APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1982, p. 832–837
0099-2240/82/100832-06$02.00/0

Vol. 44, No. 4

Susceptibility of Fecal Streptococci of Poultry Origin to Nine Growth-Promoting Agents

GUNINDRA N. DUTTA* AND LUC A. DEVRIESE

Faculty of Veterinary Medicine, State University of Ghent, B-9000 Ghent, Belgium

Received 29 March 1982/Accepted 1 July 1982

The minimal inhibitory concentrations of nine growth-promoting agents were determined by an agar-dilution method against 66 bile-tolerant streptococcal (8 *Streptococcus faecalis*, 23 *Streptococcus faecalis* subsp. *liquefaciens*, 15 *Streptococcus faecium*, and 20 carboxyphilic streptococci) strains isolated from the ceca of 52 chickens on 19 farms. Avoparcin was equally active on all groups. The natural susceptibilities against the other substances differed among the groups studied. Bacitracin and virginiamycin were more active on *S. faecium* and *S. faecalis* than on *S. faecalis* subsp. *liquefaciens*; lincomycin and the macrolide antibiotics were more active on *S. faecium* than on the other groups; and flavomycin was active on all groups except *S. faecium*. High percentages of acquired resistance were noted in all groups against bacitracin, lincomycin, and the macrolide antibiotics, oleandomycin, spiramycin, and tylosin. Resistance to nitrovin was found only among the *S. faecalis* and *S. faecium* groups.

Bacterial colonization of the intestinal tracts of chicks takes place soon after hatching, when the young chicks ingest food (18, 21). During the first 2 to 4 days, streptococci and enterobacteria colonize the small intestine and cecum and remain throughout the life of the bird. *Streptococcus faecalis* (15), *S. faecalis* and a filterable agent (9), *Streptococcus faecium* (12, 14), and a fecal filtrate in combination with *Streptococcus faecalis* subsp. *liquefaciens* and *S. faecium* (12) have been incriminated as agents in avian growth depression. However, the evidence presented so far is conflicting; different workers have failed to confirm each others' findings.

The development of intensive methods for rearing poultry has been accompanied by the use of numerous growth-promoting agents in the diet (3), most of which have antibacterial activity. Apart from the activity of dietary bacitracin on *S. faecalis* subsp. *liquefaciens* and related streptococci (2), virtually nothing is known about the activity of the growth-promoting antibacterial agents on the fecal streptococci, which are important constituents of the bacterial flora of the intestinal tract and which also appear to be important agents in avian growth depression. Knowledge about the activity of the growth-promoting agents on the various intestinal bacterial species might lead to the understanding of the mechanism by which antimicrobial agents improve growth and the efficiency of food conversion. The present investigation was undertaken to study the activity of the most commonly used growth-promoting agents on streptococci isolated from the ceca of poultry.

MATERIALS AND METHODS

Bacterial strains. Sixty-six strains of streptococci, isolated from the ceca of 52 chickens brought for autopsy from 19 farms to the Veterinary Faculty, State University of Ghent, in 1980 were used in this study. Cecal swabs were collected and inoculated onto Islam medium (16) supplemented with 30 μg of neomycin, 15 μg of nalidixic acid, and 50 μg of metronidazole per ml of the medium (23). Primary incubation was made in an incubator containing a continuous supply of 5% CO_2 at 37°C. The plates were incubated for 48 h, and colonies were picked up in duplicate Blood Agar Base (Oxoid, Basingstoke, England) plates for purification. One set was incubated aerobically at 37°C, and the other set was incubated in the CO_2 incubator. Passages were repeated on the same medium under the same incubation conditions until the cultures were considered pure. If the same culture was able to grow under both incubation conditions, then only the aerobic culture was saved for further studies. When a culture failed to grow optimally aerobically but grew optimally in the CO_2 incubator, the culture was marked separately, and all further tests were carried out in the presence of 5% CO_2 or anaerobically in a GasPak system (BBL Microbiology Systems, Cockeysville, Md.).

The following strains were included in the study.

S. faecalis CCM 2541, *S. faecalis* subsp. *liquefaciens* CCM 5613, and *S. faecium* CCM 2308 were obtained from the Czechoslovak Collection of Microorganisms (CCM), Brno, Czechoslovakia.

S. faecalis EBF 30/82, *S. faecalis* subsp. *liquefaciens* EBF 87/210, and *S. faecium* EBF 87/152 were

TABLE 1. Results of the physiological tests for identification of *Streptococcus* strains isolated from ceca of poultry

Physiological test	No. of strains showing positive reaction			
	S. faecalis (8 strains)	*S. faecalis* subsp. *liquefaciens* (23 strains)	*S. faecium* (15 strains)	Carboxyphilic streptococci (20 strains)
Growth at pH 9.6	8	23	15	17
Growth in 6.5% NaCl	8	23	15	16
Survive 60°C for 30 min	6	23	13	19
Growth at 45°C	8	23	15	20
0.1% methylene blue	8	23	10	14
Ammonia from arginine	8	23	15	4
Esculin hydrolysis	8	23	15	20
Growth on 40% bile	8	23	15	20
Reduction of tetrazolium	8	23	0	15
Resistance to K-tellurite	8	23	0	NT[a]
Hydrolysis of gelatine	0	23	0	3
Hydrolysis of starch	0	0	0	8
Fermentation of:				
Lactose	8	23	15	20
Mannitol	8	22	12	18
Melezitose	8	23	2	20
Melibiose	6	10	13	20
Raffinose	5	3	3	19
Glycerol	8	23	15	3

[a] NT, Not tested.

obtained from C. S. Impey, Food Research Institute, Colney Lane, Norwich, England.

S. faecalis subsp. *liquefaciens* 2SY4 and 2STI and *S. faecium* SY1 and 2CTI were obtained from R. Fuller, National Institute for Research in Dairying, Shinfield, Reading, England.

Physiological tests for identification. Only catalase-negative, gram-positive coccobacilli capable of growing in the Bile-Esculin Agar (Difco Laboratories, Detroit, Mich.) were considered for further identification (11). Catalase production was tested by pouring one drop of 3% H_2O_2 in a heavily grown area of a purified culture.

Tests for growth at pH 9.6, growth in medium containing 6.5% NaCl, survival at 60°C for 30 min, growth at 45°C, reduction of 0.1% methylene blue, production of ammonia from arginine, hydrolysis of esculin, growth in the presence of 40% bile, liquefaction of gelatin, and fermentation of the carbohydrates were done according to Barnes and co-workers (2).

Tests for hydrolysis of starch and reduction of tetrazolium were carried out as previously described (10). Resistance of the strains to potassium tellurite was tested by using the Tellur diagnostic tablets per the instructions of the manufacturer (A/S Rosco, Taastrup, Denmark).

Growth-promoting antibacterial agents. The growth-promoting agents, as laboratory standard powder, were obtained as follows.

Avoparcin sulfate and nitrovin (Payzone) were from Cyanamid of Great Britain Ltd., Gasport, England; bacitracin was from A. S. Apothekernes Laboratories, Oslo, Norway; oleandomycin phosphate was from Pfizer Co., Brussels, Belgium; flavomycin was from Hoechst Pharmaceuticals, Frankfurt, W. Germany; lincomycin hydrochloride was from The Upjohn Co., Kalamazoo, Mich.; spiramycin adipate was from Specia, Paris, France; tylosin base was from Eli Lilly & Co., Indianapolis, Ind.; and virginiamycin was from Smith, Kline & French Laboratories, Genval, Belgium.

The agents were dissolved and diluted in distilled water, ethanol, 0.1 N NaOH solution, or dimethyl formamide according to their solubility. Concentration of dimethyl formamide in media was kept lower than 1.6% (vol/vol) because higher concentrations are bacteriostatic. Antibiotic solutions were incorporated into agar as suggested by Ericsson and Sherris (8).

Media for susceptibility tests. Minimal inhibitory concentration (MIC) tests were carried out in Mueller-Hinton agar (Difco). The antibiotic-containing plates, except those containing nitrovin and virginiamycin, were prepared in one lot and stored in a refrigerator for 1 to 7 days. The plates were dried for 25 to 30 min in a laminar air flow after pouring. Because the activity of nitrovin and virginiamycin deteriorated rapidly in preservation, plates containing these two agents were prepared on the same day the tests were performed.

Susceptibility tests. The strains, which were able to grow optimally in the presence of 5% CO_2, were tested anaerobically in a GasPak system. The remaining strains were tested aerobically. The turbidity of the inocula was adjusted by adding the required drops from an overnight Mueller-Hinton broth (Difco) culture in 1 ml of sterile phosphate-buffered saline to match the turbidity of no. 1 McFarland standard. Inocula were applied to the plates with an inoculum replicator (Denley Tech. Ltd., Sussex, England) and incubated according to their nature overnight at 37°C. One plate of the same medium without antibiotic was also inoculated and incubated along with each batch as a control plate to compare growth.

TABLE 2. MICs of nine growth-promoting agents for eight *S. faecalis* strains from poultry

Growth-promoting agent	No. of strains with MIC (μg/ml [except bacitracin, in U/ml]) of:										
	≤0.25	0.5	1	2	4	8	16	32	64	128	>128
Avoparcin			1[a]	7							
Bacitracin			—[a]		3		2	2	1		
Flavomycin	3	3	2	—[a]							
Lincomycin					1[a]	2	1				4
Nitrovin	1	1	4[a]			1	1				
Oleandomycin		2	2[a]								4
Spiramycin			2	2[a]							4
Tylosin				2	2[a]						4
Virginiamycin			3	1[a]	3	1					

[a] MIC of *S. faecalis* CCM 2541.

The MIC for each strain was recorded as the lowest concentration of antibiotic yielding no growth, one discrete colony, or a barely visible haze as determined with the unaided eyes.

RESULTS

The results of the physiological tests for identification of the strains are shown in Table 1. The strains were classified by using the criteria listed by Sharpe (20). Four of the eight *S. faecalis* strains had a zone of inhibition in the Tellur test, but they reduced tellurite, showing a broad ring of black colonies. Five strains from the *S. faecium* group were negative in the methylene blue test. The last group of 20 strains, which showed definite growth enhancement in the presence of 5% CO_2 in air, was here called the carboxyphilic group.

Results of the MIC tests for the *S. faecalis*, *S. faecalis* subsp. *liquefaciens*, *S. faecium*, and the carboxyphilic group are shown in Tables 2, 3, 4, and 5, respectively. The MICs of the agents against the strains obtained from the CCM (hereafter called the reference strains) are indicated in the respective tables.

The strains were classified as susceptible, as low-level resistant or intermediate, or as resistant to the agents according to the MIC by the agar-dilution method. The standards of the classification were the relationship of the sensitivity of strain to that of others of the same species and the relationship of the sensitivity of strain to that of a particular standard (in this study, the reference) strain (8). When only one population was found, the strains were classified as either susceptible or resistant to an agent when their MICs were lower or higher than the concentration of the agent normally used in feed. If two populations were found, strains of the one with lower MICs were classified as susceptible, and the strains of the other population were classified as resistant. When more than two populations were found, the strains belonging to the populations between the susceptible and resistant populations were classified as low-level resistant or intermediate strains.

All but one of the *S. faecium* strains required an MIC of flavomycin of more than 128 μg/ml. The strain requiring a lower flavomycin MIC of 2 μg/ml had a dubious identification, because besides its negative blue characteristic, it also failed to ferment melibiose. The strains from C. S. Impey and R. Fuller were tested against bacitracin, flavomycin, nitrovin, and virginiamycin only. The MICs of the tested agents against these strains are not included in the tables. No difference in the MICs of the tested agents between these strains and the reference

TABLE 3. MICs of nine growth-promoting agents for 23 *S. faecalis* subsp. *liquefaciens* strains from poultry

Growth-promoting agent	No. of strains with MIC (μg/ml [except bacitracin, in U/ml]) of:										
	≤0.25	0.5	1	2	4	8	16	32	64	128	>128
Avoparcin			1	22[a]							
Bacitracin					5[a]	3	3	7		2	3
Flavomycin	21	1	1		—[a]						
Lincomycin						—[a]	4				19
Nitrovin		21	2[a]								
Oleandomycin				3[a]	1						19
Spiramycin			2	1	1[a]						19
Tylosin					4[a]						19
Virginiamycin					5	15[a]	3				

[a] MIC of *S. faecalis* subsp. *liquefaciens* CCM 5613.

TABLE 4. MICs of nine growth-promoting agents for 15 *S. faecium* strains from poultry

Growth-promoting agent	No. of strains with MIC (μg/ml [except bacitracin, in U/ml]) of:										
	≤0.25	0.5	1	2	4	8	16	32	64	128	>128
Avoparcin		2	13[a]								
Bacitracin	1[a]	4			1	1	1	2	3	2	
Flavomycin				1							14[a]
Lincomycin	1[a]						4				10
Nitrovin	1	2	1[a]			3	1	4		2	1
Oleandomycin		—[a]	5								10
Spiramycin		—[a]	1	4							10
Tylosin			1[a]	4							10
Virginiamycin	2	4[a]	4	5							

[a] MIC of *S. faecium* CCM 2308.

strains was observed, except in the case of *S. faecium* EBF 87/152 against bacitracin. This strain required an MIC of bacitracin of 8 μg/ml, which is 32 times higher than that of the reference strain, *S. faecium* CCM 2308.

Species-wise prevalence of acquired resistance among the strains against the agents is summarized in Table 6. The table does not include strains which showed natural resistance, e.g., *S. faecium* strains to flavomycin. The term "acquired resistance" is used to imply resistance in which the strains required MIC levels at least eight times higher than the MIC levels for the reference strains or the susceptible strains of the same species. It is well known that technical variables of the test may cause an MIC variation of two twofold dilutions.

DISCUSSION

Although fecal streptococci have been studied intensively and the individual species have been reasonably characterized, there are many problems of identification, especially of what may be called the intermediate forms, that is, those forms which cannot be easily assigned to one of the named species (17). We have also had such problems in naming or assigning the strains of the carboxyphilic group and the five methylene blue-negative strains included in the *S. faecium* group. These five strains were more closely related to *S. faecium* than to other fecal streptococci. The strains included in the carboxyphilic group represent a group of poorly defined microorganisms which have certain characteristics in common with the strains included by Hare (13) and Barnes and Impey (1). The strains of this group are not strictly anaerobic. Their growth is better in the presence of 5% CO_2 than aerobically.

The criteria of susceptibility, as used here, are tentative because with growth-promoting agents the interpretation criteria of susceptibility and resistance have never been established (4). The notion of susceptibility in vitro does not imply that the susceptible strains will be inhibited in vivo.

Avoparcin was active against all streptococci, irrespective of the species. Many strains were relatively unsusceptible to bacitracin. The MIC levels of bacitracin for the susceptible *S. faecalis* subsp. *liquefaciens* strains, including the reference strain, were at least 16 times higher than that for the susceptible *S. faecium* strains, including the reference strain. This is in contradiction to the results of Barnes and co-workers (2) that *S. faecalis* subsp. *liquefaciens* was more susceptible in vitro to bacitracin than *S. faecium*. It should be noted, however, that the divergence is caused by the existence of acquired resistance, which was less prevalent in *S.*

TABLE 5. MICs of nine growth-promoting agents for 20 carboxyphilic streptococcal strains from poultry

Growth-promoting agent	No. of strains with MIC (μg/ml [except bacitracin, in U/ml]) of:										
	≤0.25	0.5	1	2	4	8	16	32	64	128	>128
Avoparcin			16	4							
Bacitracin	5	1			6		4	4			
Flavomycin	15	2	1	1							1
Lincomycin	5	2					1				12
Nitrovin	17	3									
Oleandomycin	2	2		4							12
Spiramycin	1	2	1	2	2						12
Tylosin			4	1	3						12
Virginiamycin	8	3	5	4							

TABLE 6. Species-wise prevalence of acquired resistance in poultry *Streptococcus* strains against nine growth-promoting agents

Growth-promoting agent	Percentage of resistant strains[a]			
	S. faecalis (8 strains)	*S. faecalis* subsp. *liquefaciens* (23 strains)	*S. faecium* (15 strains)	Carboxyphilic streptococci (20 strains)
Avoparcin	0	0	0	0
Bacitracin	62	21	67	70
Flavomycin	25	0	0	5
Nitrovin	25	0	20+(53)	0
Lincomycin	50	83	67+(26)	60+(5)
Macrolides (oleandomycin, spiramycin, and tylosin)	50	83	67	60
Virginiamycin	0	0	0	0

[a] Figures in parentheses indicate low-level resistant or intermediate strains.

faecalis subsp. *liquefaciens* than in other groups (Table 6). The high percentage of acquired resistance to bacitracin among the strains may be due to the use of this agent in poultry feeds. This is a hypothesis, because it was not possible to trace the composition of the feeds used in the farms from which the birds originated.

The *S. faecalis* subsp. *liquefaciens* strains were susceptible to flavomycin, whereas all but one of the strains classified as *S. faecium* were resistant to the compound. The percentage of resistance in other species was not very high. This is in sharp contrast to *Clostridium perfringens* strains (5, 22), which are completely resistant, and to lactobacilli (7), which are relatively unsusceptible to flavomycin. In the cases of lincomycin against *S. faecium* and the carboxyphilic groups and nitrovin against the *S. faecium* group, the strains showed an unusual trimodal distribution of susceptibility levels. This type of strain distribution against lincomycin has been reported in *C. perfringens* from human origin (19) and animal origin (6) and in animal lactobacilli (7). Strains very susceptible to lincomycin were found only in the *S. faecium* and the carboxyphilic groups. The MICs required for inhibition of the susceptible *S. faecalis* subsp. *liquefaciens* strains were between 4 and 16 μg/ml. Resistance to nitrovin was not found among the *S. faecalis* subsp. *liquefaciens* and the carboxyphilic groups.

The percentage of resistance to the macrolide antibiotics oleandomycin, spiramycin, and tylosin was very high. Macrolide-resistant strains were also resistant to lincomycin. This high percentage of resistance to macrolide antibiotics and lincomycin among the streptococci corresponds with similarly high macrolide and lincomycin resistance rates in animal lactobacilli (7). It may be noted that antibiotics of the macrolide group and lincomycin are used therapeutically as well as for growth promotion in the poultry industry.

An extended range of MIC levels was seen in tests with virginiamycin. The MIC levels of virginiamycin against the *S. faecium* strains were the lowest. This was due to more susceptibility of the *S. faecium* strains to the virginiamycin component M, a member of the streptogramin group A antibiotics (7a).

The mechanism of bacterially induced growth depression or the mechanism of antibacterial agent-induced growth promotion is still a controversial issue. In this study, differences in the natural susceptibility levels of intestinal streptococci were found with bacitracin, lincomycin, virginiamycin, and flavomycin. Bacitracin and virginiamycin were in vitro more active on *S. faecium* and *S. faecalis* groups than on *S. faecalis* subsp. *liquefaciens* group; lincomycin and the macrolide antibiotics were more active on *S. faecium* than on others; and flavomycin was active on all groups except *S. faecium*. Thus, it appears that different growth-promoting antibacterial agents act differently on different intestinal streptococcal species. The prevalence of acquired resistance among the intestinal streptococci may cause some difficulties in obtaining reproducible results from in vivo studies with the growth-promoting antibacterial agents.

ACKNOWLEDGMENTS

We thank R. Fuller and C. S. Impey for providing the requested bacterial cultures and the Belgian subsidiaries of the pharmaceutical firms for providing the antibiotic preparations.

LITERATURE CITED

1. **Barnes, E. M., and C. S. Impey.** 1970. The isolation and properties of the predominant anaerobic bacteria of the ceca of turkeys and chickens. Br. Poult. Sci. **11**:467–481.
2. **Barnes, E. M., G. C. Maed, C. S. Impey, and B. W. Adams.** 1978. The effect of dietary bacitracin on the incidence of *Streptococcus faecalis* subspecies *liquefaciens* and related streptococci in the intestines of young chicks. Br. Poult. Sci. **19**:713–723.
3. **Bird, H. R.** 1968. Effectiveness of antibiotics in broiler feeds. World's Poult. Sci. J. **24**:309–312.
4. **Devriese, L. A., and G. N. Dutta.** 1981. Antibiotic sensi-

tivity testing: correlations between *in vitro* tests and *in vivo* situations. Ann. Rech. Vet. **12**:41–46.

5. **Dutta, G. N., and L. A. Devriese.** 1980. Susceptibility of *Clostridium perfringens* of animal origin to fifteen antimicrobial agents. J. Vet. Pharm. Ther. **3**:227–236.
6. **Dutta, G. N., and L. A. Devriese.** 1981. Macrolide-lincosamide-streptogramin resistance patterns in *Clostridium perfringens* from animals. Antimicrob. Agents Chemother. **19**:274–278.
7. **Dutta, G. N., and L. A. Devriese.** 1981. Sensitivity and resistance to growth promoting agents in animal lactobacilli. J. Appl. Bacteriol. **51**:283–288.

7a. **Dutta, G. N., and L. A. Devriese.** 1982. Resistance to macrolide-lincosamide-streptogramin antibiotics in enterococci from the intestines of animals. Res. Vet. Sci. **33**:70–72.

8. **Ericsson, H. M., and J. C. Sherris.** 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sect. B (Suppl. 217):1–90.
9. **Eyssen, H., and P. De Somer.** 1967. Effects of *Streptococcus faecalis* and a filterable agent on growth and nutrient absorption in gnotobiotic chicks. Poult. Sci. **46**:323–333.
10. **Facklam, R. R.** 1972. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. Appl. Microbiol. **23**:1131–1139.
11. **Facklam, R. R., and M. D. Moody.** 1970. Presumptive identification of group D streptococci: the Bile-Esculin test. Appl. Microbiol. **20**:245–250.
12. **Fuller, R., M. E. Coates, and G. F. Harrison.** 1979. The influence of specific bacteria and a filterable agent on the growth of gnotobiotic chicks. J. Appl. Bacteriol. **46**:335–342.
13. **Hare, R.** 1967. The anaerobic cocci, p. 284–317. *In* H. P. Waterson (ed.), Recent advances in medical microbiology. Little, Brown & Co., Boston.
14. **Houghton, S. B., R. Fuller, and M. E. Coates.** 1981. Correlation of growth depression of chicks with the presence of *Streptococcus faecium* in the gut. J. Appl. Bacteriol. **51**:113–120.
15. **Huhtanen, C. N., and J. M. Pensack.** 1965. The role of *Streptococcus faecalis* in the antibiotic effect in chickens. Poult. Sci. **44**:830–844.
16. **Islam, A. K. M. S.** 1977. Rapid recognition of group B streptococci. Lancet **i**:256–257.
17. **Jones, D.** 1978. Composition and differentiation of the genus *Streptococcus*, p. 1–49. *In* F. A. Skinner and L. B. Quesnel (ed.), Streptococci. Academic Press, London.
18. **Ochi, Y., T. Mitsuoka, and T. Sega.** 1964. Untersuchungen über die Darmflora des Huhnes III. Mitteilung: die Entwicklung der Darmflora von Küken bis zum Huhn. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **193**:80–95.
19. **Sapico, F. L., Y.-Y. Kwok, V. L. Sutter, and S. M. Finegold.** 1972. Standardized antimicrobial disc susceptibility testing of anaerobic bacteria: in vitro susceptibility of *Clostridium perfringens* to nine antibiotics. Antimicrob. Agents Chemother. **2**:320–325.
20. **Sharpe, M. E.** 1979. Identification of lactic acid bacteria, p. 233–259. *In* F. A. Skinner and D. W. Lovelock (ed.), Identification methods for microbiologists. Academic Press, London.
21. **Smith, H. W.** 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacteriol. **90**:495–513.
22. **Smith, H. W.** 1972. The antibacterial activity of nitrovin *in vitro*. The effect of this and other agents against *Clostridium welchii* in the alimentary tract of chickens. Vet. Rec. **90**:310–312.
23. **Unsworth, P. E.** 1978. Streptococci in human feces, p. 239–240. *In* M. T. Parker (ed.), Pathogenic streptococci. Reedbooks Ltd., Surrey, England.