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Growth Suppression in Early-Stationary-Phase Nutrient Broth Cultures of *Salmonella typhimurium* and *Escherichia coli* Is Genus Specific and Not Regulated by σ^S

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We have studied the growth suppression seen in early-stationary-phase LB broth cultures of *Salmonella typhimurium*. Multiplication of small numbers of an antibiotic-resistant *S. typhimurium* mutant was prevented when the mutant was added to 24-h cultures of the antibiotic-sensitive parent strain, whereas an antibiotic-resistant mutant of an *Escherichia coli* strain added to the same culture grew well. A 24-h *E. coli* culture produced a similar specific bacteriostatic inhibition against *E. coli*. In older cultures, a specific bactericidal effect similar to that observed by M. M. Zambrano and R. Kolter (J. Bacteriol. 175:5642–5647, 1993) was also observed. Whether incubated statically or shaken, sufficient nutrients were present in the filtered supernatants of 24-h cultures for small inocula of the same strain to multiply to ca. 10^9 CFU/ml after reincubation. Introduction of the *rpoS* mutation had no effect on the specific bacteriostatic inhibition. Similar specific inhibition was also observed in strains of *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter agglomerans*, and *Shigella* spp. Experiments in which the 24-h culture was physically separated from the antibiotic-resistant mutant by using a dialysis membrane were carried out. These results indicated that the inhibition might be mediated by a diffusible but labile chemical mediator.

The observed reduction in the rate of bacterial multiplication in broth cultures toward the end of logarithmic phase has been well documented in standard bacteriology texts for many years (8, 29), and at least some of the factors that cause it are understood.

A combination of composition of the growth medium and particular organisms may induce conditions which are inimical to bacterial multiplication and might even be bactericidal. The low pH that results from the fermentation of carbohydrates by different bacterial groups and the production of inhibitory concentrations of hydrogen peroxide by *Streptococcus pneumoniae* are well-known examples (29).

Limitation of nutrient concentrations is another factor that can induce a cessation of growth rate. Carbon starvation is accompanied by the induction of at least 50 bacterial proteins (12, 18, 19) and a generalized resistance to other harmful agents such as heat shock and hydrogen peroxide (16). This type of nongrowing, stationary-phase behavior is a result of the starvation conditions to which the cells are subjected. This regulon (9) is complex, and the production of different starvation-induced proteins is regulated by cyclic AMP (23), oxidative stresses (16), osmotic shock (15), and the sigma factor σ^S (RpoS, KatF) (7, 17, 20). It is, however, unclear whether the conditions that induce these changes are equivalent or similar to those present in early stationary phase in broth cultures, in which nutrient limitation might not always be a major problem.

The factors responsible for growth suppression in stationary-phase nutrient broth cultures have been of interest for a long time and were studied in the early decades of this century (for review of this early work, see reference 29). By reinoculating boiled or centrifuged cultures of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, Penfold (22) and Graham-Smith (11) deduced that nutrient depletion was not the only factor. From the work of these authors, Bail (1) developed the concept of the M concentration, a bacterial density which was constant for any particular combination of media, bacterial type, and culture conditions, the basis of which was still unknown. Topley and Wilson (29) and Hershey and Bronfenbrenner (13) suggested that decreases in oxygen concentration in cultures of increasing density produced a general retardation in growth rate. However, this still did not explain many of the earlier observations.

The present work arose out of the demonstration that intestinal colonization of newly hatched chickens by a *Salmonella* strain prevented colonization by a second *Salmonella* strain inoculated 24 h later (4, 5). The inhibition was specific; it was not induced by colonization with bacteria from other related genera or by killed salmonellae. An in vitro model of the phenomenon was developed in which 24-h nutrient broth cultures of *S. typhimurium* were inoculated with small numbers of a nalidixic acid-resistant (Nal^r) mutant of the same strain and then further incubated. It was found that growth of the *S. typhimurium* Nal^r mutant was inhibited by *Salmonella* cultures but not by those of other genera (4) and that the effect was directly related to the high bacterial density (6).

Zambrano et al. (32) and Zambrano and Kolter (31) also studied mixed cultures, in this case *E. coli*, in which a small number of mutant cells from an old stationary-phase culture in Luria-Bertani (LB) broth were able to outgrow early-stationary-phase cultures by virtue of a spontaneous *rpoS* mutation. The interesting observation was made that the older (10-day-old) LB cultures of *E. coli* were bactericidal to cells from the early-stationary-phase cultures. Introduction of a second mutation, in the *nuo* locus, abolished the ability of the *rpoS* mutant to compete in growth in LB cultures. The authors related

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their findings to nutrient starvation, although the exact explanation was not clear.

Following our earlier work (4, 6), experiments were devised to characterize in greater detail the inhibition of growth that occurs in early-stationary-phase cultures of *S. typhimurium*, *E. coli*, and other members of the family *Enterobacteriaceae* in LB broth.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* F98 (phage type 14) (3, 4, 24, 25), *S. infantis* 1326/28 (3), *S. gallinarum* 9 (25), and *E. coli* F212 (serotype O2:K1) are wild-type, prototrophic strains isolated from poultry. The *Shigella flexneri* and *S. sonnei* strains were isolated from mild cases of human dysentery. Other bacterial species, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Enterobacter agglomerans*, were isolated from healthy animals.

The method of production of spontaneous Nal^r and spectinomycin-resistant (Spc^r) mutants has been described previously (24). Previous work (4, 6, 25) indicated that these mutations had no effect on intestinal colonization or on in vitro or in vivo inhibition of multiplication.

A mutation in *rpoS* (*rpoS*::Ap) was transduced into *S. typhimurium* F98 from *S. typhimurium* ATCC 14028s, obtained from S. Libby (10), using P22 transduction (2). In addition, *S. typhimurium* C52 and an *rpoS*::Km derivative were obtained from F. Norel, Institut Pasteur, France. The phenotypes of these mutants were checked by qualitative testing for hydrogen peroxidase II activity, encoded by *katE*, which is dependent on *rpoS* activity. Peroxidase activity tested on LB plates (7, 30) was lower in the mutants than in the parent strains.

Media. Unless otherwise indicated, all broth cultures were made in 10-ml volumes of LB broth (Difco) in 25-ml universal bottles. Broth cultures reached a viable count of 3×10^9 to 6×10^9 CFU/ml after 24 h of incubation in a shaking incubator (150 rpm) at 37°C. Viable bacterial counts were made by plating decimal dilutions on MacConkey agar (CM7; Oxoid, Basingstoke, England) containing either sodium nalidixate (20 μg/ml) or spectinomycin (30 μg/ml).

Standard growth inhibition assay. The assay is described elsewhere (4, 6). Broth cultures of the bacterial strain to be tested for inhibition were inoculated with an antibiotic-resistant indicator strain to a final concentration of approximately 10^3 CFU/ml. The minority indicator strain was a 24-h LB broth culture diluted in phosphate-buffered saline (PBS) to give a starting count of 10^3 CFU/ml. The mixed cultures were reincubated as described above for periods of up to 4 days. Samples were taken at intervals to estimate the numbers of both the majority test strain and the minority indicator components of the mixture.

Separation of majority and minority strain with a dialysis membrane. LB broth cultures (500 ml in 1-liter flasks) of *S. typhimurium* F98 or *E. coli* F212 were shaken for 24 h. The indicator strain(s), *S. typhimurium* F98 Spc^r and/or *E. coli* F212 Nal^r, was diluted in LB broth before being added to the culture to produce a count of 10^3 CFU/ml. In addition, these organisms at this density in LB broth were placed in small sacs of dialysis tubing (pore size, ca. 10 to 12 kDa), each sealed with two clips. The sacs were placed in the culture, and the flasks were reincubated. Sacs and samples of the broth were withdrawn at different times, and the sacs were washed in sterile saline before viable counts were estimated.

In a second set of experiments, the diluent used for the indicator strains comprised the supernatant of 24-h LB broth cultures of the homologous organism, centrifuged and filtered through 0.45-μm-pore-size membrane filters.

RESULTS

Inhibition of growth by LB broth cultures. The inhibitory activity of LB broth cultures of *S. typhimurium* F98 and *E. coli* F212, examined at different phases of the growth curve, for the indicator mutants *S. typhimurium* F98 Spc^r and *E. coli* F212 Nal^r are shown in Fig. 1.

At 24 h, the pH of broth cultures was 7 to 7.2. The 24-h *S. typhimurium* F98 culture prevented any multiplication of the *S. typhimurium* F98 Spc^r; the viable count of the latter organism decreased slightly (Fig. 1A). In contrast, *E. coli* F212 Nal^r multiplied rapidly in the culture of *S. typhimurium* F98. The 24-h *E. coli* F212 culture prevented the multiplication of *E. coli* F212 Nal^r but allowed good growth of *S. typhimurium* F98 Spc^r. In both cases, the viable count of the heterologous species did not reach that of the 24-h culture into which they were inoculated, although they exceeded 10^8 (*S. typhimurium* F98 Spc^r in *E. coli* F212) and 10^9 (*E. coli* F212 Nal^r in *S. typhimurium* F98) CFU/ml.

At 17 days, the mean pH values of the broth cultures were 7.5 (*S. typhimurium* F98) and 8.6 (*E. coli* F212). At this time, the *S. typhimurium* F98 Spc^r indicator inoculated into the *S. typhimurium* F98 culture declined in numbers over the 4-day period of incubation from 3×10^3 to $<10^1$ CFU/ml. The *E. coli* F212 Nal^r indicator, however, multiplied in the *S. typhimurium* culture to reach a count of 3.5×10^6 CFU/ml. Similarly, the 17-day-old *E. coli* F212 culture produced reductions in the viable numbers of the inoculated *E. coli* F212 Nal^r indicator mutant but allowed *S. typhimurium* F98 Spc^r to multiply well.

At the third sampling time of 60 days, both cultures were in decline, with mean pH values of 8.9 (*S. typhimurium*) and 8.7 (*E. coli*). In both cases, both indicator strains declined in numbers after inoculation, the rate of decline being greater for the homologous strain than for the heterologous mutant.

Previous work (4, 6) indicated that the use of Nal^r or Spc^r mutations had no effect on inhibition.

Association between nutrient availability and inhibition. Bacteriostatic inhibition of *S. typhimurium* F98 Nal^r by 24-h cultures of the parent strain occurred whether the cultures of the parent strain were shaken at 150 rpm or incubated statically in air or anaerobically in boiled and cooled broth covered in a 10-mm layer of sterile mineral oil. The 24-h viable counts of the parent strain at the time of addition of the Nal^r mutant were 5.5×10^9 CFU/ml (shaken), 1.5×10^9 CFU/ml (static, aerobic), and 2.4×10^9 CFU/ml (static, anaerobic).

Because the inhibition observed in 24-h cultures occurred only between the homologous strains, the possibility of exhaustion of essential nutrients was explored by testing the ability of centrifuged, filtered (0.45-μm-pore-size filter; Schleicher & Schuell, Dassel, Germany) 24-h culture supernatants of *S. typhimurium* F98 to sustain growth of the parent strain. In this case, the bacteria added to the supernatants were diluted in PBS. After 24 h of shaking incubation, the viable counts of *S. typhimurium* F98 in filtered supernatants obtained from static and shaken cultures were 3.4×10^9 and 1.7×10^9 CFU/ml respectively. The shaken culture was filtered, inoculated, and incubated a second and a third time, and the 24-h viable counts obtained were 2.8×10^9 and 3.1×10^9 CFU/ml, respectively. Broth cultures of *S. typhimurium* F98 taken after 1, 2, 3, 4, and 8 days of incubation were centrifuged, filtered, and reinoculated with 10^3 CFU of the parent strain per ml. After 24 h of further incubation, the viable numbers obtained were all in excess of 10^9 CFU/ml.

Additional experiments not described indicated that filtration through 0.45-μm-pore-size membrane filters completely sterilized supernatants and that they added no additional nutrients to the supernatants which might be sufficient to stimulate bacterial growth.

The inhibition produced by *rpoS* mutants of *S. typhimurium*. Two *rpoS* *S. typhimurium* mutants of strains F98 and C52 were tested as 24-h cultures for their inhibitory activities against Nal^r mutants of the homologous parent strain. Complete bacteriostatic inhibition occurred. Inhibition also occurred when the parent strain was tested for inhibitory activity against the *rpoS* mutant and when the 24-h culture and the challenge strains were *rpoS* mutants of the homologous strain.

Specificity of inhibition among the *Enterobacteriaceae*. The inhibition observed in all the experiments described above was not simply a characteristic of the individual strains used. Thirteen prototophic *Salmonella* serotypes were all inhibitory for *S. typhimurium* F98 Spc^r but not for *E. coli* F212 Nal^r. Similarly, 15 of 18 field strains of *E. coli* inhibited the multiplication of *E. coli* F212 Nal^r but not *S. typhimurium* F98 Spc^r.

Selected strains from different bacterial genera within the *Enterobacteriaceae* were tested for their abilities, as 24-h cultures, to inhibit the multiplications of Nal^r mutants produced from these strains. The results are shown in Table 1. With some exceptions, inhibition did not occur between heterologous organisms. Inhibition of isogenic strains was almost complete, the increase in the $\log_{10}$ viable count of the indicator strains being less than 0.2. Multiplication of a second *S. sonnei* strain and an *S. flexneri* strain was also inhibited by *S. typhimurium*, *C. freundii*, and *E. coli* but not by *Klebsiella* or *Enterobacter* species (data not shown).

Physical separation of majority and indicator strains in 24-h cultures by dialysis tubing. The effects of separating cultures of *S. typhimurium* F98 or *E. coli* F212 from their Spc^r and Nal^r mutants by enclosing the indicator cultures in

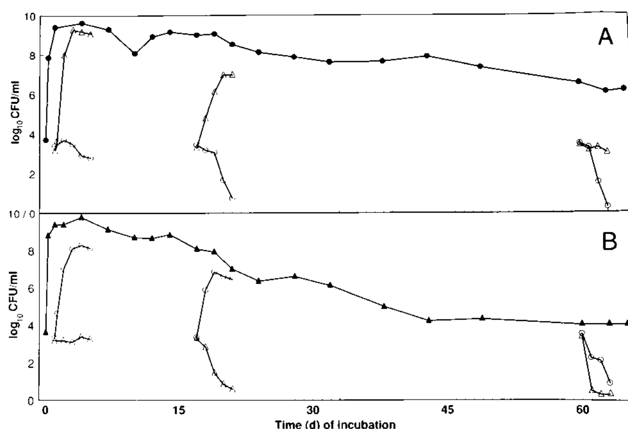

FIG. 1. Growth curves in LB broth of *S. typhimurium* F98 (A) and *E. coli* F212 (B) showing the effects of 1-, 17-, and 60-day cultures of both strains on the growth of *S. typhimurium* F98 Spc^r and *E. coli* F212 Nal^r inoculated into these cultures. Shown are $\log_{10}$ viable counts of *S. typhimurium* F98 (antibiotic sensitive) (●), *E. coli* F212 (antibiotic sensitive) (▲), *S. typhimurium* F98 Spc^r (○), and *E. coli* F212 Nal^r (△). Each point is the mean value obtained from three cultures.

TABLE 1. Homologous and heterologous inhibition produced by 24-h LB cultures of bacterial strains from different genera

Parent strain (tested as majority culture)	Increase in $\log_{10}$ viable counts/ml after 24 h of incubation in stationary-phase LB cultures of the parent strain					
	S. typhimurium F98 Nal^r	*C. freundii* 351 Nal^r	*E. coli* F212 Nal^r	*S. sonnei* C69/417 Nal^r	*K. pneumoniae* K1 Nal^r	*E. agglomerans* E145 Nal^r
S. typhimurium F98	0.1	2.9	3.7	3.5	2.3	0.5
C. freundii 351	3.7	0.1	3.5	1.4	2.0	3.3
E. coli F212	3.5	4.2	0.0	3.2	2.6	4.7
S. sonnei C69/417	4.7	4.2	5.8	0.0	4.6	5.9
K. pneumoniae K1	3.2	5.0	4.9	2.2	0.2	3.6
E. agglomerans E145	4.3	5.1	4.9	3.1	4.5	0.0
None (LB broth)	6.1	5.6	6.1	6.4	6.0	7.1

small sacs of dialysis tubing (pore size, ca. 10 to 12 kDa) are shown in Fig. 2. The culture of *S. typhimurium* F98 inhibited the multiplication of *S. typhimurium* F98 Spc^r when it was inoculated directly into the broth culture but allowed the multiplication of *S. typhimurium* F98 Spc^r when it was inoculated into the dialysis sac. The *E. coli* F212 culture prevented the multiplication of *E. coli* F212 Nal^r when it was inoculated directly into the culture, but the *E. coli* F212 Nal^r culture inoculated into the dialysis sac grew well. *S. typhimurium* F98 Spc^r inoculated both outside and inside the sac in the *E. coli* culture grew well.

Very similar results were obtained when the minority indicator strains were diluted in LB broth culture supernatants (results not shown).

DISCUSSION

For studying the behavior of cultures at different phases of the growth cycle, the system of inoculating broth cultures with small numbers of antibiotic-resistant mutants as indicator strains appears to be useful, as found previously (5, 31, 32). The method ensured that with appropriate isogenic mutants, there was essentially no difference, other than that indicated by the mutation, between the majority culture and the minority indicator cells, enabling their behavior to be monitored under different conditions.

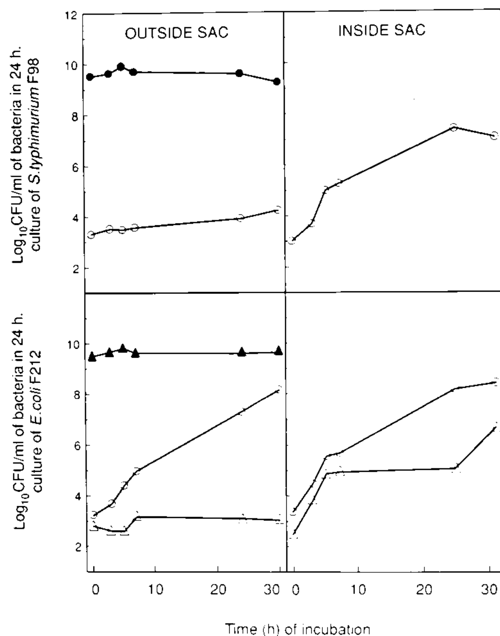

FIG. 2. Effect of separating antibiotic-sensitive 24-h cultures and inoculated antibiotic-resistant strains by using a dialysis membrane. A 24-h culture of *S. typhimurium* F98 (●) was inoculated with *S. typhimurium* F98 Spc^r (○). A 24-h culture of *E. coli* F212 (▲) was inoculated with *S. typhimurium* F98 Spc^r (○) and *E. coli* F212 Nal^r (△). The minority indicatory strains were inoculated directly into the broth (outside sac) and into broth in dialysis sacs (inside sac).

When *S. typhimurium* F98 and *E. coli* F212 were studied together in mixed cultures, we observed an inhibitory effect, specific to the homologous organism, whose intensity varied according to the phase of the growth cycle. The specific inhibitory effects were bacteriostatic in a 24-h culture and bactericidal in a 17-day culture. It was not determined whether the effects were caused by the same factor. The bactericidal effect of the 17-day culture appeared to be identical to that seen by Kolter and colleagues (31, 32). The bacteriostatic effect, which has not been observed before, occurred against homologous cells and was not the result of a pH effect, since in 24-h cultures the pH was 7 to 7.2. Thus, in 24-h LB broth cultures, entry into stationary phase by these two organisms appeared to be associated with a specific down-regulation of metabolism. The two questions to be answered from these experiments were whether nutrient depletion was involved, since the two organisms concerned were nutritionally different, and how specific the inhibitory mechanism was.

The results of several experiments indicated that nutrient shortage was not a major factor in the inhibition observed in 24-h cultures. Sufficient nutrients were present in filtered supernatants even after 8 days of incubation to allow the challenge strain to reach viable counts in excess of 10^9 CFU/ml. There was some indication that the nutrient concentration was greater in supernatants obtained from cultures which had been incubated statically than in those incubated with shaking. However, in both cases the counts obtained after reinoculation and reincubation were greater than 10^9 CFU/ml. It seems, therefore, that LB broth cultures, incubated statically or shaken moderately, enter stationary phase in the presence of considerable concentrations of nutrients that have not been fully utilized. This process is independent of *rpoS*-regulated functions indicated by the fully inhibitory effect of two independently produced *rpoS* mutants of *S. typhimurium* whose phenotypes had been confirmed. These results contrast with the observations of Weichart et al. (30) that LB cultures of mutants of *E. coli* MC4100 shaken vigorously for 24 h and then filtered require the addition of a carbon source to sustain further growth. The difference in the results is potentially very interesting. It is possible that the differences can be ascribed to the use of prototrophic wild-type strains by one group and an auxotrophic laboratory *E. coli* by another. Under the conditions described by these authors, carbon starvation did occur. However, insufficient information was given as to the vigor of the shaking and thereby the degree of aeration. It seems likely that under different growth conditions, bacterial cultures enter stationary phase for different reasons which might depend largely on the degree of oxygenation and associated nutrient depletion, mainly of a utilizable energy source.

The extent of the specificity of the bacteriostatic inhibition was not determined completely. Not all of the wild-type *E. coli*

strains inhibited *E. coli* F212. Similarly, although all *Salmonella* serotypes tested inhibited *S. typhimurium* F98, this strain does not necessarily inhibit all other salmonellae in a reciprocal fashion (4, 5). The results indicated that although inhibition between organisms within one taxon does not necessarily always occur, inhibition between organisms from different taxa does not occur. From this work, it is unclear whether the specificity concerned is at the species or genus level. Differences in nutritional requirements between *S. typhimurium* and *E. coli* could not account for the specificity because sufficient nutrients were present in 24-h LB cultures of either organism for both strains to grow.

If the metabolic down-regulation apparently occurring in early-stationary-phase LB cultures is not caused by nutrient starvation, a number of other possibilities, perhaps involving interbacterial signalling, exist. How these might involve both a bacteriostatic and a bactericidal mechanism remains to be seen. Signalling in a broth culture could result from contact between cells involving their surfaces or appendages, resulting either from motility or Brownian motion, or from the production of a diffusible signal. The experiments described have produced some apparently conflicting results. The ability of culture supernatants to sustain extensive growth indicated either that live cells were necessary or that the signal molecule was very labile or was utilized or broken down by the bacterial cells themselves. Separating the majority culture from the minority indicator culture by using a dialysis membrane suggested either that cell-cell contact was involved or that the signal molecule was very large (>12 kDa) or was easily adsorbed by the nitrocellulose membrane.

A signal molecule could be produced constitutively and accumulate solely as a result of the increasing cell density, or its production could be induced by some other nonspecific environmental factor such as oxygen tension (26, 29) or other intracellular chemicals related to changes from predominantly aerobic to mixed aerobic aerobic-anaerobic metabolism such as acetyl phosphate (21). The specificity of inhibition and reciprocity in the inhibitory behavior of the *S. typhimurium* and *E. coli* cultures suggest that these were not the specific inhibitory factors themselves.

Many examples are now known of interbacterial signalling being involved in gene regulation (14, 27, 28), a number of which involve small-molecular-weight chemical signals, such as derivatives of *N*-acetyl homoserine lactone, to control the production of a wide variety of microbial products, all as a result of a dependence of high cell density for signal accumulation and gene transcription. At the moment, therefore, it seems likely that a diffusible signal chemical whose continuous activity requires the presence of live cells is produced either constitutively or at a particular cell density.

Although not examined as thoroughly, a similar specificity occurs in vivo in the inhibition of colonization that occurs between related strains of enteric bacteria colonizing the alimentary tracts of young chickens (4, 5).

The question remains as to why enteric bacteria should reduce their growth rate when nutrients are not limiting and thereby allow the rapid growth of related but potentially competitive bacterial types. This apparently occurs in vitro and in vivo (4, 5). One possibility is that the different bacterial types do in fact occupy different metabolic niches and are not necessarily competitive. Down-regulation of metabolism in the absence of nutrient starvation could avoid the physiologically stressful effects of nutrient starvation by another form of growth regulation. In this case, provision of a higher oxygen tension and/or a reduction in bacterial density could result in a rapid resumption of growth without the major changes that might occur when cells come out of starvation conditions. Density-dependent regulation may occur in the parts of the alimentary tract such as the ceca in young animals where semi-batch growth conditions may occur and where the density of facultative anaerobes in these animals can be high. It may thus represent a strategy to allow such bacteria to have as short a lag time as possible when fresh nutrients arrive and retain their potential for competitive growth.

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