grass and to control the introduction of specified strains of bacteria into sterile grass-larva systems. Dr D. A. Barber, of the A.R.C. Radiobiological Unit, has kindly tested grasses suitable for *jurtina* and informs us that this is possible.

Our ecological approach, however, has already made it reasonable to recommend a highly economic method of search for antagonistic systems. The relatively limited range of bacteria obtained from the guts of larvae from each isolated colony, that is perhaps 12 species, could possibly include a number which have been evolved as protectors against potentially pathogenic elements of the highly varied soil, plant and faecal (*i.e.* mammalian and avian) microflora. The same could apply to any insect which forms colonies between which there is little gene-flow. The Meadow Brown Butterfly certainly fits this description, but also has the advantage of being extremely dense, especially in its island habitats, and very widespread over the palaearctic region.

At the moment it is quite uncertain how *in vivo* antagonism, which is so strongly suggested by the ecological evidence, operates. When the isolated strains of *Bacillus licheniformis*, *Staphylococcus* and *Nocardia* are tested on nutrient and blood agar against the types of pseudomonads and enterobacteria which are found in the habitat, there appears to be no inhibition. Possible explanations of the observed exclusions and specialisations could be (1) that protective bacteria in the mid- and hind-gut produce heavy-molecular inhibitors, or (2) that the *Bacillus* spp. in the Scillonian fore-guts, which have not yet been analysed, act in an inhibitory manner, or (3) that the larval gut-walls secrete some kind of inhibitory substance; no tests have yet been completed to pursue these ideas. It is to be remembered that so far only larvae which have survived severe eliminative processes have been analysed. It will be possible to extend these studies to larvae in their first and second instars taken from the field and from controlled laboratory conditions.

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