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Drug resistance of bacteria commensal with *Drosophila melanogaster* in laboratory cultures.

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Abstract

Contamination of *Drosophila* cultures with a gram-negative bacterium resistant to multiple antibiotics was encountered. Isolation of the bacterium and evaluation of sensitivity to distinct classes of antibiotic proved necessary for eradication. A plasmid conferring resistance to ampicillin was recovered after transformation into *E. coli*. DNA sequencing supported a laboratory origin for the plasmid, but found no evidence for horizontal transfer to bacteria from transgenic fruitflies.

Introduction

According to the literature, bacterial contamination of *Drosophila* cultures is not uncommon and is readily eradicated by antibiotic treatment (Ashburner, 1989). In 1994 our laboratory experienced a more serious encounter with drug-resistant bacteria. In response, antibiotics were incorporated into fly food following published examples (Ashburner, 1989), but the infection proved insensitive to either penicillin (100 µg/ml in food) or streptomycin (100 µg/ml in food). Both chloramphenicol (25 µg/ml in food) and tetracycline (tetracycline 12.5 µg/ml in food) were promising initially, but resistant infection invariably emerged one or two generations after treatment. The infecting bacterium was cultured and isolated, a more systematic survey of antibiotic sensitivity performed, and a successful treatment developed based on these findings.

Drosophila culture being very widespread, but such recalcitrant bacterial infection not being reported, it was of interest to determine the basis for the multidrug resistance. Drug resistance of human bacterial pathogens has increased in recent decades and is attributed to antibiotic exposure and selection, but antibiotics are not used routinely in *Drosophila* culture. One exception is the increasing use of G418 to select flies inheriting transgenic neomycin resistance markers, especially for a set of FRT chromosomes in wide use for inducing mitotic recombination (Xu and Rubin, 1993). Use of antibiotic resistance genes as selection markers in genetically modified organisms has led to safety concerns relating to the escape or transfer of the antibiotic resistance genes to sensitive bacterial strains from genetically modified organisms. Notably, the method used to modify *Drosophila* to neomycin resistance also inserted a linked ampicillin resistance gene and *E. coli* replication origin (*ori*) sequences from the plasmid phsneo (Steller and Pirotta, 1985). We therefore sought to test the hypothesis that bacterial β-lactam and aminoglycoside resistance observed in our laboratory had arisen by horizontal transfer of neomycin resistance, ampicillin resistance, and *ori* sequences from the *Drosophila* genome to a commensal bacterium. Our results did not support any such event.

Results

Bacteria grew as a transparent slimy exudate on the surface of food vials that seemed to trap and stifle larvae. Robust *Drosophila* cultures survived, but weak cultures or single pair matings could not be sustained, rendering many experiments frustratingly difficult. Infection was introduced by contact with flies and was never seen in fresh, unused food vials. Strains could be sterilized individually by initiating cultures with embryos dechorionated with sodium hypochlorite (bleach). This supported the notion of vertical transmission by contamination of the egg cases by maternal faeces, typical for transmission of *Drosophila* gut flora (Ashburner, 1989). Unfortunately, cured strains often became reinfected over subsequent generations. Apparently infection was readily spread on CO₂ pads, paintbrushes, or other aspects of fly handling, attempts at cleanliness notwithstanding.

Bacteria were isolated and cultured by allowing flies from infected strains to walk across sterile fly food petri plates (yeast-glucose recipe, Ashburner and Roote, 2000) and then culturing the plates at 18°C or 25°C to recover micro-organisms from the tracks. The bacterium was isolated as a slow-growing pleiomorphic gram negative bacillus, frequently recovered growing in association with yeast initially, but easily subcultured apart from yeast and not dependent on it for growth under these conditions. The organism was not identified definitively by biochemical analyses undertaken by multiple pathology services.

Antibiotic sensitivity was assessed by comparing growth on yeast-glucose solid agar in the presence or absence of each of a series of antibiotics at standard concentrations used for selection of gram negative bacteria in the laboratory. Using this assay the bacterium was found resistant to penicillin (200 µg/ml in food), streptomycin (100 µg/ml in food), kanamycin (50 µg/ml in food), erythromycin (50 µg/ml in food), and vancomycin (10 µg/ml in food), but susceptible to tetracycline (20 µg/ml in food), chloramphenicol (25 µg/ml in food), ceftriaxone (50 µg/ml in food) and spectinomycin (100 µg/ml in food). The germicides benzalkonium chloride and *o*-phenyl phenol also inhibited growth of the organism, although in our hands effective concentrations of benzalkonium chloride (0.01-0.1% in food) were toxic to flies as well as bacteria. Little consistent effect was found of varying the antifungal component of the growth media from nipagin (2.7g/L) to propionic acid/phosphoric acid (0.36% and 0.036% respectively), or of its omission.

The bacterial contaminant was resistant to both aminoglycoside (streptomycin, kanamycin) and β-lactam (penicillin) antibiotics, but susceptible to third-generation cephalosporins (ceftriaxone). Resistance emerged frequently to chloramphenicol and tetracycline. It was suspected that frequent emergence of resistance to tetracycline and chloramphenicol might be related to the fact that under the growth conditions present, these agents are bacteriostatic, not bacteriocidal, for gram negative organisms. Bacteriostatic agents inhibit growth of organisms without killing them. Thus, in the absence of additional clearance mechanisms, such as an immune system, residual organisms remain present and able to replicate if permissive conditions are subsequently restored. Bacteriostatic agents are still useful in that they prevent the out-growth of relatively small numbers of organisms such as might be introduced into *Drosophila* food by contaminated flies. This should reduce the likelihood of resistance subsequently emerging to a bacteriocidal agent.

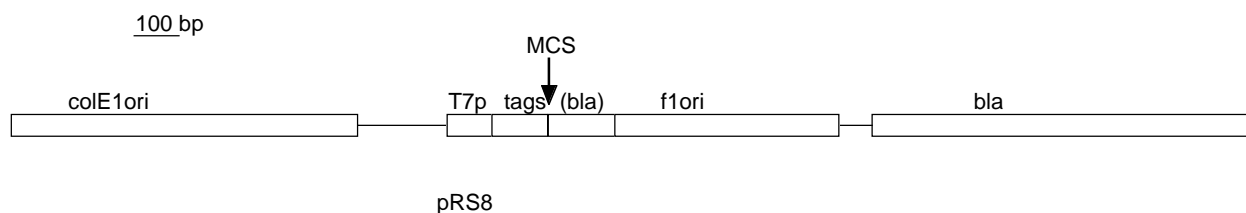
An antibiotic regimen was devised for eradication of the infection based on these findings. The bacterium was successfully eliminated by passage for one generation on yeast-glucose food containing *o*-phenyl phenol (0.1%) and tetracycline (20 µg/ml in food), followed immediately by passage for one generation on yeast-glucose food containing ceftriaxone (50 µg/ml in food), and spectinomycin, an aminocyclitol aminoglycoside that differs from other aminoglycosides in that it lacks aminosugars and

glycosidic bonds (50 µg/ml in food). Antibiotics were added as liquid food cooled below 50°C, from stock solutions in ethanol (*o*-phenyl phenol), methanol (tetracycline), or water (ceftriaxone, spectinomycin). The principal was to follow sharp reduction in the bacterial load with simultaneous exposure to two antibiotics that have distinct mechanisms of action and to which the organism was susceptible. Care was taken to remove adults and corpses from the *o*-phenyl phenol/tetracycline-containing media before emergence of the treated generation, to discard any cultures for which there was visible evidence of bacterial infection at this stage, to transfer immediately onto spectinomycin/ceftriaxone-containing media, and again to remove adults and corpses before emergence of the next, putatively-cured generation. During the *o*-phenyl phenol/tetracycline treatment step, *Drosophila* cultures were removed to a separate, previously uncontaminated environment, and all subsequent steps performed there until thorough sterilization of fly incubators and fly room had been performed (by heating of incubators and rigorous replacement of all fly room equipment except dissecting microscopes). >99% of our genetic strains survived this protocol, but not all thrived and some did not survive. Perhaps elimination of normal gut flora exposed auxotrophic mutations in some *Drosophila* strains that were otherwise inconsequential.

To investigate the basis for resistance, DNA was prepared from drug-resistant bacteria as described (Hopwood *et al.*, 1985). DNA was transformed directly into *E. coli* (strain XL1-Blue, Stratagene Corporation) and transformants selected in the presence of either 100µg/ml ampicillin or 10 µg/ml kanamycin. Only ampicillin-resistant colonies were recovered, consistent with presence of an ampicillin-resistant plasmid but not kanamycin-resistant plasmids. Ampicillin-resistant colonies were recovered at a rate equivalent to 40 copies per cell if the genome size of the unidentified bacterium is similar to that of *E. coli*. It is unknown whether this is the case.

In another approach, bacterial DNA was digested with one of the restriction enzymes *EcoRI*, *BamHI*, or *Sall*, none of which cuts the *neo^r* gene or *ori* sequence of phsneo, followed by ligation and transformation into *E. coli*. If kanamycin resistance was chromosomally encoded in a linked *neo^r-ori* segment like that of phsneo, ligation and transformation of such digests should yield kanamycin resistant colonies. No such kanamycin-resistant colonies were obtained, however.

Plasmid DNA from distinct ampicillin-positive clones fell into several different size classes. PCR primers were designed based on the beta-lactamase gene from phsneo. Such primer pairs amplified a predicted 789 bp product from 14/21 clones. One of these clones named pRS8 was selected for further analysis, because it was a member of the largest size class and because, initially, it appeared to template PCR products with primers derived from P element sequences.



A map of the DNA sequence of pRS8 is shown in Figure 1. The complete DNA sequence (2795 bp) has been deposited in Genbank under accession number (pending). The plasmid contained β-lactamase, *colE1 ori*, and bacteriophage *f1 ori* sequences similar to those of many cloning vectors. A 377 bp segment separating the *colE1 ori* and bacteriophage *f1 ori* contained a bacteriophage T7 promoter, translation start, (His)₆-tag sequence, T7-tag sequence, enterokinase site, multiple cloning site, and 159 bp sequence identical to an internal region of the β-lactamase gene. The plasmid contained no aminoglycoside-resistance gene, consistent with its failure to encode kanamycin resistance in *E. coli*.

The plasmid also lacked homology to P element transposons, to any part of the *Drosophila* genome, or to parts of the phsneo vector or FRT-containing plasmids other than the aforementioned β -lactamase and *colE1 ori* sequences that are present in many other plasmids also.

Discussion

The rising incidence of drug resistant pathogens suggests that multidrug resistant infection of *Drosophila* cultures may also become more common. Hopefully, our experiences may be helpful to other researchers dealing with such problems. We make a particular plea, however, for other researchers *not* to follow our antibiotic protocol without first isolating the particular infection and determining its specific properties. Otherwise selection of additional drug-resistance traits through inappropriate treatment is likely. Instead, the recommendation is to 1) culture the microorganism; 2) assess sensitivities to as many antibiotics as possible, representing distinct modes of action. These could include β -lactams, aminoglycosides, tetracyclines, macrolides, glycopeptides, chloramphenicol, fluoroquinolones, sulfonamides, trimethoprim, and nalidixic acid (rifampicin is not recommended because resistance emerges frequently by point mutation); 3) devise a procedure that minimizes the likelihood of selecting resistance. In our case, we first reduced the bacterial load and then simultaneously treated with two antibiotics to which the organism had been shown to be susceptible; 4) accompany the treatment with laboratory sterilization adequate to preclude reinfection, since exposing fly strains to the same antibiotics twice is a recipe for selecting resistance.

Isolation and DNA sequence of a plasmid conferring ampicillin resistance suggested that ampicillin and aminoglycoside resistance were independent. Aminoglycoside resistance did not seem to be encoded by any of the plasmids tested or by integrated plasmid genomes. Aminoglycoside resistance may have been encoded by a chromosomal resistance gene. A caveat to this conclusion is that several size-classes of ampicillin-resistance plasmids were recovered from supposedly cloned bacteria. Since each of these was isolated from the same contaminating bacteria and expressed the same antibiotic resistance pattern, it is likely that they represent derivatives from a single plasmid in the contaminant.

The DNA sequence of pRS8 strongly suggested a laboratory origin. Not only were 2.4 kb contiguously similar to widespread laboratory plasmids such as Bluescript, but a 377 bp segment containing a bacteriophage T7 promoter, translation start, in frame (His)₆-tag sequence, T7-tag sequence, enterokinase target and multiple cloning site is certainly engineered. Except for the T7 promoter, this segment resembles sequences from the yGalSET vector series, designed for inducible expression of tagged proteins in yeast (Enomoto *et al.*, 1998). Except for the multiple cloning site, this segment resembles sequences from the pRSET vector series, designed for inducible expression of tagged proteins in *E. coli* (Kroll *et al.*, 1993). No vector exactly like pRS8 has been reported, however, and the sequence revealed no clue as to its last laboratory use. The insertion of an internal fragment of an *amp^R* gene into the *Pst*I site seems unlikely to have been part of any deliberate cloning strategy.

We cannot determine from the available data whether pRS8 was selected within bacteria already commensal with a fly strain (in our laboratory or prior to arrival), or selected in a bacterium that subsequently became associated with a *Drosophila* culture. We also do not know why it was retained, given that *Drosophila* cultures are not normally exposed to ampicillin. Initially we had speculated that linkage to *neo^r* might have been responsible, since *Drosophila* strains are increasingly exposed to G418 to select for *neo^r* in transformed strains, and even that multiple antibiotic resistances could have derived from *amp^R*, *neo^r*, and *colE1 ori* sequences inserted into the *Drosophila* genome. We now reject this hypothesis, however, since we found no *neo^r* gene linked to pRS8, no sequences unique to the phsneo

plasmid or its derivative P[ry⁺, hs-neo, FRT], and no P element or other *Drosophila* sequences to indicate past residence in the *Drosophila* genome.

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