Gut Flora of *Galleria mellonella* Suppressing Ingested Bacteria

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*Streptococcus faecalis*, the only bacterium occurring almost invariably at high populations in guts of *Galleria mellonella* larvae, suppresses bacteria ingested with food by producing bacteriocin, an antibioticlike substance having a narrow range of bactericidal activity, and by releasing a lysozymelike enzyme, especially in the presence of proteolytic enzymes. The insect intestinal fluid apparently increases the activity of *S. faecalis* lytic enzyme. Unlike other organisms tested, *S. faecalis* has shown a strong bactericidal action against various species of unrelated bacteria. Microscopical examination of the sensitive organisms used as indicators has revealed changes resembling formation of protoplasts, gradually leading to destruction of bacterial cells. The insect guts could not be infected, even when the larvae had ingested a high dose of *Pseudomonas aeruginosa*, *Proteus mirabilis*, or *Bacillus thuringiensis*. The mechanism by which *S. faecalis* could suppress the ingested bacteria is suggested.

**KEY WORDS:** *Galleria mellonella*: *Streptococcus faecalis*: *Pseudomonas aeruginosa*: *Proteus mirabilis*: *Bacillus thuringiensis*: bacteriocin; lysozymelike activity; autolysin; lytic enzyme; proteases.

**INTRODUCTION**

Several investigators have claimed that the gut flora of an insect may be needed by the host for some physiological processes to extend its own metabolic faculties. Various insect species are capable of utilizing, as food, materials generally regarded as nearly or completely indigestible to other animals. Beeswax is utilized by a few animals only, the most important of which are the two cosmopolitan species of *Galleria mellonella* and *Achroia grisella*. The only bacterium occurring in all development stages of *G. mellonella* is *Streptococcus faecalis*. It is specially adapted to multiply and survive in *G. mellonella*, and is normally transmitted to the filial generation of the host (Bucher, 1963). Waterhouse (1959) and Dudziak (1975) have observed an undisturbed development of bacteria-free cultures of *G. mellonella*, indicating that the intestinal flora do not play any role in the nutrition of the wax moth larvae.

Although it is at present not possible to ultimately define the mechanisms which prevent or suppress bacterial multiplication in arthropods, the failure of infection in insect guts is attributed to the presence of a gut barrier in the host. In a recent study (Jarosz, 1975) we suggested the possibility that in the wax moth larvae *S. faecalis*, an organism capable of producing lytic activity, prevents proliferation of bacteria absorbed with food. The present paper describes further findings on the possible role of *S. faecalis* as a component of the gut barrier of *G. mellonella*, especially with regard to the production of bacteriocin and the release of lysozyme in the presence of the insect intestinal fluid.

**MATERIALS AND METHODS**

**Demonstration of bacteriocin activity.** The method of Brock et al. (1963) modified by Kalmanson et al. (1970) was employed to demonstrate the bacteriocin activity of *S. faecalis*, the isolates originating from the larvae or pupae of *G. mellonella*.

**Seeded agar test plates.** The bactericidal action of *S. faecalis* and of other species of unrelated bacteria was compared by the seeded agar test plate method. In order to examine the biocidal effect of the given organism as producer, an 18-hr broth culture was inoculated to the base layer of the medium, and incubated at 35°C until it pro-
duced numerous colonies, but no confluent lawn had yet formed. Then, the broth culture of the strain used as indicator, diluted with soft (0.7%) agar medium at the ratio of 1:10, was gently poured at a 10-ml volume over the base layer and incubated at 35°C until a solid lawn had formed on the control plate containing the indicator strain only. Both the base and upper layer were composed of agar–dextrose medium, except for Bacillus larvae, which grew on tryptone–dextrose–yeast agar medium.

The following organisms were used as both producers and indicators: S. faecalis (five strains isolated at random from the gut microflora of G. mellonella); dilutions of the intestinal content containing pure populations of S. faecalis (four larvae); Sarcina lutea; Micrococcus lysodeikticus; Bacillus subtilis ATCC 6633; Bacillus megaterium; Bacillus cereus ATCC 8145; Bacillus thuringiensis CCM 19; Bacillus lentus IP 5273; Bacillus alvei IP 5284; B. larvae (strain isolated from a honeybee larva which died of American foulbrood); Escherichia coli (colicin-sensitive strain ROW and colicin-producing strain JC. 7623 col 16); Pseudomonas aeruginosa (four laboratory strains); Proteus vulgaris OX19; Proteus mirabilis; Serratia marcescens; and three antibiotic-producing strains of Bacillus spp. Nos. 8, 26a, and 53 isolated from the gut flora of G. mellonella larvae as accidental organisms.

Effect of the intestinal fluid on the activity of S. faecalis lysozyme. The contents of 10 intestines were each collected in 1 ml of 0.033 M Sörensen buffer, pH 6.4. To deprive the intestinal fluid of its lysozyme activity, the resulting buffer mixture was passed through a CM-32 ion-exchange (Whatman Biochem. Ltd.) column previously equilibrated with the same buffer. The effluent, deprived of lysozyme activity, was sterilized using a Schot G-5 filter, and added (0.35 ml/10 ml) to agar–dextrose medium buffered with 0.02 M Sörensen buffer, pH 6.8. Assay plates, containing freeze-dried micrococcus cells (Sigma) as a substrate for lysozyme, were inoculated with S. faecalis, and incubated at 35°C for 48 hr before measuring diameters of the lytic zones. An assay plate with no intestinal fluid added served as control.

In another experiment, assay plates containing various commercially available proteases, such as trypsin, pepsin, and Pronase P (Serva), at the final concentration of 2.0 μg/ml of agar–dextrose medium, were inoculated with S. faecalis in the same manner and incubated under the same conditions.

Attempts to infect the guts of G. mellonella. Trials were conducted using larvae intensively fed honeycombs treated with insect bacterial pathogens (P. aeruginosa, P. mirabilis, or B. thuringiensis). About 5 × 10^7 viable cells/cm^2 of honeycomb surface were used to provoke infection in the larvae guts. Ten test insects, selected at random, were kept in a sterile Petri dish at 34°C for 3 hr to remove the organisms freshly absorbed with food. Then, the digestive tracts were isolated surgically, serial 1/10 dilutions were made, and viable cell counts per larva were determined. For residual activity testing, similar counts were made daily on honeycomb samples taken from a wax moth colony sprayed with an insect pathogen. All larvae were under constant observation for gross changes in behavior or for other symptoms of disease.

**RESULTS AND DISCUSSION**

**Bacteriocinogenic Activity of S. faecalis Isolates Originating from G. mellonella**

Evidence obtained in this study indicates that isolates of S. faecalis originating from G. mellonella not only exhibit lytic activity (Jarosz, 1975), but also produce bacteriocin, an antibioticlike substance having a limited spectrum of bactericidal activity. It is evident from the results presented in Table 1 that all strains of S. faecalis used as producers inhibited the growth of all strains of this organism used as indicators. As reported by others, S. faecalis isolated from other sources (Kalmanson et al., 1970) as
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</table>

*a The macrocolony of the bacteriocin producer averaged 8 mm in diameter. Diameters of zones of inhibition of S. faecalis strains used as indicators are given in millimeters. X = Trace bacteriocin activity; L = Isolates from larvae guts; P = originating from pupae.

well as S. faecalis var. zymogenes (Brock et al., 1963), an organism closely related to S. faecalis, inhibited many, but not all, strains of their own species. Moreover, the same strains used as indicators were somewhat susceptible to the action of their own bacteriocin. A similar phenomenon was described earlier by Ivánovics and Alföldi (1954), who found that a certain strain of B. megaterium was sensitive to its own bacteriocin if used at a high concentration.

**Bactericidal Action of S. faecalis against Various Species of Unrelated Bacteria**

It is of interest to note from the results presented in Table 2 that S. faecalis inhibits the multiplication of various bacterial species used as indicators, including bacteria pathogenic for the wax moth larvae (B. thuringiensis, P. aeruginosa, P. mirabilis, S. marcescens). Moreover, S. faecalis showed a bactericidal effect against all bacteria tested because in no case was there any growth of replicates. The broad spectrum of S. faecalis activity is in contrast to the very narrow activity range of bacteriocins. However, Kalmanson et al. (1970) have demonstrated that the parent bacterial forms of Gram-negative bacteria, such as P. mirabilis, E. coli, and S. marcescens, as well as Staphylococcus aureus, are resistant to bacteriocin action, but that their L-forms are generally susceptible. Microscopical examination of samples of indicator strains taken at 1-hr intervals from the site of the growth inhibition zones revealed changes resembling formation of protoplasts, gradually leading to destruction of the bacterial cells. It seems likely that the lytic enzyme produces the L-forms of unrelated bacteria used as indicators, which become sensitive to the bactericidal action of both bacteriocin and lysozyme, and this may explain the broad spectrum of the antibacterial activity of S. faecalis on assay plates inoculated with various bacterial species.

**Comparison of Antibacterial Activity of S. faecalis with That of Other Bacterial Species**

Seeded agar test plates, a very convenient means of antibacterial activity assay, were used for comparing the activity of S. faecalis with that of other bacterial species. Noteworthy is the fact that S. faecalis ap-
# Table 2

**Bactericidal Effect of Isolates of *Streptococcus faecalis* Originating from the Bacterial Flora of *Galleria mellonella* Against Various Species of Unrelated Bacteria**

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<th>Indicator strain</th>
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<td><em>Proteus mirabilis</em></td>
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* Bioassays were made by the method of Brock et al. (1963). The macrocolony of the bactericidal activity producer averaged 8 mm in diameter. Diameters of zones of inhibition of the indicator strains are expressed in millimeters.

E. coli col 16 effectively inhibited the growth of colicin-sensitive strain E. coli ROW only; others, however, had no inhibitory effect whatever.

**Effect of *S. faecalis* Lytic Enzyme and Egg White Lysozyme on Bacteria Sensitive to Bactericidal Action of *S. faecalis***

To determine if the lytic enzyme of *S. faecalis* inhibits the growth of the bacteria sensitive to the bactericidal action of this organism when assayed by seeded agar test plates, drops of the *S. faecalis* cell autolyzate obtained by the method of Shockman and Cheney (1969), containing soluble autolysin (herein referred to also as lytic enzyme), were placed on the assay plates inoculated with indicators, then incubated overnight. Autolysin did not appear to inhibit the multiplication of the organisms tested, except for *M. lysodeikticus*, *S. lutea*, *B. thuringiensis*, *B. megaterium*, *Bacillus* spp. Nos. 8, 26a, and 53, *Bacillus subtilis*, and *B. lentus* (the latter two were inhibited slightly). Crystalline egg white apparently inhibited the multiplication of all bacterial species used at a high inoculum as indicators. It is also noteworthy that the assay plates inoculated with *S. faecalis* could not be infected even with a heavy spray of tap water directed to the upper layer of soft agar, while abundant overgrowth was usually observed on plates inoculated with other bacterial species a short time after spraying. However, the biocidal activity of *S. faecalis* was not able to inhibit the growth of yeasts and molds, such as *Candida guilliermondi*, *Candida krusei*, and *Geotrichum candidum*.

Of the almost 20 bacterial species used for comparison, *B. larvae* also had a visible inhibitory effect against many species of bacteria used as indicators. This fact clarifies the observation described earlier by Holst (1945) that honeybee larvae dead of American foulbrood contain almost invariably pure cultures of *B. larvae*.

*Bacillus subtilis*, *B. thuringiensis*, *Bacillus* spp. Nos. 8, 26a, and 53, and *S. lutea* did not inhibit the multiplication of Gram-negative bacteria and of some bacilli; strain
lysozyme at a concentration of 4.70 μg/drop, used as the reference enzyme, also inhibits the growth of organisms susceptible to lytic enzyme only, *M. lysodeikticus* being more susceptible to the action of this enzyme.

**Effects of Intestinal Fluid and Proteolytic Enzymes on the Activity of S. faecalis Lytic Enzyme**

The apparent increase in the lytic activity of *S. faecalis* was noted on the assay plates supplemented with intestinal fluid of *G. mellonella* larvae (Fig. 1). In control plates without the gut contents the lytic zones were of modest size and indistinct, whereas on plates with the added intestinal fluid they were clearly outlined and apparently increased in diameter as early as 48 hr after incubation. Commercially available proteases, such as trypsin, pepsin, or Pronase P, added to the growth medium at a low concentration, also increase the bacteriolytic activity of *S. faecalis*; this is especially true of trypsin used at a concentration of 2 μg/ml. The increased lytic activity of *S. faecalis* on the assay plates with the added intestinal fluid can be ascribed to the presence of the insect proteases.

**Attempts to Infect the Guts of G. mellonella Larvae**

The intestinal contents taken from the test animals 1, 2, 3, 6, 9, and 12 days after feeding revealed no significant differences in the number and types of organisms present; only *S. faecalis* occurred at large populations in the insect guts. The larvae that had ingested a high dose of *P. aeruginosa*, *P. mirabilis*, or *B. thuringiensis* effectively eliminated them from their digestive tracts. *P. mirabilis* alone was sometimes found at low populations, never exceeding $5 \times 10^3$ cells/larva, the two other bacterial parasites occurring singly throughout the observation period. The average half-life of the cells of *B. thuringiensis*, *P. aeruginosa*, and *P. mirabilis* using day 0 as the baseline, was 3, 2, and 2 days, respectively.

The animals fed bacterial pathogens displayed no abnormal behavior or other sign of disease. There were some deaths in each test group, but they could not be attributed to the pathogen ingested. No moribund animals were found to contain any intracoelomic or gut populations of the pathogen.

**GENERAL DISCUSSION**

A very interesting fact revealed in the course of this study is the broad spectrum of the bactericidal activity of *S. faecalis*. Various bacterial species belonging to Gram-positive bacilli, Gram-negative bacteria, and micrococci, including bacterial pathogens for lepidopterous larvae, such as...
B. thuringiensis, P. aeruginosa, P. mirabilis, and S. marcescens, proved to be highly susceptible to the killing action of S. faecalis. Moreover, on comparing the antibacterial action of S. faecalis with that of other bacteria tested, it appeared that only S. faecalis had a strong bactericidal activity directed against different types of unrelated bacteria. The broad spectrum of the antibacterial activity of S. faecalis is in contrast to the narrow range of activity of both bacteriocin and lysozyme, the bactericidal factors produced by this organism (Brock et al., 1963; Conover et al., 1966). Bacteriocins are antibiotic-like substances which are usually active only against organisms of the same and of closely related species. Likewise, lysozyme is a bacteriolytic enzyme with a limited spectrum of bactericidal activity. It acts primarily on some Gram-positive bacteria.

The exponential phase cultures of S. faecalis contain an autolytic enzyme, a \(\beta\)-N-acetylmuramidase glycanydrolase (EC 3.2. 1.17), which has been found to occur basically in the cell wall fraction in an active and a latent proteinase-activable form (Conover et al., 1966; Shockman and Cheney, 1969). The treatment with a number of proteolytic enzymes, such as trypsin, pepsin, Pronase, papain, and others, increases the rate of lysis of log walls and releases a proteinase-activable form of the lytic enzyme (Shockman et al., 1967a, b; Shockman and Cheney, 1969). It seems likely that in the insect digestive tract proteases of G. mellonella also activate the latent form of autolysin to an active lytic enzyme and in this way produce favorable conditions for the killing of ingested bacteria.

Some studies have shown that the L-forms are more susceptible than the parent bacterial forms to certain antibiotics (Montgomery et al., 1966), to the killing action of serum (Kalmanson et al., 1967), to staphylococcal \(\alpha\)-toxin or streptolysin S (Bernheimer and Schwartz, 1965), and to the bactericidal action of bacteriocins (Kalmanson et al., 1970). The organisms that were totally resistant to the action of bacteriocin, such as Staphylococcus aureus, E. coli, P. mirabilis, and S. marcescens, became sensitive when their L-forms were used as indicators (Kalmanson et al., 1970). It can be assumed as a conclusion from the above findings that the latent form of autolytic enzyme released from the cell walls of S. faecalis and activated to an active lytic enzyme in the presence of insect proteases produces the L-forms of the bacteria ingested with food, which become sensitive to the killing action of both bacteriocin and lysozyme.

Finally, it is reasonable to conclude from the observations made so far and from the literature cited that in the guts of G. mellonella larvae, S. faecalis suppresses the bacteria ingested with food by the following mechanisms: (1) it produces bacteriocin, an antibiotic-like substance with bactericidal activity against S. faecalis and against the L-forms of various species of unrelated bacteria; (2) it releases a lysozymelike activity, especially in the presence of proteolytic enzymes; (3) the proteases occurring in the insect intestinal fluid increase the liberation of S. faecalis lysozyme, which, similar to insect lysozyme, (4) may produce L-forms of the ingested bacteria, which (5) become sensitive to the killing action of both bacteriocin and lysozyme. In addition, the suggested mechanism by which S. faecalis could play a role in limiting ingested bacteria is substantiated by the following facts: (1) S. faecalis is specially adapted to multiply and survive in the guts of G. mellonella; (2) it occurs almost invariably at high populations, often exceeding \(10^7\) cells/insect; (3) in the larval guts there exist favorable conditions not only for the release of S. faecalis lytic enzyme, but also for the killing action of this enzyme against Gram-negative bacteria (Mohrig and Messner, 1968). Moreover, it seems that the strains of S. faecalis inhabiting the guts of G. mellonella represent a specific ecological type of this bacterium. Unlike other
strains originating from different sources (Brock et al., 1963); Kalmanson et al., 1970), they effectively inhibited the multiplication of a broad range of bacterial species.

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REFERENCES


