Chemical Ecology of Bacterial Relationships with Fruit Flies

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Abstract: The nature of relationships between fruit flies (Diptera: Tephritidae) and bacteria has been controversial. Theories of obligate symbioses have given over to facultative mutualism, accidental, and predator-prey depending on circumstances. Fruit flies are attracted to bacteria to quench drive states including protein hunger and others that are poorly understood. Following chemical functional group characterizations indicating attractive principals were mostly chemicals containing ionizable nitrogen, a novel technique was devised to identify ammonia, 1-pyrole, acetic acid, and several amines, pyrazines, and alcohols from bacterial odors. Mixtures of these chemicals in the same concentrations as in bacterial odors were about 80-90% as attractive as the odors to Mexican fruit flies. Volatiles produced by bacteria attractive to fruit flies were found to vary with bacteria taxon at all levels of classification and with culturing medium. Interactions of attractiveness of the chemicals are consistent with the need for fruit flies to forage for various bacteria species on various substrates. The information obtained in these studies is useful for development of fruit fly lures, improvement of fly cultures, and understanding of our natural world.

Key words: symbiosis, fruit fly, Tephritidae, bacteria, semiochemical, attractant

Introduction

Relationships between fruit flies (Diptera: Tephritidae) and bacteria are poorly understood. Petri (1910) reported symbiosis of the bacterium (Pseudomonas savastanoi) that causes olive knot disease with the olive fruit fly (Bactrocera oleae) and described how the bacteria are transferred through all life stages of the fly. Later, Yamvrias et al. (1970) were unable to find P. savastanoi associated with the olive fly, casting doubt on the validity of Petri’s work. However, the presence of bacteria in the fly that were transferred through life stages has been confirmed by later workers (Girolami, 1973, 1983; Mazzini and Vita, 1981). Allen et al. (1934) described a similar symbiosis between the bacterium (Pseudomonas melophthora) that can cause apple rot and the apple maggot fly (Rhagoletis pomonella). Again, later workers were unable to identify this bacterium from the apple maggot fly (Huston, 1972; Dean and Chapman, 1973).

Despite the uncertainty regarding the identities of the bacteria studied by Petri (1910) and Allen et al. (1934), these studies became the basis for the prevailing view during most of the 20th century that fruit fly associations with bacteria were generally obligate symbioses (Buchner, 1965). As research into bacterial associations accelerated during the last quarter of the 20th century, it became more apparent that bacteria in fruit fly digestive tracts reflected bacteria found in the flies’ environment (Howard, 1989). Studies in which numerous species of bacteria were found associated with a single fly and these same bacteria could be isolated from fruit fly hosts became common (Yamvrias et al., 1970; Tsiropoulos, 1983; Fitt and O’Brien, 1985; Drew and Lloyd, 1989; Martinez et al., 1994) and the theory that bacteria maintained obligate symbioses with fruit flies began to erode. By the end of the century, few believed that any fruit fly associations with bacteria represented obligate symbioses (Howard, 1989; Drew and Lloyd, 1989).
New theories arose to replace obligate symbioses. Huston (1972) considered bacteria accidental symbiotes because the species associated with the apple maggot fly were determined by the species in its habitat. Drew et al. (1983) concluded that bacteria serve as a protein source for fruit flies after a series of studies in which Drew and his colleagues found that flies seeded their environment with bacteria then later foraged on the colonies, that bacteria were more numerous in the crop and midgut than in the feces indicating digestion, and that flies fed a diet of bacteria as their protein source produced as many eggs as those fed standard breeding diet. Robacker and Moreno (1995) hypothesized that metabolites emitted during bacterial breakdown of protein elicits attraction due to an innate neural association of bacterial odor with the presence of protein. Rather than completely abandoning close symbioses, others consider that bacteria and fruit flies engage in facultative mutualistic symbioses in some cases (Lauzon et al. 2000). Compelling evidence for these types of symbioses are formation of biofilms in fruit fly intestines, specialized organs in fly gastrointestinal tracts where bacteria are harbored, and vertical transmission of bacteria through all life stages of the flies (Stammer, 1929; Mazzini and Vita, 1981; Lauzon, 2003), as had been described originally by Petri (1910). The major difference now is that most associations are thought to be flexible. As examples, perhaps more than one bacteria species could fulfill the role of symbiont in a particular fly (Lauzon, 2003), and maybe no bacteria are necessary if diet already contains digestible nutrients (Howard, 1989). However, the theory that an obligate ‘coevolved’ bacterium symbiosis exists in the olive fly has not been disproved and continues to garner evidence (Capuzzo et al., 2005).

As a corollary to symbiosis is the question of what roles bacteria play in mutualistic symbioses with fruit flies. Among the proposed functions of bacteria are that they may biosynthesize essential amino acids (Miyazaki et al., 1968), serve as food (Drew and Lloyd, 1989) or indicators of food (Robacker and Moreno, 1995), convert unusable biochemicals into nutrients that flies can digest (Lauzon et al., 2000), detoxify allelochemicals in food and otherwise protect the gut from ingested toxins (Lauzon et al., 2003), and fix atmospheric nitrogen in the gut of flies (Behar et al. 2005). Quite possibly, different bacteria may take on different roles and maintain different levels of association within the life history of a single species of fruit fly.

Putting the question of symbiosis aside, two undeniable facts make the study of fruit fly relationships with bacteria of great relevance: bacteria are ubiquitous in fruit flies and they are attractive to fruit flies. The ubiquity of associations between fruit flies and bacteria was documented above (Yamvrias et al., 1970; Tsiropoulos, 1983; Fitt and O’Brien, 1985; Drew and Lloyd, 1989; Martinez et al., 1994). Demonstrations that fruit flies are attracted to bacteria have been published for numerous species of both flies and bacteria (Drew and Lloyd, 1989; Jang and Nishijima, 1990; Robacker et al., 1991; MacCollom et al., 1992; Epsky et al., 1998).

Despite nearly universal recognition that bacteria are attractive to fruit flies, relatively little has been accomplished regarding development of fruit fly lures from bacteria-produced attractants. One reason is that identification of chemicals produced by bacteria that are attractive to fruit flies has been nearly as problematic as determination of the nature of bacteria/fruit fly relationships. Production of ammonia by bacteria and its attractiveness to fruit flies has been common knowledge for most of the last century (Jarvis, 1931). Interestingly, several early studies suggested that ammonia was not really that attractive to fruit flies. Gow (1954) and Drew and Fay (1988) each believed chemicals produced by bacteria other than ammonia were primarily responsible. Gow (1954) even went so far as to hypothesize that the primary attractants of bacterial cultures were water soluble, nitrogen-containing chemicals other than ammonia. Unfortunately, his idea was largely ignored for 40 years as no serious studies to determine the identities of those chemicals were undertaken.
Studies done later to identify the attractive principals of bacterial fermentations showed a lack of understanding of why bacteria were attractive and therefore what type of chemicals would be involved (Robacker, 1998a). Because of the lack of understanding, bioassays were not designed properly so flies were not usually primed to respond to bacteria. Also, the chemistry methods were not appropriate for the type of chemicals that were involved. To make matters worse, often chemists performed the studies without cooperation with biologists or vice versa so chemicals were identified but not tested as attractants, or bioassays were done on bacterial fermentations but no identifications of the attractive principals were conducted. All of this led to identification of the wrong chemicals including numerous alcohols, aldehydes, ketones, pyrazines, sulfides, carboxylic acids, and aromatics.

In this paper I review work done in my lab over the past 15 years to identify chemicals produced by numerous species and strains of bacteria that are attractive to the Mexican fruit fly. Based on the results I propose a theory tying together the chemistry of bacterial odors with the ecology of fruit fly associations with bacteria.

Material and methods

Bacteria culturing

*Staphylococcus aureus* was isolated from laboratory colony Mexican fruit flies (Robacker *et al.*, 1991). *Enterobacter agglomerans* was isolated from wild *R. pomonella* and *Anastrepha ludens* (Robacker *et al.*, 2004). Most other bacteria were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). Some *Bacillus thuringiensis* strains were obtained from the Institut Pasteur (Paris, France). Most bacteria were cultured in tryptic soy broth (TSB) (Robacker *et al.*, 1991). *E. agglomerans* was cultured in Petri plates using media with glucose and uric acid (uric acid medium) (Robacker and Lauzon, 2002), casein peptone (protein medium), glucose and casein peptone (TSB), or sucrose (carbohydrate medium (Robacker *et al.*, unpublished) as the carbon sources.

Attractiveness bioassays

Two types of bioassays were used. One was a cage-top bioassay in which fruit flies in a screen cage were exposed to odor sources loaded onto filter papers and placed on the top of the cages, elevated to prevent contact chemoreception (Robacker *et al.*, 1991). The numbers of flies beneath the filter papers were counted every minute for 10 minutes and compared with the numbers that were beneath papers loaded with water, solvent, or uninoculated culturing medium as appropriate.

The second was a wind-tunnel bioassay in which odor sources were placed in the upwind end of a plexiglas tunnel screened on each end to allow airflow through the chamber and flies were released in the downwind end of the tunnel. The numbers of flies that moved upwind and contacted the odor source within 5 min of sample introduction were recorded (Robacker and Lauzon, 2002).

Chemistry

Bacterial cultures were aqueous samples for most work. Collection of volatiles from bacteria samples was done using solid phase microextraction with a PDMS fiber (Supelco, Inc., Bellefonte, Pennsylvania, U.S.A.). The fiber was exposed in the headspace above the bacteria sample then chemicals were thermally desorbed from the fiber in a heated injection port of a gas chromatograph (GC). Various GC models were used over the course of this work. For most analyses, on-column injection was conducted into a fused silica retention gap connected to the analytical GC column. The analytical column was usually a DB-1 with a 5 micrometer
film (J & W Scientific, Folsom, California, U.S.A.). Detection and identification of eluted chemicals was by flame ionization, flame thermionic, or mass spectrometry (MS). Details of methods can be found in various publications (Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker et al., 2004).

Results and discussion

Establishment of a physiological link to protein hunger
Tryptic soy broth cultures of S. aureus were more attractive to sugar-fed Mexican fruit flies than to flies deprived of sugar (Figure 1) (Robacker and Garcia, 1993). The effect increased as the period of deprivation increased. Protein deprivation had the opposite effect. Attraction of flies to S. aureus increased as the amount of protein in their adult diet diminished (Figure 2) (Robacker and Moreno, 1995). These experiments indicated that there is a physiological link between attraction of Mexican fruit flies to tryptic soy broth cultures of S. aureus and hunger for sugar and protein. This finding led to a hypothesis that bacteria odor elicits innate foraging for proteinaceous food. Sugar hunger inhibits this response because carbohydrate hunger is critical to immediate survival and the need for energy overrides the need for protein. Only after a sugar meal can flies resume protein foraging behavior. Note, however, that this relationship may not hold in cases in which attraction to bacteria may be occurring for another reason such as to replenish gut microflora (Lauzon, 2003). Evidence that finding protein is not the only reason fruit flies seek bacteria is that protein-fed Mexican fruit flies, even flies satiated on a complete breeding diet, continue attraction to S. aureus cultures at higher rates than to water controls, although at lower rates than protein-deprived flies (Robacker and Garcia, 1993; Robacker and Moreno, 1995).

![Chart showing effect of sugar deprivation on attraction of Mexican fruit flies to tryptic soy broth (TSB) cultures of S. aureus. Attractiveness = number of flies attracted to the bacteria culture relative to the number attracted to uninoculated TSB.]

Figure 1. Effect of sugar deprivation on attraction of Mexican fruit flies to tryptic soy broth (TSB) cultures of S. aureus. Attractiveness = number of flies attracted to the bacteria culture relative to the number attracted to uninoculated TSB.

Isolation and characterization of attractive principals
Experiments were conducted to determine if the attractive principals from S. aureus cultures became dissolved in the aqueous medium as cells were cultured. Pellets containing cells were
obtained from bacterial cultures by centrifugation. Supernatant was filtered to remove the remaining cells. Supernatants were >3X more attractive than resuspended pellets in cage-top bioassays indicating that attractive principals produced by the bacteria were concentrated in the aqueous medium (Robacker et al., 1993).

Figure 2. Effect of protein in adult diet on attraction of Mexican fruit flies to tryptic soy broth (TSB) cultures of *S. aureus*. Attractiveness = number of flies attracted to the bacteria culture relative to the number attracted to uninoculated TSB.

Figure 3. Effect of pH of bacterial supernatant on attraction of Mexican fruit flies to tryptic soy broth cultures of *S. aureus*. Attractiveness = number of flies attracted to a pH treatment relative to the number attracted to water.

The pH of filtered supernatants of *S. aureus* cultures was manipulated to determine the effect on attractiveness (Figure 3) (Robacker et al., 1993). Preparations with pH between 7-10
were the most attractive to sugar-fed Mexican fruit flies. This indicated that the attraction was
due to chemicals that contained protonizable nitrogen because these chemicals would be
ionized and thus nonvolatile at the lower pH's. Similar results were obtained with numerous
other species of bacteria (Robacker and Bartelt, 1997; Robacker et al., 1998).

Additional chemical functional group tests also implicated nitrogenous chemicals as the
attractive principals of bacterial odor. These included formation of nonvolatile salts of the
attractive chemicals at low pH that could be completely dried then reactivated by dissolution
in water at high pH, retention on strong cation exchange media at low pH then elution at high
pH, and lack of retention of attractive principals on reversed-phase HPLC unless paired with a
negative counter ion (Robacker et al., 1993).

Identification of attractive principals
Over 50 chemicals were identified by SPME and GC-MS from cultures of S. aureus, K.
pleumoniae, C. freundii, E. agglomerans, and bird feces, a known fruit fly food, containing
unidentified bacteria (Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker et al.,
2000; Robacker and Lauzon, 2002; Robacker et al., 2004). Chemicals that fit the profile of
attractive principals were ammonia, several water-soluble aliphatic amines including
methylamine, dimethylamine trimethylamine, 2-methylpropanamine, 2-methylbutanamine,
and 3-methylbutanamine, the aromatic amine indole, imines including 1-pyrrline and
2,3,4,5-tetrahydropyridine, and pyrazines including pyrazine, 2,5-dimethylpyrazine and
trimethylpyrazine. Among numerous other chemicals were acetic acid and other short-chain
carboxylic acids, alcohols including 3-methylbutanol, 2-ethylhexanol, and 2-phenylethanol,
phenol, 3-hydroxybutanone, and dimethylsulfide.

Cage-top bioassays were conducted to establish attractiveness of these chemicals to
Mexican fruit flies (Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker and
Lauzon, 2002; Robacker et al., 1996, 2000, 2004). Ammonia, most of the amines, 1-pyrroline,
and acetic acid were the most attractive chemicals. All chemicals containing nitrogen were
attractive to sugar-fed flies and dimethylamine, 1-pyrrline, and indole were also attractive to
sugar-starved flies. Most chemicals without nitrogen were slightly attractive to sugar-starved
flies and acetic acid and 3-hydroxybutanone were also attractive to sugar-fed flies. These
results indicate that, for bacterial odors attractive primarily to sugar-fed, protein-hungry fruit
flies, the attractive principals were mostly chemicals that contained protonizable nitrogen
including ammonia, amines, 1-pyrroline, and pyrazines.

Exceptions to this rule are known. In the case of E. agglomerans that is attractive to
sugar-starved as well as sugar-fed Mexican fruit flies, other chemicals such as 3-
hydroxybutanone probably also play important roles in attraction (Robacker et al., 2004).

Bird feces containing bacteria were attractive to both sugar-fed and sugar-starved Mexican
fruit flies and pH had little effect on attractiveness (Robacker et al., 2000). Phenol and 2-
ethylhexanol may have significant attractive effects in this case. Phenol has also been
implicated in attractiveness of K. pneumoniae (Robacker and Bartelt, 1997) and acetic acid
may contribute to attractiveness of S. aureus and Bacillus thuringiensis corenensis (Robacker
et al., 1998). These exceptions indicate that attraction of fruit flies to bacteria undoubtedly has
roots that spread far beyond need for protein.

Evaluation of the analytical method
Standard methods used to identify semiochemicals involved in insect behavior have not been
successful in identification of the attractive chemicals in bacterial odors. Collection of
volatiles above bacterial cultures with activated charcoal and extraction with organic solvent
resulted in identification of numerous organic chemicals but no amines (Lee et al., 1995;
DeMilo et al., 1996). My initial attempts to identify chemicals from bacterial odor also involved standard methods including collection of volatiles with Porapak Q, elution of chemicals from the adsorbent with organic solvent, and analysis using splitless injection onto commonly used thin-film GC columns (Robacker et al., 1993; Robacker and Flath, 1995). Although extracts prepared this way contained numerous chemicals and were very similar to the bacteria cultures as sensed by human olfaction, the extracts were not attractive to Mexican fruit flies and no amines were found by GC-MS.

Standard methods did not work because of several problems. First, the most important chemicals were dissolved in the aqueous culturing media and could not be extracted out by solvent-solvent partitioning (Robacker et al., 1993). Direct analysis of the bacteria cultures by GC was not a viable alternative because aqueous samples are notoriously difficult to analyze by GC (Robacker, 1998a). SPME with a PDMS fiber overcame these problems because organic chemicals, especially amines, have a high affinity for the fiber while water does not appreciably adsorb (Robacker, 1998a). The adsorbed chemicals can then be thermally desorbed in the GC injector without introduction of water onto the column. However, another problem was that highly polar chemicals such as short-chain amines adsorb irreversibly onto active sites in injector liners and on GC columns. This problem was reduced by using on-column injection onto a thick-film GC column (Robacker and Flath, 1995). Thick-film columns effectively cover most of the active sites so that even trace amounts of ammonia and amines pass through the column (Robacker, 1998a). In addition, thick-film columns greatly increase retention of low-molecular weight chemicals allowing baseline resolution of ammonia and short-chain amines (Robacker and Flath, 1995). Also, the absence of a solvent peak allows easy quantitation of these small molecules.

To verify that our method using SPME and a thick-film GC column successfully identified the attractive principals, synthetic mixtures of the chemicals found in the bacterial odors were constructed. Mixtures were prepared such that headspace concentrations of key nitrogenous chemicals, acetic acid, and phenol, were equal to concentrations in headspace above bacterial cultures of S. aureus, K. pneumoniae, and C. freundii. These synthetic mixtures were 81-89% as attractive as their respective bacterial cultures (Robacker and Flath, 1995, Robacker and Bartelt, 1997). A synthetic mixture of bird feces odor that contained phenol and 2-ethylhexanol in addition to nitrogenous chemicals was 96% and 80%, respectively, as attractive as the fecal extract to sugar-fed and sugar starved Mexican fruit flies (Robacker et al., 2000). Thus, our analytical method successfully determined the attractive chemicals in bacterial odors.

Variation in volatiles production with bacteria taxon

The analytical method was used to survey attractive chemicals produced by bacteria over both broad and narrow levels of classification. Volatiles were identified from seven genera representing four major taxonomic categories of bacteria: Enterbacter, Klebsiella, and Citrobacter, facultatively anaerobic, gram-negative rods; Alcaligenes, aerobic, gram-negative rods and cocci; Staphylococcus and Micrococcus, gram-positive cocci; and Bacillus, endospore-forming, gram-positive rods (Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker et al.; 1998). Within the genus Bacillus, volatiles were identified from 5 species and within B. thuringiensis they were identified from 4 subspecies (Robacker et al., 1998).

Results showed that production of volatiles was not strongly tied to bacteria relatedness. Some chemicals were produced relatively uniformly by all taxa whereas others varied at almost every level of classification. Ammonia and 2,5-dimethylpyrazine were produced in similar amounts by all of the bacteria (Table 1). Other chemicals including trimethylamine, 3-
methylbutanamine, 1-pyrroline and acetic acid varied by as much as 1000 fold from genus to genus. Within *Bacillus*, similar trends were observed among the species *B. sphaericus*, *B. subtilis*, *B. megarerum*, *B. popilliae*, and *B. thuringiensis*. Trimethylamine and 2,5-dimethylpyrazine were relatively uniform among the species while 3-methylbutanamine, cyclohexylamine, and 1-pyrroline varied widely. Even within the single species *B. thuringiensis*, chemicals such as trimethylamine and 1-pyrroline varied by as much as 20 fold while other chemicals such as 2,5-dimethylpyrazine and acetic acid varied only a little (Table 2). These results testify to the great diversity of metabolic capabilities among bacteria. They also demonstrate that some chemicals such as ammonia and 2,5-dimethylpyrazine are to be expected in volatiles of almost any bacterium that is studied. Finally results suggest that numerous other attractive chemicals are yet to be discovered.

Table 1. Concentrations (µg/ml) of chemicals in supernatant filtrates of various genera of bacteria.

|                 | Staph | Micro | Bac  | Alcal | Enter | Kleb | Citro |
|----------------|-------|-------|------|-------|-------|------|-------|
| ammonia        | 650   | 1000  | 1400 | 650   | 900   | 600  | 600   |
| TMA            | 2.5   | 0.003 | 0.02 | 0.002 | 0.003 | 0.04 | 0.02  |
| 3-MBA          | 1.6   | 0.04  | 1.3  | 0.03  | 0.01  | 0.5  | 0.08  |
| 1-pyrroline    | -     | 0.02  | 0.003| 0.003 | 0.003 | 0.2  | 0.2   |
| 2,5-DMP        | 5     | 1.9   | 2.5  | 2.3   | 3.4   | 2.8  | 4.8   |
| acetic acid    | 60    | 13    | 490  | 210   | 71    | 0    | 0     |

Abbreviations: Staph, *Staphylococcus*; Micro, *Micrococcus*; Bac, *Bacillus*; Alcal, *Alcaligenes*; Enter, *Enterobacter*; Kleb, *Klebsiella*; Citro, *Citrobacter*; TMA, trimethylamine; 3-MBA, 3-methylbutanamine; 2,5-DMP, 2,5-dimethylpyrazine.

Table 2. Concentrations (µg/ml) of chemicals in supernatant filtrates of subspecies of *Bacillus thuringiensis*.

|               | shan | kon   | darm  | cor  |
|---------------|------|-------|-------|------|
| TMA           | 0.08 | 0.16  | 0.18  | 0.01 |
| 1-pyrroline   | 0.02 | 0.001 | 0.002 | 0.002|
| 2,5-DMP       | 1.0  | 0.78  | 0.92  | 1.4  |
| acetic acid   | 660  | 380   | 320   | 1000 |

Abbreviations: shan, *B. t. shandongiensis*; kon, *B. t. konkukian*; darm, *B. t. darmstadiensis*; cor, *B. t. coreanensis*; TMA, trimethylamine; 2,5-DMP, 2,5-dimethylpyrazine.

A case involving *E. agglomerans* provides a final example of how volatiles vary with bacteria relatedness. Two strains of this bacterium that differed in their ability to metabolize uric acid, the primary nitrogen source in bird feces, were isolated from apple maggot flies
(Lauzon et al., 2000). When the two strains were cultured on a medium that contained uric acid as its principal nitrogen source, the uricase (+) strain produced more ammonia, trimethylpyrazine and 3-hydroxybutanone (not detected in the uricase (-) strain) (Robacker and Lauzon, 2002). A uricase (+) strain isolated from the Mexican fruit fly produced more indole than the uricase (+) apple maggot strain but only the apple maggot strain produced 3-hydroxybutanone (Robacker et al., 2004). Further investigation showed that the apple maggot strain contained indole-producing and no-indole cell types that alternated predominance in plated subculturings. Thus, batches of plates of this strain contained either mostly indole-producing cell types, or the other, so that volatiles produced by the strains varied widely from batch to batch of culturings. The indole-producing culturings also emitted amounts of ammonia, 3-methylbutanol, 2,5-dimethylpyrazine and 2-phenylethanol that were very similar to those emitted by the indole-producing Mexican fruit fly strain. However, they consistently produced 3-hydroxybutanone whereas the Mexican fruit fly strain never emitted this chemical. This E. agglomerans case further illustrates that production of volatiles that constitute bacterial odors cannot be predicted by bacteria taxonomy, except that certain chemicals such as ammonia are produced almost ubiquitously.

Variation in volatiles production with culturing medium
Volatile produced by bacteria also vary with the culturing medium. The apple maggot uricase (+) strain of E. agglomerans was cultured on media that differed in their primary source of nutrients (Robacker et al., unpublished). Bacteria cultured on the uric acid medium produced much more ammonia and 2-nonanol than those cultured on the other three media. Culturings on either uric acid or carbohydrate media resulted in much greater production of trimethylamine, and culturings on tryptic soy broth contained much greater amounts of 2,5-dimethylpyrazine, compared with the other media. These results indicate that volatiles produced by the same bacterium in nature are likely to differ depending on the fermentation substrate.

Chemical interactions in attraction of fruit flies to bacteria
Considering differences in volatiles produced by different bacteria species and strains of the same species and differences caused by bacteria growing on different substrates, an interesting chemical ecology question is ‘how do fruit flies manage to find the bacteria they need?’. Work in my laboratory over the past 10 years has addressed this question in the Mexican fruit fly. Part of the answer appears to be the way chemicals in the bacterial odors are perceived and the signals integrated in the central nervous system of the flies.

The approach was to quantitate attraction of sugar-fed, protein-hungry flies to one of more of the attractive chemicals in various combinations (Figure 4). As discussed above, ammonia and most of the amines were attractive when tested individually. Combinations of ammonia with either methanamine or putrescine were much more attractive than any of the three chemicals alone indicating synergistic effects (Robacker and Warfield, 1993). Putrescine was chosen for testing because it is a possible decomposition product of arginine (Wakabayashi and Cunningham, 1991) and is commonly found as a volatile emitted by fermentations of fruit and protein baits for fruit flies (Heath et al., 1995). Combinations of methanamine with putrescine were only slightly more attractive than either alone indicating additive effects (Robacker and Warfield, 1993). To some extent, various amines could substitute for methanamine with little change in attractiveness, but they could not substitute for ammonia or putrescine (not shown). Finally, 1-pyrroline was highly attractive by itself (Robacker et al., 2000) and greatly increased attractiveness of mixtures of ammonia, methanamine and putrescine (Robacker et al., 1997). 1-Pyrroline also could substitute for
putrescine in combinations with ammonia and methylamine (Robacker, 2001) (not shown). Wakabayashi and Cunningham (1991) had similar results with melon fly (*Bactrocera cucurbitae*). They presented evidence that mixtures of ammonia with either putrescine or pyrrolidine were much more attractive than any chemicals alone.

![Figure 4](image)

Figure 4. Synergistic and additive interactions of chemicals in attraction of Mexican fruit flies. Attractiveness = attraction to each chemical or combination relative to attraction to all four chemicals.

These results indicate that ammonia interacts synergistically with at least some amines that can be found as products of bacterial fermentations. Other nitrogen-containing chemicals had either additive effects with each other, no effects but could substitute for each other, or in some cases inhibitory effects with each other (Robacker, 1998b). Very little work has been directed toward interactions of chemicals that do not contain nitrogen. However, phenol added attractiveness to a mixture of the nitrogenous components of the odor of *C. freundii* (Robacker and Bartelt, 1997), as discussed above. Acetic acid enhanced the attractiveness of ammonia, methylamine and putrescine to sugar-starved, protein-starved Mexican fruit flies, but not to other hunger-state groups of flies (Robacker *et al.*, 1996). Other non-nitrogenous chemicals such as 3-hydroxybutanone have not been tested in combinations but may play roles in attraction of flies in undetermined drive states. Although the neural physiology of these interactions has not been investigated, it is probable that the observed behavioral effects are the result of both peripheral reception and central nervous system integration of antennal signals (Robacker, 1998b).

**Chemical ecology of attraction of fruit flies to bacteria**

These findings lead me to hypothesize that fruit fly appetitive search for bacterial fermentations is a function of: 1) the specific need for bacteria; 2) the chemical signature of the fermentation; and 3) the workings of the fly’s nervous system.

The specific need is the drive that triggers the fly to search for bacteria. It could include search for bacteria as food (Drew and Lloyd, 1989), as indicators of food (Robacker and
Moreno, 1995), to replenish gut microflora (Lauzon, 2003), to detoxify allelochemicals in food (Lauzon et al., 2003), etc., as discussed in the introduction.

The chemical signature of a bacterial fermentation provides information to the fly as to what type of nutrients are present. It can vary according to the species and strain of bacterium and the substrate on which the bacteria are growing. Flies searching for bacteria as protein or as indicators of protein could find the fermentations they need by responding to odors containing ammonia, amines and 1-pyrroline, i.e. common metabolic breakdown products of proteinaceous foodstuffs. Flies searching for a specific strain of *E. agglomerans* to build up their gut biofilm may respond to fermentations emitting 3-hydroxybutanone, a chemical that is emitted only by this bacterium. As a final example, flies needing a specific essential amino acid may search for chemicals that are metabolic byproducts from its biosynthesis and therefore would be attracted to bacteria actively synthesizing that amino acid.

The way the fruit fly’s nervous system interprets the blends of chemicals in bacterial odors greatly enhances the ability of the flies to find the fermentations they need. This is best demonstrated by the synergism of ammonia with amines and 1-pyrroline (Figure 4). Because ammonia is ubiquitous in bacterial fermentations (every bacterium we studied has produced this chemical), it is a focal point in the attraction process, i.e. it is attractive by itself and it synergizes attractiveness with at least some amines and 1-pyrroline. To illustrate using an example from the previous paragraph, flies would be able to locate fermentations containing protein by responding additively to ammonia, each of several amines, and 1-pyrroline. However, the synergistic neural response to these chemicals makes the searching process much more efficient. Also, the capability of at least some amines to substitute for each other in their synergism with ammonia means that flies are empowered to find a protein fermentation if it emits any of several amines.

I indicated above that specific amines may give flies information about specific amino acids. At present, there is no evidence if that occurs or if it might involve specific synergisms with ammonia or other chemicals. Also, it is not known if attractive bacteria-produced chemicals including 3-hydroxybutanone, acetic acid, phenol, and 2-ethylhexanol (and several other alcohols) act independently or synergistically with other chemicals in bacterial odors.

The effects of drive states on responses to bacterial odors should not be overlooked. These states seem to turn on or turn off the flies’ motor systems that control responses to the odors (Robacker, 1998b). In at least one study, sugar-hunger greatly depressed attraction while protein-hunger stimulated attraction of Mexican fruit flies to bacterial odors (Robacker and Garcia, 1993) indicating that need for protein was the driving force in that case. Other types of chemicals, such as 3-hydroxybutanone, may stimulate specific receptors and brain centers that are activated when flies are primed by drive states for particular bacteria that emit that chemical.

This discussion is a synthesis attempting to tie together findings from numerous studies cited earlier in this paper, and is by no means conclusive. I only hope to stimulate others to examine this hypothesis and develop research to confirm or refute my ideas.

**Benefits of these studies**

Studies of bacteria associated with fruit flies have already benefitted entomological pursuits. Information from these studies has left its mark on development of new fruit fly lures including both the BioLure MFF lure (Suterra LLC, Inc., Bend, Oregon, U.S.A.) (Heath et al., 1995) and the AFF lure (Advanced Pheromone Technologies, Inc., Marylhurst, Oregon) (Robacker and Warfield, 1993). The potential for improvement of lures by addition of novel bacteria-produced chemicals remains great. Improvement of fruit flies that are mass-reared for release of sterile males is another potential use of information from studies of fruit fly and
bacteria relationships. New studies have shown that addition of probiotic bacteria to diets fed to Mediterranean fruit flies aids reparation of the gut damaged by irradiation (Niyazi et al., 2004; Lauzon and Potter, 2005). Finally, these studies enhance understanding of our natural world. Knowledge of these complex relationships may lead to advancements in entomology, microbiology, and even medicine in ways that no one has yet imagined.

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