

VOLATILES ATTRACTIVE TO THE MEXICAN FRUIT FLY
(DIPTERA: TEPHRITIDAE) FROM ELEVEN BACTERIA TAXA

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ABSTRACT

Filtrates of 11 bacteria representing 4 higher taxonomic categories were attractive to Mexican fruit flies, *Anastrepha ludens* (Loew) (Diptera: Tephritidae) in laboratory bioassays. All bacterial filtrates were more attractive at pH 9 than at pH 5 although filtrates at pH 5 were more attractive than water controls. The effects of pH on attractiveness of filtrates were consistent with an hypothesis that attractive principals of bacterial filtrates were various nitrogen-containing compounds and carboxylic acids that became more volatile at specific pH's resulting in increased attractiveness. Volatiles produced by the bacteria were sampled by solid-phase microextraction and identified by GC and GC-MS. Attractive principals identified were ammonia, aliphatic amines, pyrazines, imines, and acetic acid. Relative amounts of most of the chemicals were not closely tied to bacteria taxonomy.

Key Words: *Anastrepha ludens*, attractants, bacteria, amines, acetic acid, solid phase microextraction (SPME)

RESUMEN

Los filtrados de 11 bacterias que representan a 4 categorías altas taxonómicas atrayeron a las mosca de la fruta mexicana, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), en bioensayos de laboratorio. Todos los filtrados bacteriales fueron más atrayentes al pH 9 que al pH 5, aunque los filtrados de pH 5 fueron más atrayentes que el testigo. Los efectos del pH sobre la atracción de los filtrados fueron consistentes con la hipótesis de que los químicos atrayentes de los filtrados bacteriales eran varios compuestos de nitrógeno y ácido carboxílico que se hacen más volátiles a pH específicos resultando en un aumento en su atracción. Volátiles producidos por las bacterias fueron colectados usando micro-extracción de fase sólida y fueron identificados por cromatografía de gas y espectrómetro de masa. Los químicos atrayentes fueron identificados como amoníaco, aminos alifáticos, pirazines, imines y ácido acético. Las concentraciones de varios químicos no estuvieron muy cercanamente relacionadas a la taxonomía de las bacterias.

Volatile chemicals from bacterial fermentations attractive to fruit flies have come under increased scrutiny during the last 10 years as possible attractants. Several papers have reported identification of ammonia from bacteria cultures (Gow 1954, Drew

& Fay 1988, Robacker & Flath 1995). Ammonia has long been known as a powerful attractant for fruit flies (Jarvis 1931). Other studies have resulted in identification of additional volatile chemicals from cultures of bacteria (Hayward et al. 1977, Lee et al. 1995, Robacker & Flath 1995, DeMilo et al. 1996, Robacker & Bartelt 1997).

Attractiveness of chemicals (other than ammonia) identified from bacterial odors has been demonstrated in only a few studies. Drew (1987) demonstrated that the bacteria-produced chemicals 2-butanone and 1-butanol were attractive to *Bactrocera tryoni* (Froggatt), purportedly because of their structural similarity to the parapheromone cue lure. Robacker & Flath (1995) and Robacker & Bartelt (1997) identified and demonstrated attractiveness for ammonia, several amines, imines, pyrazines and acetic acid from three species of bacteria.

In this research, principals attractive to the Mexican fruit fly, *Anastrepha ludens* (Loew) were identified from 11 strains of bacteria that had not been investigated before. A 3-step procedure was used. First, attractiveness of each bacterium was verified. Second, the effect of fermentation pH on attractiveness was determined to characterize the classes of chemicals involved in the attraction response. Third, chemicals that fit the attractive-principal profile as determined in the pH tests were identified and quantified.

The purposes of the work were to determine if similar bacteria produce similar attractive chemicals and conversely if dissimilar bacteria produce different, perhaps novel, chemicals. Novel chemicals, along with knowledge gained in this work of general patterns of volatiles produced by attractive bacteria, could be used in development of new lures for fruit flies.

MATERIALS AND METHODS

Insects and Test Conditions

Flies used to test attractiveness of bacterial preparations were from a culture that originated from yellow chapote, *Sargentia greggii* Coult., (Rutaceae), fruit, a native host of the fly, collected in Nuevo León, Mexico, in 1987. Fly handling and laboratory maintenance were as described in Robacker & Flath (1995). Flies were sugar-fed and protein-starved (since eclosion) because previous work indicated this physiological state maximizes attraction to bacterial odor (Robacker & Garcia 1993). Flies were used when 6-10 days old.

Bacterial Preparations

Bacteria species used in this work were: *Enterobacter cloacae* (Jordan); *Alcaligenes faecalis faecalis* Castellani & Chalmers; *Micrococcus luteus* Schroeter; *Bacillus sphaericus* Meyer & Neide; *B. subtilis* Ehrenberg; *B. megaterium* de Bary; *B. popilliae* Dutky; and *B. thuringiensis* Berliner subspecies *shandongiensis*, *coreanensis*, *konkukian*, and *darmstadiensis*. Strains obtained from the American Type Culture Collection (ATCC) (Rockville, MD) were: *E. cloacae* (ATCC strain 961); *A. f. faecalis* (ATCC strain 8750); *M. luteus* (ATCC strain 23259); *B. sphaericus* (ATCC strain 4525); *B. subtilis* (ATCC strain 6051); *B. megaterium* (ATCC strain 14581); and *B. popilliae* (ATCC strain 14706). Strains obtained from the Institut Pasteur (Paris, France) were: *B. t. shandongiensis* (strain 22001); *B. t. coreanensis* (strain 25001); and *B. t. konkukian* (strain 34001). *B. t. darmstadiensis* (strain GUAT1) was obtained from a soil sample from Guatemala (Martinez et al. 1997).

These taxa were chosen to survey volatile chemicals attractive to the Mexican fruit fly produced by bacteria over both broad and narrow levels of classification. The four

genera represent four distinct higher taxonomic categories: *Enterobacter*, facultatively anaerobic, gram-negative rods; *Alcaligenes*, aerobic gram-negative rods and cocci; *Micrococcus*, gram-positive cocci; and *Bacillus*, endospore-forming, gram-positive rods (Holt 1984). The five species of *Bacillus* and the 4 subspecies of *B. thuringiensis* allow an analysis of volatiles produced by more closely related strains.

All ATCC strains were fermented in trypticase soy broth (BBL, Baltimore, MD) in a shaker for 5 days at 30°C. *B. t. shandongiensis*, *B. t. konkukian*, and *B. t. darmstadtensis* were fermented in Bacto nutrient broth (DIFCO Laboratories, Detroit, MI) in a shaker for 3, 6, and 3 days, respectively, at 30°C. *B. t. coreanensis* was fermented in a shaker in a growth medium (medium B) developed by Dulmage et al. (1970) for 5 days at 30°C. Fermentation times and media were based on preliminary bioassays showing maximum attractiveness for these times and media. Bacterial cultures were centrifuged and the resulting supernatants were filtered to remove bacterial cells as described previously (Robacker & Flath 1995). Martinez et al. (1994) demonstrated that filtered and unfiltered cultures of several bacteria species were equally attractive indicating that the attractants were dissolved in the filtrate. Because attractive chemicals were retained in filtrate, there was no concern that bacterial cultures may have been too old to contain actively growing cells at the time they were harvested. Five fermentations of each bacterium were conducted.

Evaluation of Attractiveness of Bacterial Filtrates

Attractiveness of each bacterial filtrate was evaluated using cage-top bioassays as described in Robacker & Flath (1995) with water as the control. The three culturing media were also tested against water. Briefly, the bioassay was conducted by placing two filter paper triangles containing 10 µl of bacterial or growth-medium filtrate and two papers containing 10 µl of water on the top of an insect cage. The filter papers were raised 5 mm above the cage top using plastic rings. Each bioassay cage contained 180-200 flies. The number of flies beneath each filter paper was counted once each minute for 10 min following application of the test materials to the papers. The 11 bacterial filtrates and the three growth-medium filtrates were tested in random order. Two bioassay replications were conducted for each fermentation.

To analyze bioassay results, total flies counted at water control papers were subtracted from total flies counted at treatment papers for each cage-top bioassay to obtain a bioassay count difference. The two bioassay count differences per fermentation were averaged. The resulting fermentation-level means were then used as data points in one-way analysis of variance (ANOVA) using SuperANOVA (Abacus Concepts 1989) to compare attractiveness of the various bacteria. Means separations were conducted by Fisher's protected least significant difference method (LSD).

Effects of pH on Attractiveness of Bacterial Filtrates

For one of the five fermentations, the pH of bacterial filtrates and growth-medium filtrates was adjusted to 5, 7, and 9 with 85% phosphoric acid (Fisher Scientific, Fair Lawn, NJ) or saturated sodium hydroxide (Fisher). Attractiveness of each pH treatment was tested against water controls using cage-top bioassays. The purpose was to determine if attractive principals of the bacterial filtrates were nonionizing chemicals that would not be affected by pH or chemicals that ionize into relatively nonvolatile forms and therefore contribute little to attractiveness at certain pH's. Thus, carboxylic acids (pKa's 4-5) would contribute little to attractiveness at pH 9; ammonia (pKa 9.2) and amines (aliphatic amines, pKa's 10-11) would contribute little to attractiveness at pH 5;

imines (1-pyrroline, pKa 6.7) would be most attractive at pH > 7; and pyrazines (pyrazine, pKa 0.6) and nonionizing compounds would be volatile and attractive throughout the pH range tested (pKa's from March 1968, Amoore et al. 1975, Weast 1976).

Each replication of the experiment consisted of one cage-top bioassay for each of the three pH treatments of all filtrates, tested in random order. Ten replications of the experiment were conducted. Each bacterial filtrate or growth medium filtrate was analyzed separately by one-way ANOVA to compare pH effects. Bioassay count differences (described above) were used as data and pH means were separated by Fisher's protected LSD.

Volatiles Sampling

Chemicals were sampled in the headspace above filtrates of bacteria and uninoculated growth media by solid phase microextraction (SPME) with a 100 μ m polydimethylsiloxane-coated fiber (Supelco, Inc., Bellefonte, PA). The fiber was inserted through a septum into the headspace above 1 ml of filtrate in a 4 ml vial for 30 min at 21-23°C before analysis by GC or at 25-27°C before analysis by GC-MS.

Chemical Identifications

Two methods were used to identify chemicals. For bacteria that had volatiles profiles similar to those of three species of bacteria studied previously (Robacker & Flath 1995, Robacker & Bartelt 1997), chemicals were identified by matching GC retention times and detector response ratios with those of standards. The gas chromatograph was a Shimadzu GC-17A (Shimadzu Scientific Instruments, Inc., Columbia, MD) with flame ionization (FID) and flame thermionic (FTD) (Model FTD-17) detectors. A DB-1 capillary column (J & W Scientific, Folsom, CA) with a 5 μ m film was used. FTD/FID response ratios were obtained to establish the presence of C-N bonds. A detailed description of the GC method can be found in Robacker & Bartelt (1997).

For bacteria that had nitrogen-containing peaks not observed in previous studies, chemicals were identified by GC-MS. GC-MS data were acquired using a Hewlett Packard 5890 GC with a HP 5970 mass selective detector (electron energy = 70 eV). GC-MS identifications were based on computer matching of unknown spectra with those in the Wiley 138K Mass Spectral Database (John Wiley and Sons, New York). Identifications were authenticated by comparing spectra with those of standards for most chemicals. A DB-1 column with a 5 μ m film also was used. A detailed description of the GC-MS method can be found in Robacker & Bartelt (1997). Chemicals were sampled from headspace above unaltered filtrates, and filtrates to which sodium hydroxide was added to enhance volatilization of basic compounds.

GC Analysis of Headspace Volatiles

Relative amounts of eight attractive chemicals in the headspace of bacterial filtrates (at unaltered pH of filtrates) and the 3 uninoculated growth media filtrates were measured. This analysis was conducted to compare amounts of the various chemicals produced by different bacteria taxa.

Analyses of seven nitrogen-containing chemicals were conducted using the Shimadzu GC-17A with FTD as described above. GC peak heights were measured using Millennium 2010 Chromatography Manager software (Waters Corporation, Milford, MA). Two headspace analyses were conducted for each of five fermentations of the 11 bacteria strains and the three growth media. One headspace analysis per fermentation was also done using FID for acetic acid.

Peak heights from the 2 GC-FTD analyses were averaged to give a fermentation-level mean. A one-way ANOVA was conducted for each chemical to assess amounts in headspace above the various filtrates, using the fermentation-level means as data points. ANOVA was also conducted to analyze individual GC-FID peak heights (not means) of acetic acid in the various filtrates. Means were separated by Fisher's protected LSD.

GC Standards

Ammonium carbonate, 2-methylpropanamine, 2-methylbutanamine, 3-methylbutanamine, 2-phenylethanamine, pyrazine, methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine and trimethylpyrazine were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Methylamine HCl, trimethylamine HCl, and cyclohexylamine were obtained from Sigma Chemical Company (St. Louis, MO). Acetic acid was obtained from Fisher Scientific (Pittsburgh, PA) and 2-methylpropanoic acid and 3-methylbutanoic acid were obtained from Eastman Chemical Products, Inc. (Kingsport, TN).

Five imines were synthesized. 1-pyrroline was synthesized by acid hydrolysis of 4-aminobutyraldehyde diethyl acetal (Aldrich) according to methods of Schopf & Oechler (1936). 2, 3, 4, 5-tetrahydropyridine was synthesized by reaction of N-chlorosuccinimide (Aldrich) with piperidine (Matheson, Coleman & Bell, Norwood, OH, 98%) to form N-chloropiperidine, followed by elimination of HCl from N-chloropiperidine with KOH (Fisher) (Quick & Oterson 1976). Other imines were prepared by the general method of addition of aldehydes to primary amines (March 1968). N-isopentylidene-3-methylbutanamine was prepared by addition of 3-methylbutanal (Aldrich) to 3-methylbutanamine in methylene chloride (Fisher) at room temperature. Anhydrous sodium sulfate (EM Science, Cherry Hill, NJ) was then added to clear turbidity due to water formed as a reaction byproduct. Likewise, N-phenylmethylene-2-methylpropanamine and N-phenylmethylene-3-methylbutanamine were prepared by addition of benzaldehyde (Aldrich) to 2-methylpropanamine and 3-methylbutanamine, respectively.

RESULTS AND DISCUSSION

Attractiveness of Bacterial Filtrates

All bacterial filtrates were significantly more attractive than uninoculated media ($F = 7.8$; $df = 13,56$; $P < 0.0001$) (Fig. 1). No major differences in attractiveness occurred among the bacteria strains except that the *B. thuringiensis* group was generally less attractive than the others. In this work and in previous studies (Robacker & Flath 1995, Robacker & Bartelt 1997), cultures of many species, genera, and higher taxa of bacteria have been demonstrated attractive to Mexican fruit flies. We conclude that Mexican fruit fly attraction to bacteria cultured in aqueous laboratory media is a general phenomenon.

Effects of pH on Attractiveness of Bacterial Filtrates

The pH's of bacterial filtrates, before manipulation with phosphoric acid or sodium hydroxide, generally were between 7.8 and 9.2. These solutions contained more equivalents of bases than acids. Exceptions were *M. luteus* and *B. t. coreanensis* that had pH's of 7.1 and 5.3, respectively. The *B. t. coreanensis* filtrate contained more acids than bases. Uninoculated growth media had pH's between 6.7 and 7.0.

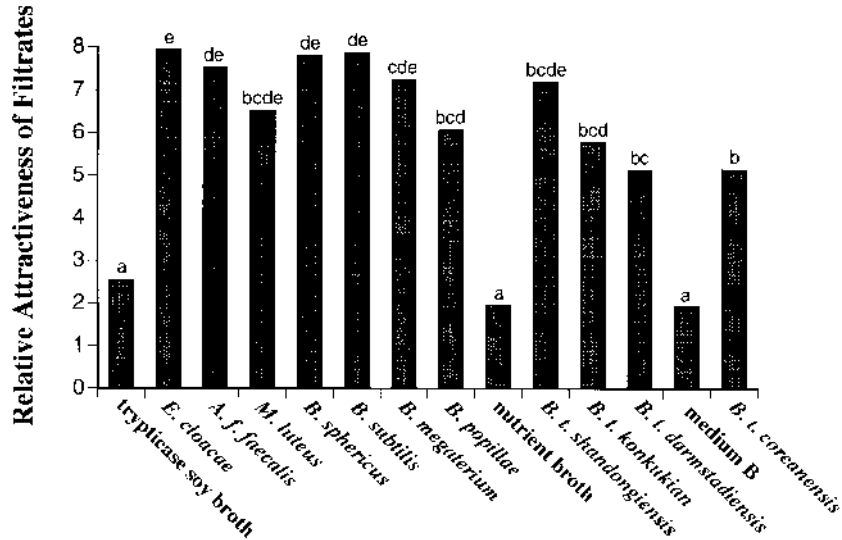


Fig. 1. Attractiveness of bacterial filtrates to *A. ludens* in cage-top bioassays. Relative attractiveness = mean counts at papers containing filtrates, divided by mean counts at papers containing water. Bars with the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

Attractiveness of all filtrates was greatly affected by changing filtrate pH (smallest $F = 9.2$; $df = 2,27$; $P < 0.001$ for nutrient broth) (Fig. 2). Most filtrates at pH 7 and all at pH 9 were more attractive than filtrates at pH 5. This is a critical result because it indicated that the most important attractive principals are compounds containing protonizable nitrogen with pKa's of 7 or above because these chemical classes would be largely ionized and nonvolatile at pH 5.

All filtrates at pH 5 except *E. cloacae* and *A. f. faecalis* were significantly more attractive than water controls, although attractiveness of most was not high compared with attractiveness at pH 7-9. However, *B. popillae* and *B. t. coreanensis* filtrates at pH 5 were much more attractive than water controls (paired *t*-test for *B. popillae*, $t = 7.5$, $df = 9$, $P < 0.001$; for *B. t. coreanensis*, $t = 6.6$, $df = 9$, $P < 0.001$). Chemicals that could account for the attractiveness of filtrates at pH 5 include carboxylic acids, pyrazines, and various nonionizing chemicals such as hydrocarbons, alcohols, aldehydes, ketones, esters, etc., that would exist largely in nonionized, volatile forms at pH 5.

Chemical Identifications

Because all filtrates were most attractive at pH 9, chemical identifications were focused on chemicals containing protonizable nitrogen. The relatively low attractiveness of most filtrates at pH 5 indicated that nonionizing chemicals probably did not play major roles in attractiveness and were not identified in this work. However, the moderate attractiveness of the naturally acidic *B. t. coreanensis* filtrate also led us to identify carboxylic acids from the bacterial volatiles.

Filtrates of *A. f. faecalis*, *B. popillae*, and *B. t. coreanensis* were analyzed by GC-MS. Nitrogen-containing chemicals and carboxylic acids that were identified are

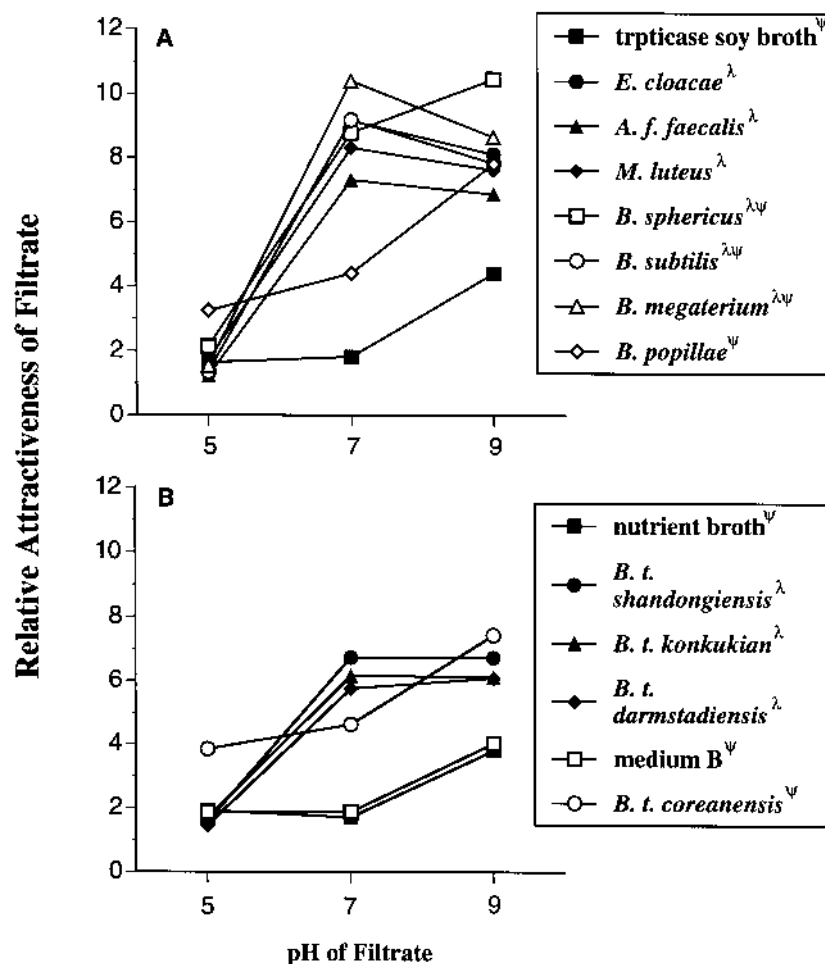


Fig. 2. Attractiveness of pH altered bacterial filtrates to *A. ludens* in cage-top bioassays. Relative attractiveness = mean counts at papers containing filtrates, divided by mean counts at papers containing water. λ indicates significant difference in attractiveness between pH 5 and 7; ψ indicates significant difference in attractiveness between pH 7 and 9; $P < 0.05$ by Fisher's protected LSD.

shown in Table 1. Of these chemicals, trimethylamine, 2-methylpropanamine, 3-methylbutanamine, 2-methylbutanamine, methylpyrazine, 2,5-dimethylpyrazine and trimethylpyrazine had been reported from one or more of the bacteria *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Citrobacter freundii* (Robacker & Flath 1995, Robacker & Bartelt 1997). Lee et al. (1995) and DeMilo et al. (1996) identified numerous pyrazines from headspace of *K. pneumoniae* and *C. freundii* including most of those in Table 1, and others. Acetic acid had been reported from headspace of *S. aureus* (Robacker & Flath 1995) and 2-methylpropanoic acid and 3-methylbutanoic acid from

TABLE 1. NITROGEN-CONTAINING CHEMICALS AND CARBOXYLIC ACIDS IDENTIFIED BY GC-MS IN HEADSPACE ABOVE UNALTERED BACTERIAL FILTRATES AND FILTRATES WITH ADDED NAOH¹.

	Aff	Aff + NaOH	Bp	Bp + NaOH	Btc	Btc + NaOH
trimethylamine	-	+	-	-	-	+
2-methylpropanamine	+	+	++	+++	+	+
3-methylbutanamine	+	++	++	+++	-	+
2-methylbutanamine	-	-	-	+++	-	-
cyclohexylamine	-	+	-	+	-	+
2-phenylethanamine ²	-	-	+	+	-	-
N-isopentylidene-3-methylbutanamine	-	-	+	++	-	-
N-phenylmethylene-2-methylpropanamine	-	-	-	++	-	-
N-phenylmethylene-3-methylbutanamine	-	-	-	+++	-	-
2,4,5-trimethyl-3-oxazoline ²	-	-	-	+	-	++
methylpyrazine	+	+	+	+	-	+
2,5-dimethylpyrazine	++	++	++	++	++	++
2,3-dimethylpyrazine	-	-	-	-	+	+
trimethylpyrazine	+	+	+	+	++	++
tetramethylpyrazine ²	-	-	+	+	+	+
ethyl dimethylpyrazine isomer ²	-	-	+	+	+	+
diethylmethylpyrazine isomer ²	-	-	-	-	+	+
acetic acid	-	-	-	-	++	-
2-methylpropanoic acid	-	-	-	-	+	-
3-methylbutanoic acid	-	-	-	-	++	-

¹Aff = *A. f. faecalis*, Bp = *B. popillae*, Btc = *B. t. coreanensis*, - = not detected above baseline, + = trace (< 500 area counts), ++ = minor (500 - 5000 area counts), +++ = major (> 5000 area counts).

²Good library match, but not verified with standard.

headspace of *K. pneumoniae* (Lee et al. 1995, Robacker & Bartelt, 1997). Cyclohexylamine, 2-phenylethanamine, N-isopentylidene-3-methylbutanamine, N-phenylmethylene-2-methylpropanamine, N-phenylmethylene-3-methylbutanamine, and 2,4,5-trimethyl-3-oxazoline had not been reported from any bacteria, to our knowledge. N-isopentylidene-3-methylbutanamine has been found in volatiles of NuLure, a protein bait for fruit flies (Flath et al. 1989).

Additional chemicals were identified by GC-FID and GC-FTD. These were ammonia, pyrazine, 1-pyrroline and 2,3,4,5-tetrahydropyridine. The latter 3 chemicals were identified by the highly sensitive GC-FTD technique. They had been identified previously by GC-MS in headspace of other bacteria (Robacker & Flath 1995, Robacker & Bartelt 1997). Ammonia was also identified from these other bacteria by GC-FID and GC-FTD. It was not identified by GC-MS because of its low molecular weight. All four chemicals were verified by GC analyses of standards.

Attractiveness of Chemicals Identified by GC-MS/GC Analyses

Many of the chemicals have been evaluated for attractiveness to Mexican fruit flies (Robacker & Warfield 1993, Robacker & Flath 1995, Robacker et al. 1996, Robacker & Bartelt 1997, Robacker et al. 1997). Ammonia, 2-methylpropanamine, 3-methylbutanamine, 2-methylbutanamine and acetic acid were 2-3 times more attractive than water controls. Trimethylamine, 1-pyrroline, 2,3,4,5-tetrahydropyridine, pyrazine, 2,5-dimethylpyrazine, and trimethylpyrazine were 1.1 to 1.5 times more attractive than water. Methylpyrazine, 2-phenylethanimine, and 3-methylbutanoic acid were not attractive. N-isopentylidene-3-methylbutanamine was not attractive to four species of fruit flies that did not include the Mexican fruit fly in olfactometer tests (Flath et al. 1989). The other chemicals in Table 1 have not been tested for attractiveness to fruit flies.

Comparison of Attractants Produced by Bacteria Taxa

Results of the GC-FTD analyses of 8 attractive components of bacterial volatiles are shown in Tables 2 and 3. Ammonia was produced in about the same amounts by all of the bacteria. Emission of most other chemicals varied greatly from strain to strain. Some generalizations can be observed in the tables regarding production of some chemicals by closely related taxa. For example, the only two bacteria that produced large amounts of 2-methylpropanamine and 3-methylbutanamine were in the genus *Bacillus*. However, the other three species of *Bacillus* produced very little of these two chemicals. Thus, chemicals were not produced in similar amounts by related taxa in many cases. In other cases, chemicals were produced by distantly related taxa but not by closely related ones. An example of this is trimethylpyrazine that was produced in relatively high amounts by *E. cloacae* and *B. t. coreanensis* but in lower amounts by other *Bacillus* and even other strains of *B. thuringiensis*.

Some of the differences in volatiles production may be attributable to differences in culturing media, but in other cases, bacteria grown on different media produced the same chemicals. For example, highest amounts of 2, 5-dimethylpyrazine were produced by bacteria cultured on trypticase soy broth and highest amounts of trimethylamine were produced by the *B. thuringiensis* strains cultured on nutrient broth. On the other hand, the trimethylpyrazine example discussed above is a case in which two bacteria grown on different media produced about the same amount of a chemical.

The discussion of similarities and differences in volatiles profiles can be expanded by including results of previous analyses of bacteria volatiles. Profiles of *K. pneumoniae* and *C. freundii* (Robacker & Bartelt 1997), members of the family Enterobacteriaceae along with *E. cloacae*, differed from the profile of *E. cloacae* (Tables 2 and 3) in amounts of 3-methylbutanamine, 2, 5-dimethylpyrazine and trimethylpyrazine but were similar with regard to several other chemicals. Also, amounts of trimethylamine, 3-methylbutanamine and acetic acid produced by *S. aureus* (Robacker & Flath 1995), a member of the Micrococcaceae along with *M. luteus*, differed dramatically from amounts produced by *M. luteus* (Tables 2 and 3). Conversely, acetic acid production by *S. aureus* was high as in *B. t. coreanensis*, a species that is not in the Micrococcaceae. These examples suggest a great diversity of metabolic pathways in bacteria that do not tie closely to currently held views of taxonomic relatedness.

Note that peak sizes do not reflect absolute amounts of different chemicals. For example, the small ammonia peaks indicate filtrate concentrations in the 100 µg/ml to 1 mg/ml range while the large 2-methylpropanamine peaks indicate concentrations only in the 1 to 10 µg/ml range (Robacker & Bartelt, 1997).

TABLE 2. PEAK HEIGHTS (MV) OF CHEMICALS IN HEADSPACE ABOVE FILTRATES OF UNINOCULATED GROWTH MEDIA AND 11 BACTERIA STRAINS GROWN ON THOSE MEDIA¹.

	ammonia	trimethyl- amine	2-methyl- propanamine	3-methyl- butanamine
trypticase soy broth	0.14 abc	0.1 a	0.2 a	0.1 a
<i>E. cloacae</i>	0.30 cde	0.6 a	0.4 a	0.2 a
<i>A. f. faecalis</i>	0.30 cde	0.4 a	0.9 a	0.6 a
<i>M. luteus</i>	0.29 cde	0.6 a	1.0 a	0.7 a
<i>B. sphericus</i>	0.32 de	0.5 a	126.1 b	61.7 b
<i>B. subtilis</i>	0.35 de	0.8 a	3.2 a	1.4 a
<i>B. megaterium</i>	0.28 cde	0.7 a	0.8 a	0.4 a
<i>B. popilliae</i>	0.25 bcd	0.6 a	107.4 b	53.6 b
nutrient broth	0.05 a	1.7 a	0.4 a	0.2 a
<i>B. t. shandongensis</i>	0.25 bcd	14.6 b	1.5 a	0.8 a
<i>B. t. konkukian</i>	0.39 de	29.7 c	0.4 a	0.3 a
<i>B. t. darmstadiensis</i>	0.44 e	34.2 c	0.5 a	0.3 a
medium B	0.09 ab	0.4 a	0.3 a	0.2 a
<i>B. t. coreanensis</i>	0.34 de	2.0 a	0.4 a	0.2 a

¹Flame thermionic detection. For a given chemical, mean peak heights followed by the same letter were not significantly different from each other by Fisher's protected LSD ($P < 0.05$, $n = 5$ fermentations).

Attractive Principals vs. pH of Filtrates

Experiments with filtrate pH indicated the importance of chemicals containing protonizable nitrogen to the attractiveness of the bacterial filtrates (Fig. 2). Ammonia, amines, imines, and pyrazines, all chemicals previously demonstrated attractive to Mexican fruit flies, were then identified from the filtrates (Tables 1-3). Also, these chemicals had been identified previously as the attractive principals of three other bacteria (Robacker & Flath 1995, Robacker & Bartelt 1997) and nonionizing chemicals identified from odor of two of those bacteria were not attractive to flies primed for response to bacterial odor. We conclude that the attractive principals of the naturally basic bacterial filtrates tested in this work (pH 7.8 to 9.2), as well as all filtrates adjusted to pH 9, were ammonia, amines, imines and pyrazines.

Two bacteria, *B. t. coreanensis* and *B. popilliae*, were also moderately attractive at pH 5 (Fig. 2). The chemical most responsible for the moderate attractiveness of these two filtrates at pH 5 probably was acetic acid. These two filtrates had the largest peak heights for acetic acid (Table 3). As discussed above, acetic acid is very attractive to Mexican fruit flies. Because the *B. t. coreanensis* filtrate was pH 5.3 before manipulation with phosphoric acid, we conclude that acetic acid played a major role in attractiveness of this filtrate at its natural pH. Pyrazines may also contribute to attractiveness of these and all filtrates at pH 5 because of their low pKa's. Possible minor roles of nonionizing chemicals at all pH levels have not been determined.

TABLE 3. PEAK HEIGHTS (MV) OF CHEMICALS IN HEADSPACE ABOVE FILTRATES OF UNINOCULATED GROWTH MEDIA AND 11 BACTERIA STRAINS GROWN ON THOSE MEDIA¹.

	pyrazine	2,5-dimethyl- pyrazine	trimethyl- pyrazine	acetic acid
trypticase soy broth	3.4 b	69.9 bcd	3.0 a	0.1 a
<i>E. cloacae</i>	5.1 bc	152.5 f	20.9 b	0.0 a
<i>A. f. faecalis</i>	4.0 b	112.1 def	4.4 a	0.1 a
<i>M. luteus</i>	5.4 bc	91.8 cde	6.2 a	0.0 a
<i>B. sphaericus</i>	4.8 bc	160.2 f	6.3 a	0.2 a
<i>B. subtilis</i>	6.4 cd	161.5 f	7.1 a	0.2 a
<i>B. megaterium</i>	5.4 bc	128.4 ef	5.7 a	0.0 a
<i>B. popilliae</i>	6.2 cd	127.4 ef	7.7 a	0.6 a
nutrient broth	0.0 a	4.1 a	0.5 a	0.0 a
<i>B. t. shandongensis</i>	0.9 a	48.2 abc	7.7 a	0.2 a
<i>B. t. konkukian</i>	3.9 b	37.5 ab	3.2 a	0.1 a
<i>B. t. darmstadiensis</i>	1.2 a	44.4 abc	3.4 a	0.1 a
medium B	8.1 de	7.9 a	0.7 a	0.0 a
<i>B. t. coreanensis</i>	8.6 e	67.7 bcd	24.5 b	3.0 b

¹Pyrazines determined by flame thermionic detection; acetic acid determined by flame ionization detection. For a given chemical, mean peak heights followed by the same letter were not significantly different from each other by Fisher's protected LSD ($P < 0.05$, $n = 5$ fermentations).

Novel Attractants from Bacterial Odors

Several novel chemicals fitting the attractive-principal profile were identified from the four major bacteria taxa that were investigated. However, the principal differences among the bacteria were quantitative rather than qualitative in that they produced mostly the same chemicals but in widely different amounts, at least when grown on laboratory media. Thus, an exhaustive investigation of bacteria species for potential new attractants would likely result in relatively few candidate compounds.

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