Serine Utilization as a Potential Competition Mechanism Between *Salmonella* and a Chicken Cecal Bacterium

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**ABSTRACT**

Our objective in this study was to use batch culture to estimate growth kinetic parameters for a growth-limiting amino acid, serine, in *Salmonella typhimurium* and *Escherichia fergusonii*, and to test these predictions for competitiveness in a mixed culture of the two organisms. Under anaerobic growth conditions, the two bacteria grew only when serine was provided as the nitrogen source. When serine was used as a carbon source in aerobic media, the maximum growth rates of the two organisms were considerably lower and the affinity constants were higher than when serine was used as a nitrogen source. The maximum growth rates of the two organisms were lower in anaerobic media than in aerobic media, but serine anaerobic affinity constants were lower than those from aerobic media. In aerobic mixed culture, *S. typhimurium* outgrew *E. fergusonii* in N-limited minimal media containing 0.1 mM serine but in N-nonlimited minimal media containing 10 mM serine, *E. fergusonii* outgrew *S. typhimurium*. In anaerobic mixed culture, *E. fergusonii* outgrew *S. typhimurium* in media containing both 0.1 mM and 10 mM serine concentrations. It appears that the cause of inhibition of *Salmonella* with serine as the variable nutrient differs depending on the oxidation-reduction status.

Key Words: *Salmonella*, amino acids, affinity constants, growth rates.

Poultry contaminated with *Salmonella* spp. is one of the primary sources of food-associated outbreaks of human salmonellosis. Newly hatched chicks are raised under controlled conditions, which can delay the establishment of the definitive cecal bacterial community, increasing the susceptibility of these chicks to salmonelae cecal colonization (40). The supply of eggs or chicks from infected parent breeder flocks can lead to a pyramidal increase in the degree of infection of progeny (8), control of *Salmonella* at early stages of production is critical. The cecum is the main site of salmonelae colonization (19), and the concept of providing newly hatched chicks with intestinal microflora from *Salmonella*-free adult chickens to decrease the incidence of salmonelae cecal colonization has been extensively reviewed elsewhere (38,40,41). Competitive exclusion by the normal bacterial flora is considered to be the main mode of prevention of colonization by various enteropathogens of the intestinal tract of man and animals (12,13,43). However, the underlying physiological mechanism(s) responsible for antagonism to *Salmonella* by the native microflora and subsequent control of its colonization in the cecum remains to be elucidated. One of the prevailing theories, nutrient exclusion, is defined as the process in which the natural microbiota utilize sufficient quantities of a nutrient for its concentration to fall below the threshold of uptake affinity by the potential pathogens (11). When as few as five different fecal bacterial isolates were cocultivated with *S. typhimurium* in continuous culture (43), Ushijima and Seto observed a significant decrease in *S. typhimurium*, and this decreased growth was associated with competition for specific amino acids (arginine, aspartic acid, serine and threonine). Of these four amino acids, only serine can be used by *Salmonella* as its sole carbon and nitrogen source when grown aerobically in pure culture (15). It is not known if this is true for the anaerobic conditions prevalent in the cecum. The objective of the present study was to use anaerobic batch culture growth to estimate growth kinetic parameters of *Salmonella* when grown on serine and test these predictions for competitiveness in a mixed culture of *Salmonella* and another organism.

**MATERIALS AND METHODS**

**Bacterial strains.**

A primary poultry isolate of *S. typhimurium* obtained from the National Veterinary Service Laboratory, Ames, IA (Accession No. 87-26254) was selected for resistance to novobiocin (NO) and nalidixic acid (NA) and maintained in media containing 25 µg NO and 25 µg NA per ml. The viable cell concentration of the challenge inoculum was counted in mixed culture and confirmed by colony counts on brilliant-green agar (BGA, Difco Laboratories, Inc., Detroit, MI) plates containing 25 µg novobiocin/ nalidixic acid/ml. A chicken cecal bacteria, *E. fergusonii*, isolated from a continuous-flow culture that had been seeded with chicken cecal contents (31), was used as the comparative organism.

**Media and growth measurement.**

Methods used for bacterial culture and media preparation for anaerobic studies were those of Bryant (3). Nitrogen-free basal medium using L-ascorbate (Sigma Chemical Co., St. Louis, MO) as the reductant was prepared as described by Ricke and Schaefer...
(34) for the anaerobic media. Nitrogen-free basal aerobic medium was identical in composition to the anaerobic medium except ascorbate, NaHCO₃, and resazurin were excluded. Growth of the two microorganisms was measured turbidimetrically at 600 nm (A₆₀₀) on a Spectronic 20 spectrophotometer (Bausch & Lomb Spectronic 20, Milton Roy Co., Rochester, NY) in aerobic and anaerobic batch culture. At least six points in the linear portions of plots of natural logarithm A₆₀₀ versus time were used for estimation of specific growth rates using linear regression analysis (34). The affinity constant (ks) and maximum growth rate (μmax) for each microorganism were estimated from Lineweaver-Burk plots of reciprocal exponential growth rate versus reciprocal initial amino acid concentrations (33). At least six growth rate points and an R² of at least 0.95 were used to estimate ks (substrate concentration at which half-maximal growth rate occurs).

**Mixed culture studies.**

*Salmonella typhimurium* and *E. fergusonii* were grown together in two different minimal media containing 0.1 mM (nitrogen limited) and 10 mM (nitrogen nonlimited) serine, respectively. One ml of each organism (2 x 10⁸ cells) grown in minimal medium (excluding nitrogen source) was added to 200 ml of the respective serine media and incubated at 37°C aerobically or anaerobically. Ten ml of culture were sampled at each of six sampling points to measure cell counts and chemical contents in the minimal media. The samples for chemical analysis were filter sterilized by passing them through 0.2 μm pore size syringe filters (Nalgene Syringe filter, Nalgene Company, Rochester, NY). *Salmonella typhimurium* was selectively isolated and quantified on brilliant green agar (BGA; Difco) plates containing 25 μg/ml NO and 25 μg/ml NA (45). *Escherichia fergusonii* was differentially enumerated as colony counts on Tryptic Soy Agar (TSA; Difco) after subtracting *Salmonella* counts (from the BGA plates). The colony counts of cells were expressed in log₁₀ colony forming units (CFU)/ml. Glucose concentration was measured by the DNS (dinitro salicylic acid) method (27). Ammonia concentration was measured by the indophenol method of Chany and Marbach (35). Volatile fatty acid (VFA) concentrations were determined by gas-liquid chromatography as previously described (7). Lactic acid concentrations were determined by the enzymatic method of Hohorst (18).

**Statistical analysis.**

Cell numbers and optical densities expressed as logarithmic functions were subjected to linear regression (least-squares) analysis with the lack of fit of the regression line determined by the methods of Draper and Smith (9). Colony counts, expressed as logarithmic functions, were analyzed by least-squares mean separations which were accomplished using the PDiff option of the GLM procedure in the SAS (statistical analysis software) program, version 6.04 (SAS Institute Inc., Cary, NC). All statistical analyses were considered significant at the P < .05 level.

**RESULTS**

**Pure culture growth kinetics.**

Maximum growth rates (μmax) and affinity constants (ks) of a primary poultry isolate of *S. typhimurium* and a chicken gastrointestinal microorganism, *E. fergusonii*, in minimal media are shown in Table 1. Under aerobic growth conditions both organisms used serine as a nitrogen (N) as well as a carbon (C) source, but they grew much faster with serine as a nitrogen source. Under anaerobic growth conditions, the two bacteria grew only when serine was provided as the nitrogen source. When serine was used as a C-source in aerobic media, the μmax of the two organisms were considerably lower than those in aerobic media using serine as an N-source (*S. typhimurium*, 0.17 h⁻¹ versus 0.57 h⁻¹; *E. fergusonii*, 0.28 h⁻¹ versus 0.81 h⁻¹) and affinities also were considerably lower (higher ks) (ks: *S. typhimurium*, 12.83 mM versus 0.79 mM; *E. fergusonii*, 9.65 mM versus 2.31 mM). The anaerobic μmax values of both organisms were lower than those in aerobic media (ks: *S. typhimurium*, 0.28 h⁻¹ versus 0.57 h⁻¹; *E. fergusonii*: 0.41 h⁻¹ versus 0.81 h⁻¹) but anaerobic affinities were greater than those in aerobic media (ks: *S. typhimurium*: 0.08 mM versus 0.79 mM; *E. fergusonii*: 0.04 mM versus 2.31 mM).

**Mixed culture growth response.**

Growth of *S. typhimurium* NO/NA and *E. fergusonii* in aerobic mixed culture is presented in Fig. 1. *Salmonella typhimurium* outgrew *E. fergusonii* in N-limited aerobic mixed culture containing 0.1 mM serine as a nitrogen source. However, in N-nonlimited aerobic mixed culture containing 10 mM serine, *E. fergusonii* predominated and the growth of *S. typhimurium* was too slow for it to persist. Growth of the anaerobic mixed culture is presented in Fig. 2. *Escherichia fergusonii* outgrew *S. typhimurium* in both N-limited and N-nonlimited mixed culture and predominated in the mixed culture.

**Mixed culture metabolite concentrations.**

Concentrations of serine and ammonia in mixed culture are presented in Fig. 3. In aerobic and anaerobic mixed culture, serine in N-limited minimal media containing 0.1 mM serine was nearly depleted and the depletion in aerobic mixed culture was higher than in anaerobic mixed culture (data not shown). High concentrations of serine remained in minimal media containing 10 mM serine (nitrogen nonlimited). In N-limited aerobic and anaerobic mixed culture containing 0.1 mM serine, detectable ammonia was not generated (data not shown). In N-nonlimited mixed culture containing 10 mM serine, significantly higher concentrations of ammonia were produced from anaerobic mixed culture (about 4 mM) than from aerobic mixed culture (approximately 0.3 mM) even though the remaining concentrations of serine in aerobic and anaerobic mixed cultures were almost identical (Fig. 3). Concentrations of

**Table 1. Growth parameters for *S. typhimurium* and *E. fergusonii* in minimal media.**

<table>
<thead>
<tr>
<th>Medium Condition</th>
<th><em>S. typhimurium</em></th>
<th><em>E. fergusonii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>μmax (h⁻¹)</td>
<td>0.57</td>
<td>0.81</td>
</tr>
<tr>
<td>Aerobic SN¹</td>
<td>0.17</td>
<td>0.28</td>
</tr>
<tr>
<td>Aerobic SC²</td>
<td>0.28</td>
<td>0.41</td>
</tr>
<tr>
<td>Anaerobic SN³</td>
<td>0.79</td>
<td>2.31</td>
</tr>
<tr>
<td>ks (mM)</td>
<td>12.83</td>
<td>9.65</td>
</tr>
<tr>
<td>Aerobic SC</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Anaerobic NA</td>
<td>0.08</td>
<td>0.04</td>
</tr>
</tbody>
</table>

¹ SN: serine used as a nitrogen source.
² SC: serine used as a carbon source.
³ When serine was used as a carbon source in anaerobic conditions the growth rates of both bacteria were too low to be accurately calculated.
Figure 1A. Mixed culture growth with 0.1 mM serine added

Figure 1B. Mixed culture growth with 10 mM serine added

Figure 2A. Mixed culture growth with 0.1 mM serine added

Figure 2B. Mixed culture growth with 10 mM serine added

Figure 3A. Aerobic mixed culture with 10 mM added serine

Figure 3B. Anaerobic mixed culture with 10 mM added serine

Figure 4. Remaining concentrations of glucose in mixed culture (pooled SEM = 0.0759 for aerobic culture and 0.0420 for anaerobic culture).

Significant quantities of lactic, pro-
pionic and butyric acids were not found in any of the mixed-culture incubations.

**DISCUSSION**

We chose to work with *E. fergusonii* as the representative cecal organism because it was one of the 11 bacterial isolates identified from a continuous-flow culture that had been shown to be protective against *S. typhimurium* cecal colonization in broiler chicks (31). In addition, for the purpose of our studies here, the metabolic similarities of *E. fergusonii* to *Salmonella* (both facultative anaerobes) made it much easier to do direct comparative in vitro studies for assessing competitive mechanisms. The theory that nutrient competition is responsible for exclusion of nonindigenous organisms originates from observations that population-control mechanisms in the intestine are similar to chemostat functions (10,17). When applying this to a gut system such as the avian cecum, a possible hypothesis holds that the populations of indigenous bacteria are controlled by substrate competition, with each species being more efficient than the rest in utilizing one or a few particular substrates, and that the population level of that species is controlled by the concentration of these few limiting substrates (11). However, determining the substrates that directly influence bacterial competition depends upon which substrates are consistently available in the cecum. We focused our efforts on serine because several lines of evidence suggest that it may be either a limiting substrate directly, or at the very least, a primary precursor to substrates that influence indigenous bacterial competition with *Salmonella*. In rats, the glycoprotein mucin has been shown to be the major endogenous carbohydrate excreted from the upper gut and characteristically contains high proportions of threonine, serine, proline, glucosamine and galactosamine (29,37). Based on the amino acid composition of the excreta of germ-free and conventional chicks, Salter and Fulford (37) saw little effect of cecal microflora on amino acid disappearance except for amino acids typically found in mucoproteins and concluded that cecal bacteria could serve an important role in the degradation of endogenous proteins and the recycling of nitrogen. More recently, Krivan et al. (23) demonstrated that *S. typhimurium* and *Escherichia coli* are capable of utilizing mouse cecal mucus total lipids, the corresponding acidic lipid fraction, and phosphatidylserine as the sole sources of carbon and nitrogen. Serine may also be an important substrate for lipid biosynthesis in strict anaerobes. Van Golde et al. (44) observed an abundance of serine-containing lipids in several strictly anaerobic rumen bacteria when grown in the presence of 14C serine even though serine-containing lipids do not accumulate in most aerobic or anaerobic bacteria. Finally, among the four amino acids that Ushijima and Seto (43) demonstrated to be subject to specific nutrient-mediated competition between *Salmonella* and fecal bacteria, only serine can be used as both the sole carbon and sole nitrogen source by *Salmonella* (15). In the present study, both *S. typhimurium* and *E. fergusonii* could use serine as a sole source of nitrogen when grown anaerobically or aerobically and, albeit at a much slower growth rate, could also use serine as a carbon source aerobically. However, neither organism could use serine as the sole carbon source anaerobically. It has been observed that when L-serine is provided as the only source of carbon for the growth of *E. coli*, addition of a small amount of glycine, L-isoleucine, and L-threonine (or L-leucine) is required (16,20). This may also explain the zero growth results under anaerobic conditions, although the ATP per unit of available carbon may simply be too low to support growth.

To predict the outcome of competition for serine between *S. typhimurium* and *E. fergusonii*, we determined growth kinetic constants of pure cultures grown on serine. This method was originally adapted from Monod (28) by Russell (36) to predict which substrates would control composition and competition of the rumen bacterial flora. Since substrate concentrations in natural environments are usually too low to permit maximum growth rates (22), it is reasonable to assume that relative substrate affinities can be a significant determinant of bacterial competitiveness in nature (30). Under aerobic growth conditions *S. typhimurium* had a 3-fold better affinity for serine than *E. fergusonii*, and this was reflected in the domination by *S. typhimurium* in the 0.1 mM serine biculture. However, when the two organisms were grown at a serine concentration well above saturation *E. fergusonii* outgrew *S. typhimurium* since it had a higher maximum growth rate. Shehata and Marr (39) found that deviations from the Monod relation occur at the higher growth rates which, they suggested, indicates that the organism's affinity for the growth-limiting substrate varies with the growth rate (33). This variance may be attributable to multiple transport systems. Although nothing is known regarding *E. fergusonii*, at least three serine transport systems have been identified for *E. coli* (16) while kinetic studies of L-serine uptake (42) indicate that *S. typhimurium* may possess only a single
transport system. This relationship did not hold for anaerobic growth conditions since *E. fergusonii* outgrew *S. typhimurium* in biculture regardless of the serine concentration. Given that affinity for serine is nearly the same for the two organisms, the inability of *S. typhimurium* to compete may be more related to the production of toxic products during the biculture fermentation. The simultaneous disappearance of serine and production of ammonia and acetate in the high-serine biculture suggests that serine was deaminated and converted to acetate. Serine is usually deaminated by a dehydratase (25), and the resulting pyruvate can be converted to acetate by many bacteria (4,6,24). The antimicrobial activity of fermentation acids has been well documented (21) and has been proposed as one of the mechanisms by which normal flora inhibit colonization by enteropathogens (1,2,7,26). In monoculture experiments not shown here, we have observed that when acetate is increased to 25 mM under these same conditions (anaerobic, pH 6.3) the lag period is increased and the growth rate is substantially decreased for *S. typhimurium* but not *E. fergusonii*.

In conclusion, it appears that the cause of inhibition of *Salmonella* with serine as the variable nutrient differs depending on the oxidation-reduction status. Under aerobic conditions, differences in growth response in coculture is consistent with differences in growth response in pure culture of *S. typhimurium* and *E. fergusonii*, but when grown anaerobically in co-culture, *S. typhimurium* is no longer competitive at either serine concentration, implying that anaerobiosis must be taken into account as a potential factor when developing theories regarding mechanisms responsible for exclusion of nonindigenous organisms. Anaerobiosis may also account for the success of some organisms in such a consortium may provide a means not only for lowering gut oxidation-reduction potential, thus ensuring an environment more suitable for colonization of the strictly anaerobic members of the culture (14,31,32), but also, for providing an environment that is more restrictive to the growth of *Salmonella*.

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REFERENCES