Enterococci in Insects

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Enterococci were obtained from 213 of 403 insects cultured during a 14-month period, in numbers from $10^2$ to $3 \times 10^7$ of insect. Insects were taken only from nonurban, wild, and cultivated fields and woods. In species of insects carrying them, enterococci were not always present in every individual cultured, and often more than one species of enterococcus occurred within a species. Enterococci were obtained from certain insects taken in the field during the dormant season, suggesting their role as overwintering agents. They were generally present in species feeding on nectar, succulent plant parts, and on and in forest litter, but not from insects feeding on less succulent leaves and stems. Streptococcus faecalis was recovered from 32%, Streptococcus faecium from 22.4%, and Streptococcus faecium var. casseliflavus from 43.5% of members of the 37 taxa of insects. S. faecalis and S. faecium var. casseliflavus exhibit a high percent of conformity to the properties published for them. The heterogeneity in properties of S. faecium is similar to that found for the species taken from plants. Many fail to grow in broth at 45 C or in broth containing 6.5% NaCl; 50% of the cultures ferment both melezitose and melibiose, and a few ferment neither sugar. The remainder ferment melibiose only. Failure to reduce methylene blue in milk by S. faecalis and S. faecium is correlated with the inability to ferment lactose. More than 93% of the cultures of S. faecalis digest casein in milk from the top downward, following the production of a soft, flowing curd. Because this property is not characteristic of S. faecalis taken from humans, the reaction in litmus milk is suggested as a means of differentiation between cultures of remote and innocent origin in nature and recent, human pollution.

The literature provides evidence that the enterococci occur randomly among some genera and species of insects, and with some degree of constancy among others. In some studies, the enterococci are clearly identifiable as Streptococcus faecalis or as Streptococcus faecium, intermediate forms (23), and possibly as Streptococcus faecium var. casseliflavus (10; Cosenza, B., and A. E. Girard, Bacteriol. Proc., p. 45, 1970). They may be recovered from various parts of the insect body (16). Geldreich et al. (18) have obtained them in high numbers from members of the orders Diptera and Coleoptera. They are abundant in the intestinal tracts of the wax moth and the hornworm (5, 6), and transmissible to succeeding generations via the eggs (4, 8). They have been recovered from ichneumonid parasitoids (7), larvae and pupae of moths and pine sawflies (9, 34), flies and roaches (19), muscid flies (30), desert locusts (35), stable flies (20), from Anopholes but not two other genera of mosquitoes (21), and yellow mealworms (22, 37). They have been incriminated as possible agents of insect diseases (12, 14, 31, 32; Cosenza and Gerard, see above). Their use as an insecticidal aerosol has been proposed (13). Some studies have been concerned with the insects in intimate association with human and warm-blooded animal life (19, 20, 30), and others have dealt with insects which are primarily plant feeders (9, 22, 35, 37).

It has been suggested (25) that insects play a role in the seasonal introduction of enterococci to wild and cultivated plants. This study was undertaken to determine more precisely, through enumeration, speciation, and studies of distribution among insects, their role both in dissemination and as an overwintering agent for the bacteria.

MATERIALS AND METHODS

Collection of insects. All insects were taken from agronomic and wild areas to avoid fields harboring domesticated animal life and human habitation. Some insects were in association with particular habitats, as Anthonomus grandis on cotton plants and Epicauta sp. on soy bean plants. The majority of insect specimens were taken in any convenient manner, including the use of nets, bottles, and forceps,
and they were placed immediately into sterile glass containers. All specimens either were cultured promptly upon return to the laboratory or were frozen at -21°C for periods of 2 to 7 days. A total of 403 individuals representing 36 genera of insects was cultured.

Culture. Each of the larger insects was homogenized singly in a 15-ml tissue homogenizer in a volume of water equal to 10 times the weight of the insect. Several individuals of the smaller insects were homogenized as one sample to obtain the necessary volume for quantitation of fluid for culture. From the homogenates, serial dilutions were made in azide-dextrose (AD) broth and plated on KP agar by the surface spreading technique. Plates of MMRS agar (36) were also streaked from tubes of AD broth with growth at the highest dilution. Colonies were selected from both plate media to the medium of Mundt and Johnson (27), and cultures were maintained in this medium for routine culture and storage.

Identification of cultures. Cultural conditions and criteria employed for identification of the cultures have been described earlier (26, 27). All sugar fermentations and ability to grow at 10°C and at 45°C, in 6.5% NaCl broth, and at pH 9.6 were determined in phenol-red meat-extract broth base (PR). Additional media were bile-esculin agar (BE, reference 18) upon which enterococci and but few other bacteria produce growth with blackening (16), and malic acid medium (36), in which S. faecalis and its variants are usually anaerogenic in the presence of glucose, but S. faecium and its variants are usually not. The ability to decarboxylate tyrosine was determined with the basal medium of Deibel, Lake, and Niven (11) to which brom-cresol-purple and 3% gelatin were added; the medium was heated and then tubed in 6-ml quantities in screw-capped tubes. Incubation of the medium through 6 days was practiced because some enterococci decarboxylate tyrosine very slowly with the late restoration of the alkaline color of the indicator. S. faecium var. casseliflavus was recognized by pigment formation on streaked plates of 4% sucrose-Trypticase soy agar (Baltimore Biological Laboratory) after incubation for 4 days. Except as noted, all media were incubated at 37°C.

RESULTS

Distribution of enterococci. Enterococci were recovered from 53% of 403 cultural assays (Table 1). The greater number of individuals, representing the orders Coleoptera, Hymenoptera, and Lepidoptera, as compared with numbers in the remaining orders, indicate the relative frequency in occurrence of members of the various orders in the environments at the time of sampling.

Enterococci were present in all specimens of the adults and pupae of Camponotus spp. taken from forest litter, of Carabidae taken from forest soils, and of Chrysobothris, Epicauta, and adult and larval forms of Phylophaga taken from flowering agronomic plants. They were present in 40 to 50% of the specimens of Apis and Bombus spp. taken from flowers, Monophadnoïdes geniculatus taken from raspberry canes, members of the taxa Diabrotica and Coccinellidae, and the majority of Chauliognathus sp. The weevils, Antho- nomus grandis and Odontopus calceatus, and the wasp, Polistes, were devoid of enterococci.

Except for the leaf-feeding Hyphantria cunea, 85 and 100% of the larval forms of Lepidoptera yielded enterococci. These were taken from green and also overwintering corn plants. The succulent floral structures of the corn plant frequently bear enterococci in high numbers (23). Enterococci were recovered from adults of Papilio and Pieris, but not from three specimens of related Nymphalidæ.

Members of the families Acrididæ and Gryllidæ of the order Orthoptera associated with grassy areas yielded enterococci, but the few specimens of Microcentrum and Stagmomantis did not. Two of the four specimens of the order Hemiptera and none of the 75 specimens in the order Diptera, Homoptera, and Isopota yielded the bacteria on culture.

Seasonal fluctuation in populations and persistence in dormancy. Representatives of 11 of the 37 taxa of insects listed in Table 1 were encountered during more than one collection period; these are shown in Table 2, together with the month of sampling and other pertinent data. Total numbers of enterococci per gram of insect weight increased with increasing seasonal temperature. This is consonant with earlier observations made with plants. The value of the two regressions may be questioned, because one specimen of each was cultured. Enterococci were present in 90% of specimens of insects taken during the dormant season in January, in numbers to 3.9 \times 10^7/g of body weight. Attempts to reproduce survival during dormancy by frozen storage of insects taken during the late summer were unsuccessful; specimens of M. geniculatus and Z. grandiosella taken in the field during January harbored enterococci, whereas those placed into storage did not.

Distribution of enterococcal species. Little consistency is seen in the occurrence of S. faecalis, S. faecium, and S. faecium var. casseliflavus within any species of insect (Table 3). Each taxon may occur singly or together with either of the other two or with other spherical bacteria as Aerococcus, Leuconostoc, and Pedicoccus, and presence of any taxon may vary with the time of sampling. Of the 37 taxa of insects from which enterococci were obtained, S. faecalis was recovered from
TABLE 1. Occurrence of enterococci in insects

| Insect taxon       | Recovery* of streptococci | Insect taxon       | Recovery* of streptococci |
|--------------------|---------------------------|--------------------|---------------------------|
|                    | No. samples   | No. + | Percent |                    | No. samples   | No. + | Percent |
| Order Coleoptera   |              |      |         | Order Coleoptera   |              |      |         |
| Anthonomus grandis (boll weevil) | 5  0  0 |      |         | Monophasdnoides geniculatus (raspberry sawfly) | 18  8  44 |
| Family Carabidae (ground beetles) | 8  8 100 |      |         | Monophasdnoides geniculatus larvae | 5  5 100 |
| Chauliognathus (soldier beetle) | 10 9 90 |      |         | Polistes (paper wasp) | 5  0  0 |
| Chrysobothris femorata (flaished apple borer) | 10 10 100 |      |         | Family Ichneumonidae (ichneumon wasp) | 1  1 100 |
| Family Coccinellidae (lady beetles) | 8  4 50   |      |         | Order Isopera |              |      |         |
| Diabrotica undecimpunctata (cucumber beetle) | 5  2 40   |      |         | Reticulotermes flavipes (termite) | 20  0  0 |
| Epicauta (blister beetle) | 10 10 100 |      |         | Order Lepidoptera |              |      |         |
| Lucanus elaphus (stag beetle) | 3  0 0 |      |         | Hyphantria cunea (fall webworm) | 15  0 0 |
| Odontopus calceatus (poplar weevil) | 32 0 0 |      |         | Heliothis zea (corn earworm) (larvae) | 5  5 100 |
| Phyllophaga (may beetle) | 5  1 100 |      |         | Family Nymphalidae (nymphalid butterflies) | 3  0 0 |
| Phylophaga larva (grubworm) | 5  5 100 |      |         | Family Noctuidae (noctuid larva) | 1  0 0 |
| Saperda calcarata (poplar borer) | 1  0 0 |      |         | Ostrinia nubilalis (European corn borer) | 20 17 85 |
| Order Diptera       |              |      |         | Papilio (swallowtail butterfly) | 2  2 100 |
| Tabanus (horsefly)  | 1  0 0 |      |         | Pieris rapae (cabbage butterfly) | 10 8 80 |
| Order Hemiptera     |              |      |         | Unidentified moth | 1  0 0 |
| Acrosternum (green stink bug) | 4  2 50   |      |         | Unidentified furryworm | 1  0 0 |
| Order Homoptera     |              |      |         | Diatraea grandiosella (sweet corn borer) (Larvae) | 22 22 100 |
| Family Aphididae (aphids) | 50 0 0 |      |         | Order Orthoptera |              |      |         |
| Family Cicadellidae (leafhoppers) | 7  0 0   |      |         | Family Acrididae (shorthorn grasshopper) | 4  2 50 |
| Order Hymenoptera   |              |      |         | Family Gryllidae (cricket) | 10 10 100 |
| Apis mellifera (honeybee) | 20 8 40  |      |         | Microcentrum (longhorn grasshopper) | 3  0 0 |
| Bombus (bumblebee)  | 6  3 50 |      |         | Stagmomantis (praying mantia) | 1  0 0 |
| Camponotus (carpenter ant) | 60 60 100 |      |         | Total | 403 212 53 |
| Camponotus pupae    | 10 10 100 |      |         |                |              |      |         |

* Recovery on KF or MMRS agar, or both.

32%, S. faecium from 22%, and S. faecium var. casseliflavus from 44% of members of the taxa during the 14-month study. S. faecalis was never recovered from any insect taken during October through March. Only one of 30 other dormant individuals yielded cultures of S. faecalis when specimens were cultured during the active season, suggesting that this bacterium is not commensal with members of these taxa.

**Properties of the enterococci.** S. faecalis and S. faecium var. casseliflavus exhibit a high percent conformity to the properties established for the enterococci by Sherman et al. (33), to growth in ethyl violet (EV) broth, and in typical pigmentation on BE and tellurite agars and agar containing 1,3,5-triphenyltetrazolium chloride agar (TTC, reference 1). The small percent nonconformity may be within the limits of deviation for a species noted by Deibel (10). The heterogeneity noted earlier among S. faecium obtained from plants (10, 28) exists also among cultures obtained from insects. The colonies of the pigmented variant on TTC agar are neither red nor white as described by Barnes (1), but pink, and are recorded as negative. Many cultures of S. faecium failing to grow in EV broth are sensitive to the sodium azide as determined by inoculation of PR-glucose broth with and without sodium azide. All S. faecalis taken from insects are anaerobic in malate-glucose broth, as are 20% of S. faecium and 9% of cultures of the pigmented variant. S. faecalis is variable and S. faecium var. casseliflavus were usually negative in the ability to decarboxylate tyrosine, but the majority of isolates of S. faecalis responded typically.

Either a transient reduction of litmus in
milk or no reaction is produced by those cultures not fermenting lactose. Certain nonfermenting cultures of *S. faecalis* and of *S. faecium* either reduce methylene blue in milk weakly or bring about no reduction. Nonfermenting cultures of *S. faecium* var. *casseliflavus* reduce the methylene blue, further evidence suggesting the stronger reducing ability by members of this taxon as compared with those of *S. faecium*.

The lactose-fermenting cultures of *S. faecium* and of the pigmented variant produce either a reduced, acidic reaction in litmus milk or a reduced, hard, acidic curd. Less than 2% of the cultures of *S. faecalis* of insect origin produce the characteristic acid-proteolytic mode of digestion described in *Bergey's Manual* (3), and more than 93% of the cultures produce a soft, rennet-like or flowing curd (RSCD). These results differ from those reported by Geldreich et al. (18); however, they have captured specimens of insects from environments which this study sought to avoid.

Few *S. faecalis*, 51% of *S. faecium*, and 93% of the pigmented cultures ferment raffinose. Both named species studied by Orla-Jensen (31) ferment the sugar, the former weakly. Fermentation of raffinose is not a characteristic of *S. faecalis* of human origin (2). Orla-Jensen's figures suggest a weak fermentation of arabinose by *S. faecalis*, and none of the cultures studied by Deibel et al. (11) ferment it. Nearly 90% of the insect-derived cultures ferment arabinose weakly, as demonstrated by change in color of the indicator in PR broth, and a reduction in pH of 0.9 unit, as determined electrometrically for approximately 100 cultures.
### Table 3. Percent of cultures of enterococci responding positively to selected characteristics

| Characteristic | S. faecalis | S. faecium | S. faecium var. casei flavus |
|----------------|-------------|------------|-----------------------------|
| No. of cultures | 314-359 | 176 | 148-500 |
| Growth: | | | |
| On bile-esculin agar | 98.3 | 91.2 | 100.0 |
| At 10°C | 95.7 | 91.6 | 100.0 |
| At 45°C | 99.7 | 73.6 | 98.2 |
| In 6.5% NaCl | 98.6 | 66.0 | 99.1 |
| At pH 6.6 | 71.4 | 68.8 | 97.4 |
| On tellurite agar | 94.9 | 18.2 | 86.2 |
| In ethyl violet broth | 100.0 | 83.1 | 99.6 |
| NH₃ from arginine | 86.0 | 34.0 | 86.0 |
| Methylene blue reduced in 48 hr | 92.0 | 64.2 | 97.0 |
| Tetrazolium reduced | 98.4 | 17.6 | 4.4 |
| Litmus milk: | | | |
| Reduced (only) | 4.8 | 30.8 | 26.3 |
| Reduced, acid | 0.0 | 23.9 | 20.4 |
| Reduced, acid curd | 0.0 | 46.2 | 53.3 |
| Reduced, acid curd digested | 1.9 | 0.0 | 0.0 |
| Reduced, soft curd digested | 93.3 | 0.0 | 0.0 |
| Tyrosine decarboxylated | 99.4 | 30.2 | 5.8 |
| Gas from malate | 0.0 | 79.7 | 90.8 |
| Fermentation of: | | | |
| Raffinose | 5.9 | 51.0 | 93.0 |
| Arabinose | 88.5 | 99.3 | 99.4 |
| Melitose | 93.8 | 43.0 | 5.7 |
| Melibiose | 9.4 | 81.3 | 99.4 |
| Sorbitol | 81.0 | 63.0 | 22.0 |

* Number of cultures studied: *Streptococcus faecalis*, 314-359; *S. faecium*, 176; *S. faecium var. casei flavus*, 148 cultures for determining fermentation of raffinose, arabinose, melezitose, melibiose, and sorbitol, and 500 cultures for other properties.

Among the 314 cultures of *S. faecalis*, the general pattern is the fermentation of melezitose but not melibiose. Those cultures fermenting melibiose also ferment melezitose. Among 176 cultures of *S. faecium*, 88 or 50% fermented both sugars, 41% fermented melibiose but not melezitose, and the remainder fermented neither sugar. More than 99% of 148 cultures of the pigmented variant tested fermented melibiose, 3% fermented both sugars, and a few cultures fermented neither. The differences from results obtained by Orla-Jensen (29) and Deibel et al. (11) are a probable reflection in differences of origins of the cultures studied.

### DISCUSSION

The results of this study suggest that insects play a role in the distribution of enterococci within the plant world during the growing seasons of the year. Absent from plants during winter and early spring (25), enterococci appear upon these with new outgrowth and flowering, and are recoverable with increasing frequency with the advance of the season. The cycle corresponds with the extent of insect activity in nature. The apparent randomness in the distribution of the taxa of the enterococci also bears a similarity to that on plants and animals in a wild environment (23-25).

Insects have the potential for being overwintering agents of the enterococci. The enterococci, however, also are constant residents of wild, nonlactating as well as lactating animals, including *Mammalia*, *Amphibia*, and *Reptilia* (24). In selected environments a logical visualization is the reseeding of plants via insects which are both coprophagous and floriphagous and further spreading by insects which are primarily nectarophagous, with reproduction of the enterococci on plants to the extent that environmental conditions permit. Even among insects the life pattern may determine the nature of the intestinal flora. Except for *Reticulotermes* sp., insects feeding on nectarine foods or dwelling in the humid environment of soil or in deep ground cover harbor enterococci in this study, whereas members of *Odontopus*, *Hyphantria*, *Aphididae*, and *Cicadellidae*, which feed and dwell on less succulent leaves and stems did not.

The prominent feature of *S. faecalis* taken from agronomic and wild plants and from wild animals (24, 25) during earlier studies and from insects is the RSCD reaction in litmus milk. The curd is rennet-like, soft, and flowing, except for a very few cultures not fermenting lactose which produce a grainy curd prior to digestion. The pH of the milk during 1 to 4 days of incubation with several hundred cultures was approximately pH 4.6 to 6.6. Digestion proceeds in stratiform fashion from the top downward terminating in a small, plastic residue reminiscent of sweet-curdling digestion. The reaction is unknown or rare among cultures of human origin (26) and cultures from human sources which we have studied. Therefore, cultures of *S. faecalis* exhibiting this reaction appear to be part of the natural microflora of nature. The reaction in litmus milk then becomes an excellent marker in analytical laboratories to distinguish between *S. faecalis* introduced to materials in the remote past by methods characteristic to nature and *S. faecalis* of recent and human origin.

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