JOURNAL OF BACTERIOLOGY, Aug. 1970, p. 447-456

Vol. 103, No. 2
Printed in U.S.A.

# Selective Outgrowth of Fimbriate Bacteria in Static Liquid Medium

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Received for publication 14 February 1970

Competitive mixed cultures were grown from inocula of a large number of bacteria of a genotypically nonfimbriate (*fim*$^-$) strain of *Salmonella typhimurium* and a small number of a genotypically fimbriate (*fim*$^+$) variant strain that formed type 1 fimbriae and had been derived from the *fim*$^-$ strain by phage transduction. The *fim*$^+$ strain differed from the *fim*$^-$ strain in fermenting L-rhamnose (*rha*$^+$), and the viable *fim*$^+$ and *fim*$^-$ bacteria present in the cultures after different periods at 37 C were counted differentially in platings on rhamnose media. When the cultures were grown under aerobic static conditions in tubes of nutrient broth, the *fim*$^+$ bacteria rapidly outgrew the *fim*$^-$ bacteria, so that, although starting as a small minority (e.g., 1 in $10^7$), they approached or surpassed the number of the *fim*$^-$ in 48 hr. A pellicle consisting of fimbriate bacteria was formed on the surface of the broth between 6 and 24 hr, and it is thought that the advantage of access to atmospheric oxygen enjoyed by these bacteria in the pellicle enabled them to outgrow the *fim*$^-$ bacteria confined in the oxygen-depleted broth. The *fim*$^+$ bacteria did not show selective outgrowth in mixed cultures grown in broth aerated by continuous shaking, in static broth incubated anaerobically in hydrogen, and on aerobic agar plates, i.e., under conditions not allowing an advantage from pellicle formation. The outgrowth of *fim*$^+$ bacteria in aerobic static broth was prevented by the addition of $\alpha$-methylmannoside, a substance that inhibits the adhesive and early pellicle-forming properties of bacteria with type 1 fimbriae. A motile flagellate (*fla*$^+$) variant of a *fim*$^-$ *fla*$^-$ strain of *S. typhimurium* outgrew its parent strain in mixed cultures in aerobic static broth, but the selective advantage conferred by motility was weaker than that conferred by fimbriation.

Many bacteria that form the filamentous appendages termed fimbriae or pili (8) vary between a fimbriate and a nonfimbriate phase, and the influence of cultural conditions in determining the variation has been studied in *fim*$^+$ strains of *Escherichia coli* (11), *Shigella flexneri* (10, 12), and *Salmonella* species (9, 16). These organisms form fimbriae of type 1, a type that gives the bacteria adhesive, hemagglutinating, and pellicle-forming properties that are sensitive to inhibition by D-mannose and $\alpha$-methylmannoside (7, 9, 16). Duguid and Gillies (10) found that inocula from nonfimbriate-phase cultures of *fim*$^+$ strains of *S. flexneri* gave rise to cultures containing mainly fimbriate-phase bacteria when carried through several serial subcultures in tubes of broth incubated under aerobic and static conditions at 37 C for periods of 24 to 72 hr. The converse change took place when subculture was in anaerobic broth, in broth aerated by shaking, or on aerobic plates. Since several successive subcultures were required to complete the change of phase, they concluded that the effect of the cultural conditions was to favor the selective outgrowth of spontaneously originating phase variants and not directly to influence the synthesis of fimbriae. The advantage of the fimbriate over the nonfimbriate bacteria in aerobic static broth was thought to lie in their ability to establish themselves rapidly in a pellicle on the surface of the broth, where their growth was promoted by the supply of atmospheric oxygen.

The responses of motile salmonellae in changing their fimbrial phase are similar in direction, but faster than those of *S. flexneri* (9). Thus, a nonfimbriate-phase inoculum may yield a fimbriate-phase culture in a single 48-hr period of growth in aerobic static broth. Since such a change might be considered too rapid to be the result of selection, we tried to obtain proof that these conditions of culture are strongly selective for fimbriate bacteria. We grew *fim*$^+$ and *fim*$^-$ strains of

*Salmonella typhimurium* in competition with one another in mixed cultures and observed the relative amounts of their growths. The two strains were marked with different rhamnose-fermentation properties so that their bacteria could be counted separately by plating on a rhamnose-containing medium. The *fim*$^-$ strain, which was incapable of varying spontaneously into a fimbriate phase, was selected from the "FIRN" group of *S. typhimurium* strains (9, 15), which have the combination of characters: nonfimbriate, not fermenting inositol in 24 hr, and not fermenting L-rhamnose in 24 hr (*rha*$^-$). The *fim*$^+$ strain was *rha*$^+$, i.e., it fermented rhamnose in 6 to 10 hr ("FRP" strain). In a few experiments, the *fim*$^+$ strain was unrelated to its FIRN competitor, but in most it was a variant which had been derived from the FIRN strain by transduction in *fim* and mutation in *rha*.

Most observations were made with motile flagellate (*fla*$^+$) strains, but some were made with *fla*$^-$ strains to determine the influence of motility on competitive growth.

## MATERIALS AND METHODS

**Bacteria.** The characters, sources, and derivations of the strains of *S. typhimurium* are shown in Table 1. Strains S90 and S6356 were supplied by B. A. D. Stocker, S206 by H. A. Wright, S625 and S635 by J. E. Wilson, and S7471 by E. S. Anderson. Their phage types in the scheme of Callow (5), determined by Anderson, were, respectively, 4, 13, 2, 14, 13, and 1. Strains S90 and S206 were FRP and the other wild-type strains were FIRN. All strains were non-colicinogenic. Strain S90, a line from Lilleengen's (14) phage-type strain LT2, was non-gas-producing, fermented trehalose only after 2 or more days, and fermented inositol within 24 hr. The other strains produced gas in fermenting glucose, fermented trehalose within 24 hr, and did not ferment inositol within 24 hr. Strains S6351, S6352, S6353, S6354, S6355, S6357, and S6358 were lysogenic for phage P22 as a result of having been exposed to it in a transduction procedure. A subculture from a single colony of each strain was grown on a slant of Dorset's egg medium, stored at ambient temperature, and used to prepare the inocula for the experimental cultures.

**Culture media.** Phosphate-buffered broth was a mixture of equal volumes of double-strength nutrient broth and phosphate buffer (*p*H 7.0), mixed just before use and steamed at 100 C for 90 min. The double-strength broth contained 20 g of peptone (Oxoid), 20 g of meat extract (Oxoid, Lab-Lemco), and 10 g of NaCl per liter of water, and the buffer contained 7 g of $KH_2PO_4$ and 13 g of $Na_2HPO_4$ per liter of water. Nutrient agar was the phosphate-buffered broth solidified by the addition of 10 g of Oxoid Ionagar per liter. Sugar broth for competition experiments (Table 5) was the buffered broth with 0.2% (w/v) of the test sugar, sterilized separately from the broth. Rhamnose eosin-methylene blue (EMB) agar contained 3 g of peptone, 0.6 g of $K_2HPO_4$, 0.4 g of eosin yellow, 0.065 g of methylene blue, and 10 g of Davis New Zealand agar per liter of water; the *p*H was 6.8 and 5 g of L-rhamnose was added per liter. Rhamnose minimal agar was the minimal medium of Davis and Mingioli (6) without glucose or citrate, but with 3 g of L-rhamnose and 10 ml of Oxoid nutrient broth no. 2 added per liter. Media for fermentation tests contained 10 g of test sugar, 10 g of peptone, and 5 g of NaCl per liter of water, and Andrade's acid-fuchsin as *p*H indicator. D-Glucose and L-rhamnose were Analar grade from British Drug Houses Ltd., D-mannose was bacteriological grade from Gurr Ltd., and α-methylmannoside (methyl-α-D-mannopyrano-

TABLE 1. *Characters and origin of strains of Salmonella typhimurium used in the competitive growth experiments*

| Strain no. | Flagella produced (*fla*) | Fimbriae produced (*fim*) | Rhamnose fermented (*rha*) | Lysogenic for A-type phage | Source or derivation |
|---|---|---|---|---|---|
| S90 | + | + | + | − | Anaerogenic line of FRP strain LT2 |
| S206 | + | + | + | − | Wild-type FRP strain from patient |
| S625 | + | − | − | + | Wild-type FIRN strain from fowl |
| S635 | − | − | − | − | Wild-type FIRN strain from fowl |
| S7471 | + | − | − | − | Wild-type FIRN strain from patient |
| S625F | + | + | + | + | From S625, by transduction of *fim*$^+$ and selection of a *rha*$^+$ mutant |
| S6351 | − | − | − | + | From S635, by lysogenization with phage P22 |
| S6352 | + | − | − | + | From S635, by transduction of *fla*$^+$ |
| S6353 | + | − | + | + | From S6352, by selection of a *rha*$^+$ mutant |
| S6354 | − | + | − | + | From S635, by transduction of *fim*$^+$ |
| S6355 | − | + | + | + | From S6354, by selection of a *rha*$^+$ mutant |
| S6356 | + | − | − | − | From S635, by transduction of *fla*$^+$ without lysogenization with P22 |
| S6357 | + | + | − | + | From S6356, by transduction of *fim*$^+$ |
| S6358 | + | + | + | + | From S6357, by selection of a *rha*$^+$ mutant |

side) and L-sorbose were from Koch-Light Laboratories.

**Transductions.** Phage P22 (19) was used for the transduction of *fim* and *fla*. It was propagated in a soft-agar culture of a line of the *fim*$^+$ *fla*$^+$ strain S90 and the harvested lysate was pasteurized at 55 C for 60 min. The *fim*$^+$ transductant strains, S625F, S6354, and S6357, were derived from FIRN strains S625, S635, and S6356 by mixing FIRN bacteria with the phage lysate, subculturing the mixture serially for two or more 48-hr periods in aerobic static broth, and isolating a *fim*$^+$ clone by plating and picking a colony. The *fla*$^+$ transductant strains, S6352 and S6356, were isolated from the *fla*$^-$ strain S635 by methods already described (18).

**Rhamnose mutants.** Spontaneous *rha*$^+$ mutants were derived from FIRN strains S625 and S635, and their *fim*$^+$ and *fla*$^+$ transductants. Because such mutants cannot be selected from FIRN strains by direct plating on rhamnose minimal medium (15), they were obtained by a two-step procedure. A *rha*$^w$ (rhamnose-weak) mutant was first selected from the FIRN strain by culturing it in rhamnose-peptone-water at 37 C, until, after several days, it formed acid. The fermented culture was plated on nutrient agar and a colony was picked to yield a *rha*$^w$ clone, which was recognized by its property of fermenting rhamnose in peptone water in 10 to 24 hr. Plating of this clone on rhamnose minimal medium produced a few *rha*$^+$ colonies in 2 to 3 days.

**Competition experiments: absence of bactericidal effect.** Each pair of strains to be grown together in mixed culture was first tested to show that neither strain produced a lytic phage or bacteriocin active against the other. A broth culture of either strain was pasteurized at 55 C for 60 min; a 1-ml amount was added to 10 ml of an exponential-phase broth culture of the nonlysogenic strain Q1 of Boyd (2). The mixture was incubated overnight and then pasteurized; dilutions were inoculated on to a lawn of the other strain on an agar plate and, after incubation, the lawn was examined for plaques. Bacteriocin production was tested by the method of Gillies (13), each strain being set against the other, first as "producer" and then as "indicator."

**Growth conditions.** Cultures were incubated at 37 C (i) under aerobic static conditions in 10-ml volumes of the phosphate-buffered broth in test tubes (15 by 1.3 cm) stoppered with cotton wool, which were left unmoved in the incubator until the time of their single, final examination; (ii) under anaerobic static conditions in similar tubes of broth, each incubated in a separate jar of hydrogen until the time of its single, final examination; the jar contained an indicator to confirm that it remained anaerobic; (iii) under aerobic shaken conditions in 40-ml volumes of the buffered broth in 500-ml conical flasks held on a reciprocating shaker (100 rev/min) to give continuous aeration; and (iv) on aerobic plates, 9 cm in diameter, containing 20 ml of buffered nutrient agar.

**Inocula for the mixed cultures.** Each competing strain was plated on nutrient agar and a colony was subcultured in aerobic static broth at 37 C for 24 hr. One drop of the subculture was inoculated into each of several tubes of 10 ml of phosphate-buffered broth and four drops were inoculated into each of several flasks of 40 ml of broth. The cultures were incubated for 3.5 hr, at which time they contained about $10^8$ viable bacteria per ml and were ready for use as inocula. The cultures of the *fim*$^+$ strains contained a mixture of fimbriate-phase and nonfimbriate-phase bacteria.

The mixed cultures were grown from inocula of a small number of *fim*$^+$ bacteria, termed the "challenging" organisms, and a large number of *fim*$^-$ bacteria, termed the "challenged" organisms. One 0.02-ml drop of a selected high dilution of a 3.5-hr culture of the challenging strain was added, per 10 ml, to each of a series of 3.5-hr tube and flask cultures of the challenged strain. The dilution of the challenging culture was made in fresh broth just before it was used. The mixture of organisms, still in the broth in which the challenged organisms had been grown for 3.5 hr, was incubated for the period of the experiment. Parallel series of cultures were seeded from dilutions of the challenging culture calculated to give ratios of challenging to challenged organisms of about $1:10^4$ (high-level challenge) and $1:10^7$ (low-level challenge). Plates were seeded by spreading each of them with the combined deposits of bacteria centrifuged from two 10-ml volumes of broth mixture prepared as for the liquid cultures. Control, pure cultures were grown from separate inocula of each strain in the same numbers as used for the mixed cultures.

**Examination of the cultures.** This was done after incubation for 0, 6, 24, and 48 hr. Because the procedure of examination disturbed the culture, a separate parallel culture from each series was examined after each different period of incubation. Tube cultures were first inspected for the presence of a pellicle on the surface of the broth and then homogenized by pipetting to disperse any pellicle or deposit. Growth on plates was harvested into a volume of broth (20 ml) equal to that of the agar in the plate. A series of tenfold dilutions in broth was made from the harvested culture and a 0.1-ml volume of each dilution was spread on each of two plates of rhamnose EMB medium and two plates of rhamnose minimal medium. After incubation for 48 hr, the *rha*$^+$ (dark red) and *rha*$^-$ (pale red) colonies on the EMB plates were counted, and after 72 hr, the *rha*$^+$ (large) and *rha*$^-$ (very small) colonies on the minimal medium. When the number of *rha*$^+$ bacteria was large, approaching that of the *rha*$^-$ bacteria, the numbers of each type were estimated from the counts on the EMB plates; however, when it was relatively small, the *rha*$^+$ bacteria were counted on the minimal medium and the *rha*$^-$ on the EMB. At least 12 colonies of each type were subcultured and tested for fimbriation, motility, and fermentation of rhamnose in peptone water.

The residue of each mixed and control culture was killed by the addition of 0.25% (w/v) formaldehyde and examined for optical density at 530 nm in a Unicam spectrophotometer to give a measure of the total amount of growth. A portion of it was examined for hemagglutinating power (HP) with guinea pig red blood cells (9) to give a rough indication of the proportion of fimbriate-phase bacteria present. Some of

the cultures were examined for the presence of fimbriae and flagella with the electron microscope (9).

## RESULTS

**Competition between unrelated fim$^+$ and fim$^-$ strains.** When fimbriate strain S90 or S206 was grown in mixed culture in aerobic static broth with unrelated nonfimbriate strain S7471 from an inoculum of bacteria only 0.00001% as numerous as that of S7471, its growth was selectively favored to the extent that its bacteria comprised 10 to 40% of the viable population of the culture after 48 hr. This outgrowth of the *fim*$^+$ bacteria was associated with the formation of a pellicle on the surface of the broth after 6 to 12 hr and the development of hemagglutinating activity in the culture, which indicated that many of the *fim*$^+$ bacteria were in the fimbriate phase. Seventy-eight *rha*$^+$ and 78 *rha*$^-$ colonies were picked from the EMB platings of the mixed cultures of strains S90 and S7471 grown for 48 hr. All of the *rha*$^+$ clones were found to be fimbriate, not to produce gas from glucose, and to ferment inositol and rhamnose within 24 hr, but trehalose only after 2 or more days. All of the *rha*$^-$ clones were found to be nonfimbriate, to produce gas, not to ferment inositol or rhamnose within 24 hr, but to ferment trehalose within 24 hr. Since these two opposite sets of properties are characteristic of strains S90 and S7471, respectively, and clones with a hybrid set were not detected, it is concluded that the *rha*$^+$ and *fim*$^+$ characters were not transmitted from the challenging (S90) to the challenged (S7471) bacteria by a mechanism of infective heredity.

When mixed cultures of strain S90 or S206 with strain S7471 were grown in broth under anaerobic static or aerobic shaken conditions, or on aerobic plates, the proportion of the *fim*$^+$ bacteria again increased during incubation for 48 hr, but the extent of the increase (about $10^4$-fold) was much less than that in the aerobic static broth (about $10^6$-fold), and the cultures did not develop hemagglutinating activity. This moderate selection of *fim*$^+$ bacteria in cultures grown otherwise than in aerobic static broth may have been due to some difference in character between the competing strains other than the difference in fimbriation. The *fim*$^+$ strains S90 and S206 differed in phage-type from the *fim*$^-$ strain S7471 and it is possible that they produced a phage active against S7471 that was not detected by our method of testing. To avoid, therefore, the possible effects of such character differences, we made all the succeeding experiments with pairs of *fim*$^+$ and *fim*$^-$ strains that were closely related to one another.

**Competition between related fim$^+$ and fim$^-$ strains.** Tables 2 and 3 show the results of a typical experiment in which the *fim*$^+$ *rha*$^+$ strain S625F was grown as challenging organism in mixed culture with the *fim*$^-$ *rha*$^+$ strain S625, from which it had been derived by transduction and mutation. The *fim*$^+$ strain was tested at the two inoculum levels of 1.4 and 1,400 bacteria per ml in cultures containing $40 \times 10^6$ *fim*$^-$ bacteria per ml. In aerobic static broth, the *fim*$^+$ bacteria grew up from the low- and high-level inocula until, after 48 hr, they made up, respectively, 86 and 89% of the total bacterial population. As judged from the viable counts at 48 hr, their multiplication in the culture with the low-level inoculum was $1.2 \times 10^9$-fold, whereas that of the *fim*$^-$ bacteria was only 6.5-fold.

The selective outgrowth of the *fim*$^+$ bacteria was associated with the development of hemagglutinating activity in the mixed culture and an increase in the HP value from 0 at 0 hr to 200 at 48 hr. A surface pellicle was formed sometime between 6 and 24 hr. By 48 hr the total amount of growth, measured by turbidity (1.88), was as great in the mixed cultures as in the control cultures of the *fim*$^+$ strain alone, and three times greater than that (0.64) in the control culture of the *fim*$^-$ strain alone. Thus, the mechanism enabling the fimbriate bacteria to give greater growth in aerobic static broth than the nonfimbriate bacteria was as effective when the fimbriate bacteria were grown in mixed cultures with nonfimbriate bacteria as when they were grown separately in pure cultures. As is characteristic in *fim*$^-$ *fla*$^+$ strains of *S. typhimurium* (9), the pure culture of strain S625 formed a surface pellicle at a late stage of growth, between 24 and 48 hr.

In contrast with their behavior in aerobic static broth, the *fim*$^+$ bacteria showed no ability to outgrow the *fim*$^-$ bacteria in broth incubated under anaerobic static or aerobic shaken conditions, or on aerobic plates. They did not give even a moderate outgrowth, like that given by the *fim*$^+$ strains S90 and S206 when grown under these conditions with the unrelated *fim*$^-$ strain S7471. Thus, in the mixed cultures grown from the inocula containing 0.0035% of *fim*$^+$ S625F bacteria (1,400/ml), the proportion of *fim*$^+$ bacteria present after 48 hr was only 0.0031% in anaerobic broth, 0.002% in shaken broth, and 0.0037% on an aerobic plate. No pellicles were formed under these conditions, and in pure cultures the *fim*$^+$ bacteria did not give significantly greater amounts of growth than the *fim*$^-$ (Table 3).

**S6358 versus S6352.** Similar results to the above were obtained in a comparable experiment (Table 4, top section) made with a pair of strains derived from the *fim*$^-$ *rha*$^-$ strain S635, which differs

TABLE 2. *Amount of growth, presence of pellicle, hemagglutinating power (HP), and differential viable counts in mixed cultures of "challenged" fim⁻ rha⁻ strain S625 and "challenging" fim⁺ rha⁺ variant strain S625F grown under different conditions at 37 C*

| Conditions of growth | Level of *fim*⁺ inoculum | Time of incubation | Amt of growth[a] | Pellicle | HP | Viable count ($10^6$ bacteria)/ml of | |
|---|---|---|---|---|---|---|---|
| | | | | | | *Fim*⁻ bacteria | *Fim*⁺ bacteria |
| | | *hr* | | | | | |
| Aerobic static broth | Low | 0 | 0.14 | − | | 40[b] | 0.0000014 |
| | | 6 | 0.37 | − | 0 | 350 | 0.00011 |
| | | 24 | 0.52 | + | 2 | 500 | 17.5 |
| | | 48 | 1.88 | + | 200 | 260 | 1,650 |
| | High | 0 | 0.14 | − | | 40 | 0.0014 |
| | | 6 | 0.39 | − | 0 | 400 | 0.032 |
| | | 24 | 0.92 | + | 200 | 670 | 1,430 |
| | | 48 | 1.88 | + | 200 | 190 | 1,530 |
| Anaerobic static broth | Low | 0 | 0.14 | − | | 40 | 0.0000014 |
| | | 48 | 0.29 | − | 0 | 75 | 0.00003 |
| | High | 0 | 0.14 | − | | 40 | 0.0014 |
| | | 48 | 0.29 | − | 0 | 130 | 0.0042 |
| Aerobic shaken broth | Low | 0 | 0.14 | − | | 40 | 0.0000014 |
| | | 6 | 3.21 | − | 0 | 5,200 | 0.0002 |
| | | 24 | 3.66 | − | 0 | 8,700 | 0.001 |
| | | 48 | 3.76 | − | 0 | 3,700 | 0 |
| | High | 0 | 0.14 | − | | 40 | 0.0014 |
| | | 6 | 3.85 | − | 0 | 5,600 | 0.11 |
| | | 24 | 3.88 | − | 0 | 10,700 | 0.10 |
| | | 48 | 4.00 | − | 0 | 3,600 | 0.07 |
| Aerobic plates | High | 0 | 0.14 | | | 40 | 0.0014 |
| | | 6 | 3.34 | | 0 | 3,300 | 0.140 |
| | | 24 | 4.70 | | 0 | 15,000 | 0.27 |
| | | 48 | 5.40 | | 0 | 7,000 | 0.26 |

[a] Turbidity values (optical density) for 0 hr are measurements made on a suspension in 10 ml of broth of the amount of bacteria that was inoculated per 10 ml of broth or agar medium.

[b] Multiply each value by $10^6$.

from S625 in phage type and in being *fla*⁻. The challenged *fim*⁻ strain, S6352, and the challenging *fim*⁺ strain, S6358, had both been made flagellate and motile by transduction (Table 1). The *fim*⁺ bacteria were tested at an inoculum level of 330 bacteria per ml in cultures containing $130 \times 10^6$ *fim*⁻ bacteria per ml. In aerobic static broth, the extent of their apparent multiplication in 48 hr was $3.4 \times 10^5$ times greater than that of their *fim*⁻ competitors, and this outgrowth was associated with the formation of a pellicle between 6 and 24 hr and the development of strong hemagglutinating activity (HP = 100 at 24 hr, 500 at 48 hr). The turbidity of the mixed culture at 48 hr (1.84) was similar to that of a pure control culture of the *fim*⁺ strain and much greater than that (0.77) of a pure culture of the *fim*⁻ strain. In a mixed culture in aerobic shaken broth, the *fim*⁺ bacteria did not outgrow the *fim*⁻ bacteria, hemagglutinating activity was absent, and the turbidity at 48 hr (3.61) was similar to that in pure shaken cultures of the *fim*⁺ (3.60) and *fim*⁻ (3.54) strains.

**Influence of motility.** Experiments were made with other variants of strain S635 (Table 1) to determine whether motility was necessary for the selective outgrowth of *fim*⁺ bacteria. The *fim*⁺ *fla*⁻ strain S6355 was used as challenging organism and the *fim*⁻ *fla*⁻ strain S6351 as challenged organism (Table 4, middle section). Selection of the fimbriate phase is slower in *fla*⁻ than in *fla*⁺ organisms, and since it was desired to test the fimbriate phase of the challenging organism, each of the competing strains was carried through two serial 48-hr cultures in aerobic static broth before

TABLE 3. *Amount of growth, presence of pellicle and hemagglutinating power (HP) in pure, control cultures grown from similar inocula of the fim⁻ strain S625 and the fim⁺ strain S625F to those used for the mixed cultures described in Table 2*

| Conditions of growth | Time of incubation | *Fim*⁻ bacteria | | | *Fim*⁺ bacteria, low-level inoculum | | | *Fim*⁺ bacteria, high-level inoculum | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Growth | Pellicle | HP | Growth | Pellicle | HP | Growth | Pellicle | HP |
| | *hr* | | | | | | | | | |
| Aerobic static broth | 0 | 0.14 | − | | 0 | − | | 0 | − | |
| | 6 | 0.36 | − | 0 | 0.06 | − | 0 | 0.11 | − | 0 |
| | 24 | 0.49 | − | 0 | 0.53 | + | 20 | 0.76 | + | 5 |
| | 48 | 0.64 | + | 0 | 1.88 | + | 200 | 1.88 | + | 200 |
| Anaerobic static broth | 0 | 0.14 | − | | 0 | − | | 0 | − | |
| | 48 | 0.31 | − | 0 | 0.26 | − | 10 | 0.27 | − | 10 |
| Aerobic shaken broth | 0 | 0.14 | − | | 0 | − | | 0 | − | |
| | 6 | 3.40 | − | 0 | 0.08 | − | 0 | 0.54 | − | 0 |
| | 24 | 3.42 | − | 0 | 3.65 | − | 5 | 3.60 | − | 5 |
| | 48 | 3.52 | − | 0 | 3.84 | − | 2 | 3.75 | − | 2 |
| Aerobic plates | 0 | 0.14 | | | | | | 0 | | |
| | 6 | 3.58 | | 0 | | | | 0.78 | | 0 |
| | 24 | 4.68 | | 0 | | | | 4.94 | | 25 |
| | 48 | 4.68 | | 0 | | | | 5.00 | | 25 |

it was grown in the 3.5-hr broth cultures used to inoculate the experiments. Despite being *fla*⁻, the fimbriate-phase *fim*⁺ bacteria were found to outgrow their *fim*⁻ *fla*⁻ competitors in the mixed cultures in aerobic static broth and the extent of their apparent multiplication in 96 hr was $9 \times 10^5$ times greater than that of the latter organisms. In another experiment (*not shown*), the *fim*⁺ *fla*⁻ strain S6355 was inoculated into the mixed cultures in the form of a nonfimbriate-phase 3.5-hr broth subculture from a 24-hr broth culture, and it did not give any outgrowth over the *fim*⁻ *fla*⁻ strain during 48 hr. The ability of *fim*⁺ bacteria to give selective outgrowth was thus shown to be dependent on their being in the fimbriate phase.

**S6353 versus S6351.** To determine whether the property of motility by itself had any effect in promoting selective outgrowth, an experiment was made in which neither of the competing strains was fimbriate (Table 4, bottom section). The *fim*⁻ *fla*⁺ strain S6353, tested as challenger, was found to multiply to a greater extent ($3.3 \times 10^8$ times) than the challenged *fim*⁻ *fla*⁻ strain S6351 during 48 hr in mixed culture in aerobic static broth. This degree of outgrowth is substantial, though considerably less than that shown by *fim*⁺ over *fim*⁻ bacteria. A "late" pellicle was formed on the broth between 24 and 48 hr, and the selective outgrowth of the *fla*⁺ bacteria may thus have been due to their aerotaxis and growth in the pellicle. The same inoculum of *fla*⁺ bacteria did not show any outgrowth over the *fla*⁻ bacteria in cultures incubated aerobically with shaking.

**Influence of mannose and α-methylmannoside.** D-Mannose and α-methylmannoside are exceptional among sugars in having a strong inhibiting effect on the ability of bacteria with type 1 fimbriae to agglutinate red blood cells (9, 10) and form an "early" (6 to 18 hr) pellicle on broth (16). We took advantage of this effect to prove the role of early pellicle formation in promoting selective outgrowth of fimbriate bacteria in aerobic static broth by making competition experiments in broths containing 0.2% (w/v) of these sugars. Control cultures were grown in broths containing D-glucose and L-sorbose, sugars known not to inhibit hemagglutination and early pellicle formation. One of the inhibiting sugars (mannose) and one of the noninhibiting sugars (glucose) were known to be fermented and utilized in broth cultures within 12 hr, and the other sugars not to be fermented or utilized within 48 hr (16). Because of the buffering of the broth with phosphate, the production of acid from the mannose and glucose did not lower the *p*H levels (final values, 6.5 to 6.8) sufficiently to affect the amount of growth. The *fim*⁺ *fla*⁺ strain S625F, as challenging strain, was inoculated at the level of 12 bacteria per ml into tubes of 10 ml of sugar broth containing $39 \times 10^6$ to $54 \times 10^6$ bacteria per ml of the *fim*⁻ *fla*⁺ strain S625 as challenged strain, and the results are given in Table 5.

In the glucose and sorbose broths, the *fim*⁺

TABLE 4. *Amount of growth and differential viable counts in mixed cultures of pairs of variant strains from strain S635 differing in fimbriation and flagellation genotypes grown in aerobic static and aerobic shaken broths at 37 C*

| Competing strains | | Conditions of growth | Time of incubation | Amt of growth | Viable count ($10^6$ bacteria)/ml of | |
|---|---|---|---|---|---|---|
| Challenged (*rha*⁻) | Challenging (*rha*⁺) | | | | Challenged bacteria | Challenging bacteria |
| | | | *hr* | | | |
| S6352, *fim*⁻ *fla*⁺ | S6358, *fim*⁺ *fla*⁺ | Aerobic static broth | 0 | 0.16 | 130 | 0.00033 |
| | | | 6 | 0.37 | 530 | 0.0053 |
| | | | 24 | 0.71 | 380 | 165 |
| | | | 48 | 1.84 | 1,030 | 880 |
| | | Aerobic shaken broth | 0 | 0.16 | 130 | 0.00033 |
| | | | 6 | 3.27 | 4,200 | 0.0012 |
| | | | 24 | 3.48 | 4,600 | 0.0029 |
| | | | 48 | 3.61 | 4,700 | 0.0093 |
| S6351, *fim*⁻ *fla*⁻ | S6355, *fim*⁺ *fla*⁻ | Aerobic static broth | 0 | 0.19 | 65 | 0.00017 |
| | | | 6 | 0.35 | 270 | 0.00080 |
| | | | 24 | 0.46 | 195 | 4.8 |
| | | | 48 | 0.48 | 86 | 74 |
| | | | 72 | 0.66 | 105 | 185 |
| | | | 96 | 1.41 | 190 | 450 |
| S6351, *fim*⁻ *fla*⁻ | S6353, *fim*⁻ *fla*⁺ | Aerobic static broth | 0 | 0.15 | 94 | 0.00024 |
| | | | 6 | 0.38 | 380 | 0.00080 |
| | | | 24 | 0.55 | 500 | 0.0016 |
| | | | 48 | 0.69 | 540 | 4.5 |
| | | Aerobic shaken broth | 0 | 0.15 | 94 | 0.00024 |
| | | | 6 | 2.91 | 6,900 | 0.016 |
| | | | 24 | 3.00 | 3,800 | 0.011 |
| | | | 48 | 2.94 | 4,900 | 0.011 |

bacteria showed nearly as great a selective outgrowth as they had done in the experiments in broth without added sugar (cf. Table 2) and the extent of their apparent multiplication in 48 hr was about $10^7$ times greater than that of the *fim*⁻ bacteria. A pellicle was formed on these cultures between 6 and 24 hr. In the broths with mannose and α-methylmannoside, the formation of the pellicle was delayed and the outgrowth of *fim*⁺ bacteria inhibited. The effect of the mannose was only slight, presumably because it was completely removed by fermentation within 12 hr. Nevertheless, the proportion of *fim*⁺ bacteria present in the mannose broth after 24 and 48 hr (0.11 and 12.3%) was less than that in the glucose broth (1.77 and 69%). The α-methylmannoside, which was neither fermented nor utilized, had a much greater effect, and almost entirely prevented selective outgrowth of the *fim*⁺ bacteria. As judged from the viable counts at 48 hr, the multiplication of the *fim*⁺ bacteria was only 20-fold, whereas that of the *fim*⁻ was ninefold. The amount of growth at 48 hr was much less in the α-methylmannoside broth (turbidity 0.70), in which pellicle formation was delayed, than in the sorbose broth (1.07) in which a pellicle was formed early.

## DISCUSSION

Our findings support the conclusions of Duguid and Gillies (10) that the conditions of culture in aerobic static broth are selective for fimbriate-phase (phenotypically fimbriate) bacteria and that the mechanism of selection depends on the special ability of fimbriate bacteria rapidly to establish themselves in a pellicle on the surface of the broth, where their growth is promoted by the free supply of oxygen. Duguid and Gillies studied the emergence of fimbriate-phase bacteria in pure, nonfimbriate-phase cultures of *fim*⁺ strains of *S. flexneri* and considered that it depended on the selective outgrowth of spontaneously originating fimbriate-phase variants, but a possible alternative explanation of their findings was that the cultural conditions directly induced the nonfimbriate-phase bacteria to form fimbriae. Our observations on the outgrowth of *fim*⁺ *rha*⁺ strains of *S. typhimurium* in mixed

TABLE 5. *Amount of growth and differential viable counts in mixed cultures of "challenged" fim⁻ rha⁻ strain S625 and "challenging" fim⁺ rha⁺ variant strain S625F grown in aerobic static broths containing 0.2% (w/v) of different carbohydrates*

| Carbohydrate in broth[a] | Time of incubation | Amt of growth | Viable count ($10^6$ bacteria)/ml of | |
|---|---|---|---|---|
| | | | *Fim⁻* bacteria | *Fim⁺* bacteria |
| | *hr* | | | |
| D-Glucose (not inhibiting; utilized) | 0 | 0.06 | 54 | 0.000012 |
| | 6 | 1.28 | 1,530 | 0.00090 |
| | 24 | 0.90 | 700 | 12.6 |
| | 48 | 1.41 | 500 | 1,110 |
| L-Sorbose (not inhibiting; not utilized) | 0 | 0.06 | 51 | 0.000012 |
| | 6 | 0.27 | 520 | 0.00048 |
| | 24 | 0.53 | 390 | 15.5 |
| | 48 | 1.07 | 560 | 810 |
| D-Mannose (inhibiting; utilized) | 0 | 0.06 | 39 | 0.000012 |
| | 6 | 1.06 | 1,730 | 0.0019 |
| | 24 | 0.93 | 860 | 0.94 |
| | 48 | 1.34 | 970 | 137 |
| α-Methylmannoside (inhibiting; not utilized) | 0 | 0.06 | 54 | 0.000012 |
| | 6 | 0.28 | 440 | 0.00047 |
| | 24 | 0.61 | 540 | 0.00045 |
| | 48 | 0.70 | 480 | 0.00024 |

[a] Properties in parentheses: inhibiting, preventing the formation of an early, fimbrial-type pellicle; utilized, eliminated by fermentation within 12 hr; not utilized, not fermented within 48 hr.

cultures with closely related *fim⁻ rha⁻* strains cannot be explained as having been due to the induction of fimbrial synthesis in the *fim⁻* organisms. They prove that there was a selection of the *fim⁺ rha⁺* bacteria, since the *fim⁻ rha⁻* bacteria were incapable of varying to the *fim⁺ rha⁺* genotype under the conditions of the experiments. Mutation to a *fim⁺* or *rha⁺* genotype was not observed in any of our control, pure cultures of the *fim⁻ rha⁻* strains, nor in any of many similar cultures of such FIRN strains examined previously (9, 15). The possibility that the *fim⁻ rha⁻* bacteria in the mixed cultures acquired the *fim⁺* or *rha⁺* genotype from the small inoculum of *fim⁺ rha⁺* bacteria by a mechanism of infective heredity was excluded by the finding that none of 228 *rha⁺* and 228 *rha⁻* clones isolated from the mixed cultures had the genotype *fim⁺ rha⁻* or *fim⁻ rha⁺*. The possibility that the *fim⁺* and *rha⁺* genes were transmitted together on a single transfer factor was excluded by the finding that there were no bacteria with hybrid sets of characters among the 78 *rha⁺* and 78 *rha⁻* clones isolated from the mixed cultures of strains S90 and S7471, which differed from one another in three observed characters additional to those of *fim* and *rha*.

The great strength of the selective effect of culture in aerobic static broth is seen from the finding, e.g., that an inoculum of as few as 14 *fim⁺* bacteria mixed with one of $4 \times 10^8$ *fim⁻* bacteria in 10 ml of broth grew out so rapidly that in 48 hr the *fim⁺* bacteria outnumbered the *fim⁻* ones (Table 2). Such an effect seems fully strong enough to explain why nonfimbriate-phase cultures of *fim⁺* strains of *Escherichia coli* (11), *S. flexneri* (10), and salmonellae (9) are changed into the fimbriate phase during one or a few serial 48-hr subcultures. Fimbriate-phase bacteria appear to arise by spontaneous variation from nonfimbriate-phase bacteria in pure cultures of *fim⁺* strains (3), and presumably these variants are exposed to selection in the pure cultures in the same way as has been demonstrated for the *fim⁺* bacteria in the mixed cultures with *fim⁻* bacteria. The observation that our mixed cultures developed strong hemagglutinating activity showed that many of the *fim⁺* bacteria in them were in the fimbriate phase. Evidence to show whether the selection applied to the fimbriate or the nonfimbriate phase of the *fim⁺* bacteria could not be obtained in the experiments with the *fla⁺* strains of *S. typhimurium*, in which the phase change was very rapid. The change was slower in *fla⁻* strains, and it was found that the *fim⁺ fla⁻* strain S6355 outgrew its related *fim⁻ fla⁻* strain when it was inoculated into the mixed culture from a fimbriate-phase culture,

but not when it was inoculated from a nonfimbriate-phase one. This observation indicates that it is only the fimbriate phase that is selected.

Although the conventional conditions of aerobic culture in broth have been shown to be strongly selective for fimbriate bacteria, there is evidence that these conditions may not be optimal and that selection may be even stronger when the concentration of oxygen above the broth is less than that in air. Thus, when $5.2 \times 10^8$ bacteria per ml of the *fim*$^+$ strain LT2 were grown with $32 \times 10^6$ per ml of the *fim*$^-$ strain S7471 in static broths incubated (i) in air, and (ii) in a jar from which 90% of the air had been evacuated, the numbers of the *fim*$^+$ bacteria increased so that their representations in the populations at 48 hr were, respectively, 23% (HP of mixture = 20) and 42% (HP = 600) (D. C. Old, Ph.D. Thesis, University of Edinburgh, 1963).

**Mechanism of selection.** The failure of the *fim*$^+$ bacteria to give proportionally greater multiplication than the closely related *fim*$^-$ bacteria in the mixed cultures in anaerobic static broth, aerobic shaken broth, and aerobic plates shows that the advantage conferred by the fimbriae is effective only in the special conditions of culture in static liquid medium under air. Previous observations (9, 10, 12, 16) on pure cultures showed that the yield of fimbriate-phase bacteria exceeded that of nonfimbriate-phase bacteria of the same strain, or that of a closely related *fim*$^-$ strain, only when the cultures were grown in aerobic static broth. After 6 to 10 hr in such broth, the fimbriate-phase cultures formed a surface pellicle consisting of fimbriate bacteria and then underwent a large secondary phase of growth, which was absent in the nonfimbriate cultures. Our mixed cultures of *fim*$^+$ and *fim*$^-$ *S. typhimurium* bacteria formed a pellicle at a time, between 6 and 24 hr, somewhat later than the time of pellicle formation in pure *fim*$^+$ cultures (6 to 10 hr) but considerably earlier than that in pure *fim*$^-$ ones (24 to72 hr). The reason the *fim*$^+$ outgrew the *fim*$^-$ bacteria was probably the same as that suggested (10) to explain why fimbriate-phase bacteria gave greater growth than nonfimbriate-phase bacteria in pure cultures. It is thought that the fimbriae enabled the bacteria to float on the surface of the broth and thus to establish themselves rapidly in a pellicle, where they were able to use atmospheric oxygen for energy production at a period when the *fim*$^-$ bacteria in the depths of the broth had exhausted both the dissolved oxygen and the substrates capable of yielding energy by anaerobic processes. This conclusion that the selection of the *fim*$^+$ bacteria depended on the ability of their fimbriate phase to form an "early" pellicle is supported by the finding that selection did not take place in broth containing $\alpha$-methylmannoside, which inhibits the early pellicle-forming properties of type 1 fimbriae.

Another factor that in some experiments contributed to the selection of *fim*$^+$ bacteria was a tendency for the *fim*$^-$ bacteria to die during the later stages of culture, after 24 hr, when the *fim*$^+$ bacteria were still multiplying (Table 2). The high death rate of the *fim*$^-$ bacteria may have been due to their anaerobic, energy-starved condition in the depths of the broth. Brinton (4) described a marked example of this differential effect in a mixed culture of *fim*$^+$ and *fim*$^-$ strains of *E. coli* incubated for 14 days.

**Influence of motility.** The experiments with variants of strain S635 showed that the outgrowth of *fim*$^+$ over *fim*$^-$ bacteria was faster when the competing strains were motile than when they were nonmotile. This effect of motility may explain why a single 48-hr period of culture in aerobic static broth usually suffices to select the fimbriate phase of a motile *fim*$^+$ salmonella organism (9), whereas two, three, or more serial subcultures may be required to do the same with nonmotile *S. flexneri* (10) and *fim*$^+$ *fla*$^-$ salmonellae such as strain S6355. Motile salmonellae migrate aerotactically when grown in oxygen-depleted medium under air (1), and motile fimbriate bacteria may thus be assisted to reach the surface of static broth and form a pellicle more rapidly than nonmotile fimbriate bacteria.

The finding that the *fim*$^-$ *fla*$^+$ strain S6353 outgrew its *fim*$^-$ *fla*$^-$ relative, S6351, showed that even in the absence of fimbriae the property of motility was advantageous for growth in aerobic static broth. Smith and Doetsch (17) demonstrated a similar selective advantage for motility in *Pseudomonas fluorescens*. In *S. typhimurium*, the selection due to motility was weaker than that due to fimbriation. The outgrowth of the motile *fim*$^-$ bacteria probably depends on their being able to migrate aerotactically to the surface of the broth and so to form a pellicle at an earlier stage (24 to 72 hr) than that at which nonmotile *fim*$^-$ bacteria could form one.

ACKNOWLEDGMENTS

We thank E. S. Anderson, B. A. D. Stocker, J. E. Wilson, and H. A. Wright for giving us strains and information about their properties, and I. Corneil for assistance with preliminary experiments.

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