Selected faecal bacteria and nutrients essential for antagonism of *Salmonella typhimurium* in anaerobic continuous flow cultures

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Summary. As few as five of the species of bacteria commonly found in human faeces—*Escherichia coli*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Bacteroides ovatus* and *Fusobacterium varium*—when grown together in anaerobic continuous flow cultures exerted antagonistic effects on *Salmonella typhimurium* as great as those given by mixed bacteria from extracts of human faeces. In a single culture, the population of *S. typhimurium* was $c. 10^8$ cfu/ml but in mixed cultures with the five antagonistic bacteria or mixed faecal bacteria it was reduced to $c. 10^3$ cfu/ml. Antagonism appeared to be the result of competition for the growth limiting amino acids, arginine, serine, threonine and aspartic acid. Optimal manifestation of antagonism required the presence of carbon sources fermentable only by antagonistic bacteria, such as lactose 0.1%, w/v, sucrose 0.1% (w/v) and starch 0.2–0.3% w/v. These carbohydrates promoted the growth of the antagonistic bacteria, particularly *E. coli* and *B. ovatus*. However, an increase in concentration by several fold of any one of four growth-limiting amino acids in the medium diminished the antagonistic effects and the population of *S. typhimurium* rose 10^2 – 10^3 -fold.

Introduction

Competitive exclusion by the normal bacterial flora is considered to be the main mechanism of elimination of various enteropathogens from the intestinal tract of man and animals. This principle has been applied successfully to eliminate food-poisoning *Salmonella* spp. from the intestinal tract of animals.¹ Food poisoning by *S. typhimurium* and *S. enteritidis* has been increasing recently in England² and the USA,³ partly through an increase in eggs and chicken meat contaminated by salmonellae. One of the best ways to prevent this has been to administer orally to newly hatched chickens a mixture of pure cultures of bacteria from the caecal flora of adult chickens.⁴ However, the main bacterial species and the precise mechanisms responsible for the competitive exclusion of salmonellae are unknown.

The anaerobic continuous flow (CF) culture method has been considered to be an excellent tool for in-vitro studies of interactions among colonic bacteria.^{5,6} Through its use we found that *S. typhimurium* was the most resistant of several enteropathogens to the antagonistic effects of some bacteria from human faeces.⁷ In this study, identification was made of nutrients and a group of bacterial species of the normal human faecal flora essential for the antagonism of the growth of *S. typhimurium* in anaerobic CF cultures.

Materials and methods

Bacterial strains

Bacteria used were *Escherichia coli* strain, SU 5034, ATCC 25922, ATCC 11775 and IFO 12713, *S. typhimurium* strain LT-2, *Enterobacter aerogenes* strain IFO 13534, *Bacteroides ovatus* strain SU 39, *Fusobacterium varium* strain ATCC 8501 and *Enterococcus faecalis* strain IFO 12969. Most strains were obtained from the American Type Culture Collection (ATCC) and Institute for Fermentation, Osaka (IFO). Others were stock cultures from this laboratory (SU: Shiga University). Some of the biological characteristics of these bacteria are shown in table I.

Inocula

Bacteria were cultivated in semi-solid (agar 0.1% w/v) maintenance medium⁸ at 37°C for 18–20 h in air. Cultures were suspended in sterile saline at a concentration of $c. 5 \times 10^8$ cfu/ml (McFarland tube No. 3–4). Fresh human faeces was suspended in sterile saline (1:9) and homogenised by a Teflon homogeniser. The preparation was allowed to settle for 10 min and 0.1 ml of the upper layer was inoculated into tubes (12 × 120 mm) of semi-solid maintenance medium and incubated at 37°C for 20 h. Cultures were suspended in sterile saline to the same density as that of the pure

Table I. Some characteristics of the bacterial strains used

Organism	Fermentation* of			Consumption of amino acids in MCM medium			
	sucrose	lactose	sorbose	arginine	serine	threonine	aspartic acid
<i>E. coli</i> SU 5034	+	+	+	+†	+	+	+
ATCC 25922	—	+	+	+	+	+	+
ATCC 11775	—	+	+	—	+	+	+
IFO 12713	—	+	—	—	+	+	+
<i>S. typhimurium</i> LT-2	—	—	—	+	+	+	+
<i>Ent. aerogenes</i> IFO 13534	+	+	—	—	+	+	+
<i>B. ovatus</i> SU 39‡	+	+	+	—	+	+	+
<i>F. varium</i> ATCC 8501§	—	—	—	—	+	+	+
<i>Ec. faecalis</i> IFO 12969	+	+	—	+	+	—	—

* +, Fermentation; —, No fermentation.

† Residual quantities of most of these amino acids in single anaerobic static cultures containing MCM medium (glucose 0.05%, starch 0.2%) were $\leq 10 \mu\text{M}$.

‡ Starch fermenting strain.

§ Glutamic acid utilising strain.

culture suspensions. A portion (1.5–2 ml) of the cell suspension was used as the inoculum.

Analysis of the interaction of different bacteria in the anaerobic CF culture system

Analysis of the interaction of different bacteria was made in an anaerobic CF culture system (fig. 1).⁹ The CF culture was incubated at 37°C and stirred by a Teflon covered magnetic stirring bar (20 mm long,

6 mm diam.) at 300–400 rpm to prevent sedimentation and clumping of bacterial cells. The dilution rate of the culture medium was 0.05/h. MCM (mock contents of the caecum of mice) medium^{7, 11} without acetic, propionic, butyric, lactic and succinic acids was the basal medium for cultivation of bacteria in the CF cultures. In the preliminary study,¹² a medium optimally suited for demonstrating antagonistic effects on *S. typhimurium* in anaerobic CF cultures had been devised. This was the basal MCM medium supplemented with starch 0.2% w/v, glucose 0.05% w/v, lactose 0.1% w/v, sucrose 0.1% w/v and sorbose 0.05% w/v. The number of viable cells of each bacterial species co-cultivated in the CF cultures was determined by counting on selective media.

Selective media

The selective media for viable counting of bacteria have been reported previously.^{8, 13} *E. coli* was recovered on ALPPL medium without penicillin and lincomycin but supplemented with sorbose 0.05% w/v to stimulate growth of some *E. coli* strains. *Ent. aerogenes* was counted in ASC medium supplemented with ampicillin 1 $\mu\text{g}/\text{ml}$ instead of streptomycin and colimycin.

Analysis of residual amino acids in culture media

Bacteria were removed from the static or CF culture media by centrifugation at 4000 *g* for 15 min and the clear supernate was filtered through membranes of 0.45- μm pore size. One portion of the filtrate was mixed with two portions of sulphosulthilate solution 5% w/v and centrifuged at 4000 *g* for 10 min. The clear supernate (100 μl) was applied to a JLC-200A automatic amino-acid analyser (Japan Electron Optics, Co. Ltd, Tokyo) equipped with glass column (6 $\times$ 120 mm) packed with cation exchange resin, CK-10U (Mitsubishi Kasei Co. Ltd).

Fig. 1. Schematic figure of the anaerobic continuous flow culture system.⁹ A, growth vessel (Erlenmeyer flask) filled with c. 120 ml of culture medium (excess culture medium is expelled spontaneously through a siphon, N); B, fresh medium reservoir; C, collecting flask; D, rubber bladder filled with nitrogen; E, magnetic stirrer; F, oxygen-absorbing bottle filled with steel wool treated with CuSO_4 1% in distilled water with Tween 80 0.05%, pH ≤ 2 ;¹⁰ G, peristaltic pump; H, port for inoculation and sampling of bacteria; I, gas outlet needle with cotton wool plug; J, plastic cap for preventing contamination of the port; K, nitrogen gas inlet tube with cotton wool plug (the air of the vessel, B, was expelled by flushing with nitrogen gas and then the tip of the tube was capped tightly with a rubber stopper); L and M, air inlet and outlet tubes with cotton wool plug; P, cotton wool plug to prevent contamination of bottle B.

Results

Comparison of the antagonistic effects of five selected species and mixed human faecal bacteria in CF cultures containing optimum MCM medium

The final population level of *S. typhimurium* growing alone in the CF culture system containing optimum MCM medium was $c. 1.2 \times 10^8$ cfu/ml. However, the final population of *S. typhimurium* in CF cultures co-cultivated with either the selected five faecal bacterial species, including arginine-consuming *E. coli* strain SU 5034, or mixed faecal bacteria was $c. 10^3$ cfu/ml (fig. 2). These results indicate clearly that the mixture of the five faecal species showed antagonistic effects as potent as those produced by mixed faecal bacteria. The omission of any one of the five bacterial species resulted in a decrease in antagonistic effect (table II). Thus manifestation of optimum antagonistic effects required the presence of all five bacterial species, but not of other bacteria.

Influence of sugars in the media on the antagonistic effect

When the five antagonistic bacterial species and *S. typhimurium* were co-cultivated in the CF culture system containing optimum MCM medium without

Table II. Influence of omission of one of the five antagonistic bacterial species on the population level of *S. typhimurium* in anaerobic CF cultures

Strain	Final population ($\log_{10}$ cfu/ml)* in culture no.					
	1	2	3	4	5	6
<i>S. typhimurium</i>	3.06	4.96	4.13	5.26	5.98	5.73
<i>E. coli</i> SU 5034	8.43	—†	8.71	8.27	7.98	8.68
<i>Ent. aerogenes</i>	7.76	8.3	—	6.86	7.94	6.32
<i>Ec. faecalis</i>	7.16	7.72	7.54	—	7.30	6.8
<i>B. ovatus</i>	9.46	9.26	9.61	9.38	—	9.41
<i>F. varium</i>	3.83	6.26	5.89	5.13	6.74	—
pH of cultures	6.26	6.23	6.24	6.31	6.61	6.30

* Mean of population at 2 and 3 days.

† Strain omitted.

any of the five carbohydrates, the antagonistic effects disappeared almost completely and all bacteria co-cultivated persisted in a narrow population range of $c. 3 \times 10^6$ – 2×10^7 cfu/ml (fig. 3).

Sucrose and lactose were fermented by the antagonistic bacteria but not by *S. typhimurium*. The population of *S. typhimurium* co-cultivated with the five antagonistic bacterial species in CF cultures containing optimum MCM medium without lactose and sucrose was $c. 10^6$ cfu/ml (fig. 4A). The population of

Fig. 2. Comparison of antagonistic effects of five species of faecal bacteria and mixed faecal bacteria on *S. typhimurium* in anaerobic CF cultures containing optimum MCM medium. A, viable counts of *S. typhimurium* (1), *B. ovatus* (2), *E. coli* SU 5034 (3), *Ent. aerogenes* (4), *Ec. faecalis* (5) and *F. varium* (6) when all were grown together. B, viable counts of *S. typhimurium* (1) and all aerobic (2) and anaerobic (3) bacteria of three samples (a, b, c) of human faeces when cultured together.

Fig. 3. The viable counts of *S. typhimurium* (1), *B. ovatus* (2), *E. coli* (3), *Ent. aerogenes* (4), *Ec. faecalis* (5) and *F. varium* (6) when grown together in optimum MCM medium without carbohydrates in the CF culture system.

S. typhimurium gradually decreased as the concentration of these sugars was increased and it reached the lowest level of $c. 10^3$ cfu/ml in the presence of lactose and sucrose 0.1%.

Starch was fermented only by *B. ovatus*. The population of *S. typhimurium* in the CF culture

containing optimum MCM medium devoid of starch was $c. 10^5$ cfu/ml. The population of *S. typhimurium* gradually decreased with an increase in starch concentration and reached the lowest level of $c. 10^3$ cfu/ml in the presence of 0.2–0.3% of starch (fig. 4B).

Comparison of antagonistic effects of arginine-positive and -negative *E. coli*

In the mixed CF cultures containing the optimum MCM medium and the five faecal bacterial species, the population of *S. typhimurium* was $\geq 10^2$ -fold lower in the presence of arginine-positive *E. coli* than in the presence of arginine-negative *E. coli* (fig. 5A).

In the mixed CF cultures with the five faecal bacterial species including arginine-negative *E. coli*, the population of *S. typhimurium* was 10^1 – 10^2 -fold lower in the optimum MCM medium devoid of free arginine than in the optimum medium containing $1400 \mu\text{M}$ of free arginine (fig. 5B).

Influence of increasing concentrations of growth limiting amino acids on the antagonistic effect

When the concentrations in the optimum MCM medium of any one of the four growth limiting amino acids—arginine, serine, threonine and aspartic acid—was increased by $2000 \mu\text{M}$, the antagonistic effect of the five faecal bacterial species including arginine-positive *E. coli* on *S. typhimurium* decreased and the population of *S. typhimurium* increased 10^2 – 10^3 -fold (fig. 6A).

The final population of *S. typhimurium* in both of

Fig. 4. Influence of carbohydrate concentration in the optimum MCM medium on the population level of *S. typhimurium* in mixed anaerobic CF cultures with the five faecal bacterial species. A, lactose and sucrose: 1, 0%; 2, 0.01%; 3, 0.05%; 4, 0.1%. B, starch: 1, 0%; 2, 0.1%; 3, 0.2%; 4, 0.3%.

Fig. 5. Comparison of the antagonistic effects of the five faecal bacterial species on *S. typhimurium* in optimum MCM medium when: **A**, the *E. coli* representative strain was arginine negative, IFO 12713 (1), ATCC 11775 (2) and arginine positive ATCC 259221 (3), SU 5034 (4); **B**, the *E. coli* strains included both arginine negative strains IFO 12713 (1) and ATCC 11775 (2), in the optimum MCM medium with (A) and without (B) 1400 µM arginine.

Fig. 6. Influence of four growth-limiting amino acids on the populations of *S. typhimurium* in mixed CF cultures with the five faecal bacterial strains shown in fig. 2A. **A**, addition of 2000 µM of arginine (1), serine (2), threonine (3) and aspartic acid (4), to the optimum MCM medium (5); **B**, addition of various amounts of arginine—95 µM (2), 345 µM (3), 595 µM (4), 2095 µM (5)—to the optimum MCM medium without free arginine (1).

the CF cultures containing MCM medium with and without 95 μM free arginine was the same. Additional increases of arginine resulted in a gradual elevation of the *S. typhimurium* population to a maximum in the presence of 595 μM arginine (fig. 6B).

Residual amino acids in the CF culture media

In the mixed CF cultures containing MCM medium supplemented with either 95 or 595 μM arginine, concentrations of most of the four growth-limiting amino acids were $\leq 5 \mu\text{M}$ (table III).

Table III. Residual concentration of the four growth-limiting amino acids after growth in mixed anaerobic CF cultures of five antagonistic faecal bacterial species and *S. typhimurium*

Amino acid	Concentration of growth-limiting amino acid (μM) in		
	CF medium with arginine (μM)		uninoculated medium with 95 μM arginine
	95	595	
Arginine	1.1	1.7	89
Serine	<1.0	2.7	136
Threonine	5.3	2.6	300
Aspartic acid	4.6	14.6	76

Discussion

With only five of the bacterial species commonly found in human faeces—*E. coli*, *Ent. aerogenes*, *Ec. faecalis*, *B. ovatus* and *F. varium*, antagonistic effects against *S. typhimurium* could be produced in anaerobic CF cultures as potent as those given by mixed bacteria from extracts of human faeces. It appeared that this was achieved by competition for growth-limiting nutrients, such as arginine, serine, threonine and aspartic acid.

Hudault *et al.*¹⁴ showed potent antagonistic effects of caecal flora of conventional chickens on *S. typhimurium* in gnotobiotic mice and chickens. Regrettably however, they did not find any combinations of bacterial species that showed antagonistic effects on *S. typhimurium* as potent as those of whole caecal flora.

In attempting to develop a pure-culture treatment, many authors reported that chickens were successfully protected against colonisation by *S. typhimurium* when given a pure culture of, e.g. *Ec. faecalis*,¹⁵ or *Clostridium* spp.,¹⁶ or *E. coli*,¹⁷ or various *Salmonella* spp.¹⁸ However, Impey *et al.*⁴ suggested that not only was a limited number of strains unlikely to confer complete protection but that they may disturb the ecological balance of the intestinal tract. They found a group of bacterial strains which, when administered orally, prevented colonisation by *S. typhimurium* of the caeca of newly hatched chickens. The treatment conferred

the same protection as that given by a suspension of the caecal contents of adult chickens. The group comprised as many as 48 different bacteria including several unidentified isolates.

Most previous studies, including the latter on the antagonistic effects of intestinal bacteria on *S. typhimurium*, have been made in conventional animals and thus it is difficult to distinguish between the role of the inoculated bacteria and that of bacteria already present in the intestinal tract. The mechanisms by which colonic bacteria normally antagonise *S. typhimurium* and other food poisoning *Salmonella* spp. are difficult to study *in vivo* even if gnotobiotics are employed, because precise manipulation of the growth environment is not possible. Anaerobic CF cultures can reproduce many of the bacterial interactions that occur in the large intestine and the mechanisms which regulate *in vivo* the bacterial population.^{5,6} Moreover, the various factors that influence these interactions are more easily controlled.

In mixed anaerobic CF cultures, potent antagonistic effects on *S. typhimurium* were manifest only in the presence of all five bacterial species, including an *E. coli* strain that had the ability to utilise arginine, serine, threonine and aspartic acid. *S. typhimurium* also actively utilised these amino acids. Substitution of the arginine-utilising *E. coli* strain by an arginine-negative *E. coli* strain provoked a massive decrease in antagonistic effect. In this situation, the amount of arginine available to *S. typhimurium* may be increased and, thus, this bacterium may be able to reach a higher population density. This assumption was supported by the fact that, in optimal MCM medium without free arginine, both clusters of the five antagonistic bacteria including either an arginine-positive or an arginine-negative *E. coli* strain showed similar potent antagonistic effects.

In addition, the presence of some carbohydrates, such as sucrose, lactose and starch, which were fermented only by antagonistic bacteria, was also necessary for manifestation of the potent antagonistic effects. Inclusion of these carbohydrates in the MCM medium promoted a large increase in the population of the antagonistic bacteria, especially *E. coli* and *B. ovatus*, and thereby a diminution in the amount of growth-limiting nutrients.

Even in the optimal conditions for manifestation of the antagonistic effects, i.e., in the presence of arginine-consuming *E. coli* and carbohydrates fermentable by only the antagonistic bacteria, an increase of several-fold of only one of the growth-limiting nitrogen sources—arginine, serine, threonine or aspartic acid—in the optimal MCM medium resulted in a sharp decrease in the antagonistic effects, and the population of *S. typhimurium* increased 10^2 – 10^3 -fold.

An increase in the free arginine concentration of the optimum MCM medium from 0 to 95 μM did not influence the population of *S. typhimurium* in the mixed CF cultures. However, further increases in the arginine concentration promoted growth of

S. typhimurium to the maximum level at an arginine concentration of 595 μM . The residual amount of most of the four growth-limiting amino acids in the CF cultures in optimum MCM media supplemented with either 95 μM or 595 μM arginine was $\leq 5 \mu\text{M}$. In these culture environments of low concentrations of growth-limiting amino acids, the five antagonistic bacterial species and *S. typhimurium* competed with one another, particularly for arginine, and this resulted in c. 10^3 -fold difference in the population of *S. typhimurium*.

These results indicate clearly that the mechanism of antagonism of the five bacterial species on *S. typhimurium* was the competition for growth-limit-

ing amino acids. They verify the hypothesis of Freter *et al.*⁶ that populations of the colonic flora are controlled by one or a few growth-limiting nutrients.

Omission of any one of the five bacterial species from the mixed CF cultures resulted in a massive decrease of the antagonistic effects. Thus each one contributed to the inhibition of growth of *S. typhimurium* by a mechanism not yet determined.

As suggested by Freter *et al.*,^{5,6} the anaerobic CF culture method can reproduce many of the bacterial interactions that occur in the colon, and these results may contribute to the development of methods for the reduction of food poisoning outbreaks.

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