Comparison of Salmonella typhimurium and Selected Facultative Chicken Cecal Bacteria Survivability after Specific Amino Acid-Limited Batch Growth

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ABSTRACT

The objective of this in vitro experimentation was to compare the survivability of Salmonella typhimurium strains and selected facultative chicken cecal bacteria after specific amino acid-limited growth on either serine, threonine, arginine, or aspartate. Survivability of Salmonella typhimurium and chicken cecal bacteria was estimated by measuring the rate of decrease of viable cell numbers and calculating the average time for 50% of the cells to become nonviable (50% survival time, ST50). Two S. typhimurium strains, LT2 and a primary poultry isolate (NO/NA), and three selected facultative chicken cecal bacteria, Citrobacter freundii, Escherichia coli and Escherichia fergusonii, were grown aerobically at 37°C to stationary phase on carbon-limited or nitrogen-limited minimal media. All organisms remained viable longer (P < 0.05) on serine media than on any of the other media tested. When serine was used as a nitrogen source in minimal media the ST50 of C. freundii and E. fergusonii were significantly longer than those of the two S. typhimurium strains. It appears that when media are limited in the same nutrient, the ability to sustain viability varies among facultative bacteria derived from the chicken cecum.

Key words: Salmonella, cecal facultative bacteria, chicken, amino acids, survivability

Poultry contaminated with salmonella are one of the primary sources of food-associated outbreaks of human salmonellosis. The cecum is the main site of salmonellae colonization (20); in newly hatched chicks raised under controlled conditions the establishment of the definitive cecal bacterial community can be delayed, and this delay can increase the susceptibility of these chicks to salmonellae colonization (35). This is due to the fact that the composition and complexity of the cecal microflora is in a dynamic state for the first 5 to 6 weeks of life (1, 11, 35) until the definitive microbial community is stabilized. Recently, Nisbet et al. (27) found that the administration of a continuous-flow (CF) derived bacterial culture originating from the cecal contents of a mature broiler maintained on a diet containing 5% lactose significantly decreased Salmonella typhimurium colonization in the ceca of broiler chicks and hastened the establishment of the native cecal microflora. The concept of providing naïve chicks with intestinal microflora from Salmonella-free adult chickens to decrease the incidence of salmonellae cecal colonization (28) has been extensively reviewed elsewhere (34, 35, 36) and competitive exclusion by the normal bacterial flora is considered to be the primary method of preventing enteropathogen colonization in the intestinal tract of man and animals (9, 10).

Although adult birds possessing a stable gut microflora are relatively resistant to infection by salmonellae, hens undergoing feed-deprived induced molt are more susceptible to intestinal infection by S. enteritidis and shed more organisms (18, 19). Feed removal may be a critical factor as fasted chickens (17, 21), mice (23, 37, 38) and ruminants (3, 12) exhibit increased levels of Salmonella in normally hostile gastrointestinal environments. Hentges (15) has suggested that drastic dietary changes may disturb the intestinal microflora sufficiently enough that they can no longer impede colonization by pathogenic organisms. In support of this, several researchers have noted that Salmonella spp. and other enteropathogens grow much better in rumen fluid collected from cattle after a 24- or 48-h fast than from recently fed cattle (5, 22, 30). Although nutrient limitation of specific amino acids (Arg, Asp, Ser, Thr) has been suggested as a mechanism by which chicken cecal microbiota competitively inhibit Salmonella spp. (39) in the gastrointestinal tract, it is not known whether such conditions could influence salmonellae survival. The objective of this study is to compare the survivability of Salmonella typhimurium strains and selected facultative chicken cecal bacteria after growth on specific amino acid-limited media.

MATERIALS AND METHODS

Bacterial strains

A primary poultry isolate of Salmonella typhimurium obtained from the National Veterinary Service Laboratory, Ames, IA (Accession # 87-26254) was selected for resistance (41) to novobiocin (NO) and nalidixic acid (NA) and maintained in media containing 25 µg
NO and 25 μg NA per ml. *S. typhimurium* strain LT2 (ATCC 15277) was obtained from the American Type Culture Collection (Rockville, MD). Facultative chicken cecal bacteria examined in this study included *Citrobacter freundii*, *Escherichia coli*, and *E. fergusonii*, all of which were isolated from a continuous-flow culture seeded with chicken cecal contents (26).

### Media and growth methodology

Methods used for bacterial culture and media preparation for anaerobic studies were those of Bryant (4). Nitrogen-free basal anaerobic medium using L-ascorbate (Sigma Chemical Co., St. Louis, MO) as the reductant is shown in Table 1 and was prepared as described by Ricke and Schaefer (31). Nitrogen-free basal aerobic medium was identical in composition to the anaerobic medium except ascorbate, NaHCO₃, and resazurin were excluded. All bacteria were grown at 37°C and growth 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 ln A₆₀₀ versus time were used for estimation of growth rate by linear regression analysis (32). Carbon-limited growth (20 mM glucose, high nitrogen concentration) was experimentally determined for each nitrogen source as the nitrogen concentration at which growth rate no longer responded to an increase in nitrogen concentration. Nitrogen-limited growth (20 mM glucose, low nitrogen concentration) was experimentally determined for each nitrogen source as the nitrogen concentration at which growth rate increased when nitrogen concentration was increased.

### Survivability measurements

Total cell counts were determined as a direct microscopic count using a Petroff-Hauser counting chamber (Hausser Scientific Partnership, Horsham, PA) on diluted samples. Viable cell counts were determined by serial 10-fold dilutions in phosphate buffer (0.31 mM KH₂PO₄ and 0.22 mM NaOH, adjusted to pH 7.2) followed by plating various dilutions on recovery medium (trypsin soy agar) (Difco Laboratories, Detroit, MI). The plates were incubated at 37°C for 24 h aerobically. Viable total cells were counted for a period of at least 5 days after stationary phase from liquid cultures incubated at 37°C during that time period. STₜₒ was defined as the time for 50% of the initial viable population to become nonviable and was calculated from the fractional turnover (m) of the regression line of viable cell number where STₜₒ = ln 2/m (24, 25).

### 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 (8). Colony counts expressed as logarithmic functions were analyzed by least squares mean separations, which were accomplished by 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 considered differences significant at the P < 0.05 level. Viability data, expressed as logarithmic functions, were analyzed by nonlinear regression (least squares).

### RESULTS

#### Comparison of survivability after growth on specific amino acids

To characterize minimal media effects on bacterial survivability, we compared viability of cells after carbon- or nitrogen-limited growth. Bacteria were grown aerobically to stationary phase on minimal media containing 20 mM glucose; the concentrations for the respective nitrogen sources are given in Table 2. Limited growth response for either nitrogen or carbon was experimentally determined for each nitrogen source as the concentration at which growth rate no longer responded to an increase in the corresponding limiting nutrient. Since growth rate and affinity differences were observed among the bacteria (data not shown), the concentrations of the respective nitrogen sources used for survivability determinations were varied to achieve comparable growth responses during exponential growth. When overall nitrogen-limiting conditions were examined (data pooled for all nitrogen sources) only *C. freundii* remained viable significantly longer (P < 0.05) than the other four organisms (data not shown), while after carbon-limited growth conditions, *E. fergusonii* remained viable longer than *C. freundii* and *S. typhimurium* NO/NA but not any of the other bacteria (data not shown).

The STₜₒ values (expressed as hours) for the five microorganisms grown in minimal media containing various nitrogen sources are shown in Table 2. The STₜₒ values for *S. typhimurium* strains were not significantly (P > 0.05) different among various media. *C. freundii* remained viable longer (P < 0.05) after growth on Ser as the nitrogen source than on either NH₄Cl or Arg but not after growth with Asp or Thr as the nitrogen source or Ser as the carbon source. *E. fergusonii* survived longer (P < 0.05) after growth on Ser as the nitrogen source than on either Arg or Asp as the nitrogen source or Ser as the carbon source, but not after growth on NH₄Cl or Thr as the respective nitrogen sources. When compared to the other bacteria, *C. freundii* and *E. fergusonii* survived longer after growth on Ser as the nitrogen source than either *S. typhimurium* NO/NA or *S. typhimurium* LT2, but were not significantly different from each other or from *E. coli*.

#### Effect of anaerobic growth conditions

In order to examine the effect of prior aerobic and anaerobic growth conditions, we chose to compare poultry

### TABLE 1. Composition of growth media

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral salt solution</td>
<td>500 ml/l</td>
</tr>
<tr>
<td>Pfennings’s mineral solution</td>
<td>10 ml/l</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 mM</td>
</tr>
<tr>
<td>Resazurin (0.1%)</td>
<td>1 ml/l</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>3.6 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>30.75 mM</td>
</tr>
<tr>
<td>Nitrogen source</td>
<td>Variable</td>
</tr>
</tbody>
</table>

* Mineral salt solution contained (g/l): 2. K₂SO₄; 27, K₂HPO₄; 9.4, KH₂PO₄; 0.2, MgSO₄·7H₂O; 5, NaCl.
* Pfennings’s mineral solution contained (g/l): 5, EDTA-Na₂; 2, FeSO₄·H₂O; 0.03, H₂BO₂; 0.02, CoCl₂·6H₂O; 0.01, ZnSO₄·7H₂O; 0.003, MnCl₂·4H₂O; 0.003, NaMoO₃·2H₂O; 0.002, NiCl₂·6H₂O; 0.001, CuCl₂·2H₂O.
* Resazurin, ascorbic acid, and NaHCO₃ were used in anaerobic culture media only.
* NH₄Cl, Ser, Arg, Asp, or Thr; when serine was provided as a carbon source, glucose was replaced by serine and NH₄Cl was added as the nitrogen source.
TABLE 2. The mean ST<sub>so</sub> values for five microorganisms aerobically grown in minimal media containing various nitrogen sources

<table>
<thead>
<tr>
<th>Organisms</th>
<th>NH&lt;sub&gt;4&lt;/sub&gt;Cl&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Arg</th>
<th>Asp</th>
<th>Thr</th>
<th>Ser (N)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ser(C)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhimurium NO/NA</td>
<td>62.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>130.90</td>
<td>53.01</td>
<td>122.54</td>
<td>80.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.84</td>
</tr>
<tr>
<td>S. typhimurium LT2</td>
<td>63.35</td>
<td>53.28</td>
<td>62.53</td>
<td>66.05</td>
<td>76.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.63</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>72.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75.52&lt;sup&gt;y&lt;/sup&gt;</td>
<td>115.59&lt;sup&gt;y&lt;/sup&gt;</td>
<td>137.46&lt;sup&gt;x&lt;/sup&gt;</td>
<td>217.44&lt;sup&gt;x&lt;/sup&gt;</td>
<td>178.24&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>E. coli</td>
<td>81.02</td>
<td>106.48</td>
<td>90.16</td>
<td>146.54</td>
<td>164.61&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>102.56</td>
</tr>
<tr>
<td>E. fergusonii</td>
<td>121.91&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>92.25&lt;sup&gt;y&lt;/sup&gt;</td>
<td>83.17&lt;sup&gt;y&lt;/sup&gt;</td>
<td>121.28&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>233.69&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>79.55&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentrations of nitrogen sources used: Asp, 0.1 and 5 mM; NH<sub>4</sub>Cl, 0.05 and 5 mM; Arg, (Salmonella spp., 0.05 and 5 mM; cecal bacteria, 0.05 and 20 mM); Ser as a nitrogen source, 0.1 and 10 mM for all bacteria except S. typhimurium NO/NA (0.1 and 10 mM); Ser as a carbon source, 1 and 50 mM; Thr, Salmonella spp., 0.1 and 10 mM, cecal bacteria, 100 and 200 mM.

<sup>b</sup> Ser was used as a nitrogen (N) or carbon (C) source.

<sup>c</sup> Means in the same column not followed by common letters differ significantly (P<0.05): A, B.

<sup>d</sup> Means in the same row not followed by common letters differ significantly (P<0.05): x, y.

The mean ST<sub>so</sub> values for the two organisms after experiencing anaerobic starvation were not (P>0.05) different after aerobic culture were significantly (P<0.05) higher than when the organism was grown under anaerobic conditions. The ST<sub>so</sub> values for S. typhimurium NO/NA undergoing either aerobic or anaerobic starvation were not significantly (P>0.05) different. Even though the ST<sub>so</sub> value for E. fergusonii was significantly (P<0.05) higher than that of S. typhimurium NO/NA under aerobic starvation conditions, ST<sub>so</sub> values for the two bacteria were not significantly (P>0.05) different after experiencing anaerobic starvation.

TABLE 3. The mean ST<sub>so</sub> values for S. typhimurium NO/NA and E. fergusonii grown in aerobic or anaerobic batch minimal media

<table>
<thead>
<tr>
<th>Organisms</th>
<th>ST&lt;sub&gt;so&lt;/sub&gt; (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>S. typhimurium NO/NA</td>
<td>67.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>E. fergusonii</td>
<td>146.26&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Pooled standard error of the mean = 21.76. Each mean is the average of 6 samples.

<sup>b</sup> Means in the same column not followed by common letters differ significantly (P<0.05): A, B.

<sup>c</sup> Means in the same row not followed by common letters differ significantly (P<0.05): x, y.

**DISCUSSION**

This study was designed to test whether survivability of S. typhimurium and facultative chicken cecal bacteria are influenced differently by growth-limiting minimal nutrient conditions. We chose to work with these particular facultative as representative cecal organisms for several reasons. First of all, they are part of the 11 bacterial isolates previously identified from a defined continuous-flow culture that had been shown to be protective against S. typhimurium cecal colonization in broiler chicks (26). Furthermore, given the extremes of aerobic and anaerobic environments encountered, facultative organisms are more likely to survive horizontal transfer in a poultry house. Finally, for the purpose of our studies here, the metabolic similarities of these organisms to Salmonella spp. made it much easier to do direct comparative in vitro studies for assessing survivability under specific nutrient limiting conditions.

Microbial growth in the environment is influenced by nutrient concentration, and ecosystems are typically devoid of one or more essential nutrients (14). We focused on four amino acids (Asp, Arg, Ser, and Thr) as the specific nutrient limitations because Ushijima and Seto (39) demonstrated that when as few as five different human fecal isolates were cocultivated with S. typhimurium in continuous culture, a decrease in the S. typhimurium population was observed specifically associated with these amino acids. In earlier work, we have suggested that serine utilization may be a potential competition mechanism between S. typhimurium and E. fergusonii that could be predicted by growth kinetics (13). In this study, the longer survivabilities for two of the three cecal facultative bacteria grown under aerobic serine nitrogen-limited conditions when compared to S. typhimurium indicates that serine limitation may also influence the makeup of the microbial consortium outside the gut. Since serine originating from the gut mucoproteins might be expected to be always present in the feces regardless of diet (2, 29, 33), then the organisms with the best capabilities of survival under these conditions would also be most likely to remain viable after excretion from the bird.

It has been suggested (16) that a portion of the indigenous cecal population may be much less resilient to the sudden removal of feed to induce molt and thus lead to a cecal ecosystem that is more vulnerable to opportunistic invaders such as Salmonella spp. Although it is not known which component of the chicken cecal population might be most subject to alteration during molting, Dawes (7) has suggested that "unbalanced growth" due to nutrient limitation may in fact lead to formation of endogenous reserve materials that

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sustain viability of bacteria during starvation. Strict anaerobes such as rumen bacteria have poor survivability capabilities when subjected to limiting nutrient conditions (24, 25, 40), but Tannock and Savage (37) observed that total anaerobic counts in the gut of starved mice remained constant while numbers of facultative organisms fluctuated greatly. Our data suggests that the ability to sustain viability may vary among facultatives limited by the same nutrient after aerobic growth. However, based on the comparison between *E. fergusonii* and *S. typhimurium* NO/NA (Table 3), this may not hold true for anaerobic growth conditions. Therefore, horizontal transfer and successful colonization by *Salmonella* spp. in molting adult birds may be the result of a higher susceptibility to *Salmonella* spp. colonization because of a selective loss of one or more of the indigenous facultative organisms. It has been noted (6) that cecal bacteria from a CF culture obtained from adult chickens can rapidly become established in the ceca of naive chicks following contact with the vent lips, and subsequently increase chick resistance to *S. enteritidis*. Based on our observations in this study, the CF culture facultatives in the ceca that appear to possess better aerobic survival traits would more likely remain viable longer in the poultry house environment after excretion from the bird. More importantly, the ability of individual facultative members of this protective consortium to remain viable longer under such conditions might also enhance the likelihood of reinoculation of birds by these same microflora.

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**REFERENCES**