Effect of 2-Nitrofluorene, 1,2-Dimethylhydrazine, and Azoxymethane on *Salmonella typhimurium* Mutants in the Gastrointestinal Tract of Gnotobiotic Rats

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ABSTRACT

Gnotobiotic Sprague-Dawley rats, associated with *Salmonella typhimurium* strain TA1538, may also be associated with either *Lactobacillus plantarum* or *Bacteroides vulgatus* as well as with both of these strains. After the ingestion of 2-nitrofluorene (3.4 mg), the fecal concentration of his* revertants is greatly elevated in all groups of rats except those associated with strain TA1538 and *B. vulgatus*. The concentrations of the various bacteria were measured at eight sites in the gastrointestinal tract. *B. vulgatus* achieved high concentrations in the stomach when it was associated with strain TA1538, but its concentration was not as high when it was associated with *L. plantarum* together with strain TA1538. The concentration of *B. vulgatus* in individual rats correlated negatively with their response to 2-nitrofluorene. *B. vulgatus* readily reduces 2-nitrofluorene to 2-aminofluorene, a reaction which is negligible in cultures of *L. plantarum* and strain TA1538. Since 2-aminofluorene is less mutagenic than 2-nitrofluorene, *B. vulgatus* appears to diminish the revertant response by removing the more potent mutagen from within the gastrointestinal tract.

Other Ames *Salmonella* tester strains (TA1535, TA100, and TA98) can also be maintained in association with otherwise germ-free rats. The feces of animals associated with strains TA1535 and TA100, however, show a variable increase in the concentration of his* revertants in response to the ingestion of experimental colon carcinogens, e.g., 1,2-dimethylhydrazine (21 mg/kg) and azoxymethane (19 mg/kg).

INTRODUCTION

An association of the histidine auxotroph of *Salmonella typhimurium* (strain TA1538) can be maintained for several months within the gastrointestinal tract of otherwise germ-free rats. The bacteria achieve concentrations of greater than $10^7$/g of contents in the forestomach and greater than $10^9$/g of contents of the lower bowel and in the feces. Carcinogens which cause strain TA1538 to revert to histidine independence in the *in vitro* assays developed by Ames will, when ingested, cause an increased concentration of histidine-independent revertants to appear in the feces. In contrast, the number of revertants in the feces is not increased by the ingestion of structurally related compounds which are not mutagenic to the bacteria *in vitro* and for which no evidence of carcinogenicity exists.

This host-mediated assay offers a possible means of studying the relationship of the bacterial flora to the formation of reactive intermediates that may be related to gastrointestinal carcinogenesis.

After the ingestion of the gastrointestinal carcinogen 2-nitrofluorene (4), the revertant response of strain TA1538 in the feces is ordinarily quite striking. The response is considerably diminished, however, when 2 constituents of the normal rat flora, *Lactobacillus plantarum* and *Bacteroides vulgatus*, are associated in addition to strain TA1538 (5). The diminished revertant response might be explained by certain characteristics of these bacteria. For example, *B. vulgatus* is capable of reducing 2-nitrofluorene to 2-aminofluorene (5) and thus might diminish the response by decreasing the exposure of the *Salmonella* tester strain to the more potent mutagen. On the other hand, colonization of various sites within the gastrointestinal tract by either *B. vulgatus* or *L. plantarum* might displace strain TA1538 from the site(s) where mutagenesis occurs. These possibilities suggest that an examination of the response to 2-nitrofluorene in the presence of only one of the 2 additional bacteria may elucidate the mechanism of the blunted revertant response.

This paper reports studies on the effect of these additional bacteria on the response of the strain TA1538-associated rat to 2-nitrofluorene. In addition, studies are reported of the use of other Ames *Salmonella* tester strains in this host-mediated assay to detect mutagenic activity with experimental colon carcinogens, such as 1,2-dimethylhydrazine and azoxymethane.

MATERIALS AND METHODS

**Animals and Microbial Methods.** Germ-free Sprague-Dawley rats (45 to 47 days old) and steam-sterilized rat-mouse 7RF diet were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). Germ-free rats were associated with *S. typhimurium* strains TA1538, TA98, or TA100 (obtained from Dr. Bruce Ames, University of California, Berkeley, Calif.) and were housed in stainless steel metabolism cages maintained in a sterile, isolated environment (6).

Germ-free rats were associated with strain TA1538 and 14 days later with either *L. plantarum* (V.P.I. 0516) or *B. vulgatus* (V.P.I. 9520), at which time the concentration of revertants in the feces of the animals was less than 200 revertants/g. Some animals associated with strain TA1538 and *L. plantarum* were also associated with *B. vulgatus* 8 days after their prior expo-
sure to *L. plantarum* (5). Samples of feces were collected daily, and both the his− mutants and the his+ revertants were enumerated on the day of collection (6). The feces of gnotobiotic animals were also assayed daily on selective media (8) to enumerate *L. plantarum* and *B. vulgatus*. Fourteen days after the ingestion of the carcinogen, the rats were sacrificed, and the bacteria were enumerated at various sites in the gastrointestinal tract (6, 8).

**Feeding of Chemicals.** 2-Nitrofluorene (Eastman Organic Chemical Co., Rochester, N. Y.) was dissolved in acetone and sterilized by filtration through a 0.2-μm Fluoropore filter (Millipore Filter Corp., Bedford, Mass.) before it was added to the germ-free diet. The acetone was permitted to evaporate for 1 hr before the mixture was given to the animals. 1,2-Dimethylhydrazine dihydrochloride (Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in aqueous 1 mM EDTA and neutralized to pH 6.8 with NaOH prior to dilution to the specified concentration. This solution and aqueous solutions of azoxymethane (Ash Stevens, Inc., Detroit, Mich.) and methyl(acetoxy-methyl)nitrosamine (a gift of Dr. P. P. Roller, Carcinogen Metabolism and Toxicology Branch, National Cancer Institute, Bethesda, Md.) were filtered through a 0.2-μm FHLP filter (Millipore) before passage into the isolators. Samples (1.0 ml) of solutions of dimethylhydrazine, azoxymethane, and methyl(acetoxy-methyl)nitrosamine were administered by gastric intubation. This procedure was greatly facilitated by retarding the reactions of the rats by exposing them to Fluothane vapor (Ayerst Labs, Inc., New York, N. Y.) for approximately 10 sec prior to intubation.

Statistical comparison of means was based on Student’s *t* test. The correlations were evaluated using the Pearson product-moment test.

**RESULTS**

The Effect of Additional Bacteria on the Mutagenic Response of *S. typhimurium* TA1538 in Rats Ingesting 2-Nitrofluorene. When gnotobiotic rats previously associated with *Salmonella* strain TA1538 were additionally associated with either *L. plantarum* or *B. vulgatus*, the fecal concentration of *Salmonella* remained between $10^8$ and $10^{10}$/g, as it had in the presence of both strains or in their absence (5). Both *L. plantarum* and *B. vulgatus* achieved concentrations in the feces of between $10^8$ and $10^{10}$/g.

Each group of gnotobiotic rats, except those associated with strain TA1538 and *B. vulgatus*, showed a significant increase in the fecal concentration of his+ revertants after the ingestion of a single dose of 3.4 mg of 2-nitrofluorene (Chart 1). Even after a larger challenge with 2-nitrofluorene (34.0 mg, administered 8 days later), the rats associated with strain TA1538 and *B. vulgatus* showed only a weak response, in no case greater than 1400 revertants/g of feces. Previous studies had shown that the higher dose of 2-nitrofluorene was capable of provoking a substantial revertant response in animals associated with *L. plantarum* in addition to *B. vulgatus* and strain TA1538 (5).

One explanation for the lack of a response in these animals is that *B. vulgatus* metabolizes 2-nitrofluorene and thus decreases the exposure of the *Salmonella* tester strain to the mutagen. *In vitro* experiments show that *B. vulgatus*, but not strain TA1538 or *L. plantarum*, can reduce 2-nitrofluorene to 2-amino-5-fluorene (5). Chart 2 compares the response of strain TA1538 to 2-nitrofluorene and 2-amino-5-fluorene. Only with 2-amino-5-fluorene is mutagenic activity dependent upon the presence of a liver microsomal preparation. This observation suggests that in the gastrointestinal tract *B. vulgatus* converts 2-nitrofluorene to 2-amino-5-fluorene which, at this site, is less mutagenic, resulting in a decreased revertant response of strain TA1538 *in vivo*.

This hypothesis, however, does not explain why those animals associated with *L. plantarum* in addition to *B. vulgatus* and strain TA1538 show a high revertant response. A possible explanation is that *L. plantarum* establishes itself within the gastrointestinal tract in a way that displaces *B. vulgatus* from the location(s) where it metabolizes 2-nitrofluorene to the less potent 2-amino-5-fluorene. This possibility was examined by determining the distribution of *L. plantarum* and *B. vulgatus* at

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various levels of the gastrointestinal tract of sacrificed animals with different bacterial associations (Chart 3). Only in the stomach was the concentration of *B. vulgatus* significantly higher in unresponsive rats (associated with strain TA1538 and *B. vulgatus*) than in the responsive ones (additionally associated with *L. plantarum*) ($p < 0.01$). The concentrations of *B. vulgatus* in the stomachs of individual rats were examined to see if they correlated with the revertant response that had earlier been observed in the same rats. Chart 4 shows the existence of a significant inverse correlation (coefficient of correlation, $r = -0.69$; $p < 0.05$).

The concentrations of *B. vulgatus* in the cecum and colon were also examined to see if these concentrations correlated with the revertant response. Neither rats associated with strain TA1538 and *B. vulgatus* nor those additionally associated with *L. plantarum* showed such a correlation. This correlation may not have been revealed under these circumstances, however, because rats were sacrificed 8 days after the revertant response had been observed. A day-to-day variation is found in the concentration of *B. vulgatus* in the feces, and such variation, if it occurred in the colon, might obscure the relationship.

A second possible explanation for the effect of *B. vulgatus* on the revertant response is that this strain displaces strain TA1538 from the location in the gastrointestinal tract where contact between mutagen and tester strain makes possible the reverse mutational response. If this were the case, then it might be reflected in a lowered concentration of strain TA1538 in the feces of rats associated with *B. vulgatus* in comparison with that of other rats. As indicated in Table 1, however, there is no difference in the fecal concentration of strain TA1538 among the various groups of rats after the ingestion of 2-nitrofluorene. The possibility that strain TA1538 was displaced by *B. vulgatus* was also examined by comparing the salmonella concentrations at various levels of the gastrointestinal tract in the different groups of rats which were sacrificed 14 days after the ingestion of 2-nitrofluorene (Chart 5). At no site above the colon does the association with *B. vulgatus* alone cause a significant depression of the concentration of strain TA1538. In the colon, however, the concentration of strain TA1538 is lowest in the animals associated with strain TA1538 and *B. vulgatus* ($p < 0.01$ compared to the animals associated only with strain TA1538 or additionally with *B. vulgatus* and *L. plantarum*, but $p > 0.5$ compared to the animals associated with strain TA1538 and *L. plantarum*). Even when statistically significant, this decreased concentration of strain TA1538 does not appear to be of sufficient magnitude to account for the approximately 10- to 100-fold decrease that had been observed earlier in the revertant response.

### Table 1

<table>
<thead>
<tr>
<th>Bacteria associated</th>
<th>Salmonella strain TA1538 (log$_{10}$/g feces)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA1538</td>
<td>8.24 ± 0.81$^*$</td>
</tr>
<tr>
<td>TA1538 + <em>L. plantarum</em></td>
<td>8.45 ± 0.44</td>
</tr>
<tr>
<td>TA1538 + <em>L. plantarum</em> + <em>B. vulgatus</em></td>
<td>8.20 ± 0.47</td>
</tr>
<tr>
<td>TA1538 + <em>B. vulgatus</em></td>
<td>8.18 ± 0.12</td>
</tr>
</tbody>
</table>

$^*$ Mean values from 6 rats in each group ± S.D.

Chart 3. The concentration of *L. plantarum* or *B. vulgatus* in the gastrointestinal tract of germ-free rats associated additionally with strain TA1538, strain TA1538 and *L. plantarum*, or strain TA1538 and *B. vulgatus*. The values presented are the mean ± S.D. for 6 animals in each group. The locations in the gastrointestinal tract are: A, forestomach; B, stomach; C, duodenum; D, E, and F, various levels in the small intestine; G, cecum; and H, colon.

Chart 4. The relationship between the highest concentration of his$^+$ revertants observed during the 4-day period after ingestion of a single dose of 3.4 mg of 2-nitrofluorene and the concentration at sacrifice of *B. vulgatus* in stomach contents. Six rats were associated with strain TA1538 and *B. vulgatus* (○), and 4 rats were associated with strain TA1538, *B. vulgatus*, and *L. plantarum* (○).
Furthermore, if the action of *B. vulgatus* were the result of its displacement of strain TA1538 from the colon, then a correlation might be expected between the concentration of strain TA1538 in the colon of any animal and the revertant response in that animal. A correlation of this kind was not observed for animals additionally associated with *B. vulgatus*. Of course, the interpretation of this result is subject to the possibility that a correlation might be obscured by variations in the bacterial concentrations that occur in the interval between the ingestion of the carcinogen and the time that the rats are sacrificed.

The Association of Germ-free Rats with *Salmonella* Strains TA1535, TA100, and TA98 and the Response of These Strains to the Feeding of Various Colon Carcinogens. Ames *Salmonella* tester strains TA1535, TA100, and TA98 were each found to associate with otherwise germ-free rats and to reach concentrations throughout the gastrointestinal tract and in the feces comparable to those observed previously for rats associated with strain TA1538.

In rats associated with strain TA1535, the revertant response following the administration of 2-nitrofluorene and the experimental colon carcinogens 1,2-dimethylhydrazine and azoxymethane is shown in Chart 6. No response was observed after the ingestion of 2-nitrofluorene, and the responses to 1,2-dimethylhydrazine and azoxymethane were variable. The experiment conducted initially showed an increased revertant response after each ingestion of 1,2-dimethylhydrazine. However, this result was not reproducible in a second group of rats associated with strain TA1535 as indicated in Chart 6.

The response to these carcinogens in 3 different groups of rats associated with strain TA100 is presented in Chart 7. The fairly uniform response of the first group of rats to 1,2-dimethylhydrazine and azoxymethane was not confirmed, however, in a second group of rats associated with strain TA100. A third group of rats appeared to respond to the colon carcinogen methyl(acetoxymethyl)nitrosamine.

When 6 rats were associated with strain TA98, the fecal concentration of his* revertants remained above 1000/g, the highest concentration of revertants observed with any *Salmo-

![Chart 6. The concentration of his* revertants in the feces of rats associated with strain TA1535 in response to the ingestion of various carcinogens. One group of 6 rats (B) was given 3.4 mg of 2-nitrofluorene (2-NF), followed by 3 single doses of 1,2-dimethylhydrazine (21 mg/kg) at 2 weeks (DMH-1), 5 weeks (DMH-2), and 8 weeks (DMH-3) after the initial ingestion of 2-nitrofluorene. Azoxymethane (AOM; 18 mg/kg) was administered 3 weeks after the final dose of 1,2-dimethylhydrazine. A second group of 6 rats (C) was given 2 single doses of 1,2-dimethylhydrazine (21 mg/kg) separated by a 2-week interval and 3 weeks after the second dose received a single dose of azoxymethane (18 mg/kg). Plotted are the greatest concentrations of his* revertants in the feces collected during 24-hr intervals for the 4 days following ingestion of the carcinogen. The number of revertants in a fecal sample taken 1 day prior to the feeding is the control (CONT.).](https://cancerres.aacrjournals.org/content/29/6/1013)
feces undergoes an increase. This increase is greatly diminished, however, when the rats are associated with \textit{B. vulgatus} in addition to strain TA1538. When the association also includes \textit{L. plantarum}, the revertant response to 2-nitrofluorene is indistinguishable from that when only strain TA1538 is associated. The explanation for these observations apparently rests with the ability of \textit{B. vulgatus} to reduce the more mutagenic 2-nitrofluorene to the less mutagenic 2-aminofluorene (5), a metabolic reaction that is negligible with either strain TA1538 or \textit{L. plantarum}. An additional observation, that \textit{L. plantarum} tends to displace \textit{B. vulgatus} from the stomach, accounts for the finding that the revertant response is normal when both \textit{L. plantarum} and \textit{B. vulgatus} are associated with strain TA1538. The distribution of the various bacteria in the stomach and the capacity of the various bacteria to reduce 2-nitrofluorene to 2-aminofluorene are compatible with the view that \textit{B. vulgatus} decreases the revertant response by metabolizing 2-nitrofluorene to the less potent mutagen, 2-aminofluorene. Furthermore, the presence of \textit{B. vulgatus} (\textit{B. fragilis} ssp. \textit{vulgatus}) within the gastrointestinal tract should also blunt mutagenesis by 2-aminofluorene if it is formed in vivo, since \textit{B. vulgatus} reduces the \textit{N}-hydroxy derivatives (7) which are intermediates in the activation of this kind of carcinogen by the liver (3).

The site of mutagenesis in the gastrointestinal tract is still unclear. The lag of at least 24 hr between the ingestion of 2-nitrofluorene and the appearance of revertants in the feces is compatible with the time required for revertants formed in the stomach to traverse the gastrointestinal tract. The occurrence of mutagenesis in the stomach is also consistent with the observation that ingested mutagens, which do not require mammalian enzyme activation, \textit{e.g.}, 2-nitrofluorene, are more potent in provoking the fecal revertant response than those that require mammalian activation, \textit{e.g.}, 2-acetylaminofluorene.\textsuperscript{4} The occurrence of mutagenesis in the stomach or elsewhere in the upper bowel as a result of the ingestion of 2-nitrofluorene is also compatible with the observation that 2-nitrofluorene is a carcinogen for the upper but not the lower bowel.

Base-pair mutants such as strain TA100 and TA1535 are like strain TA1538 in having the capacity to associate with the germ-free rat. Unlike strain TA1538, however, these strains may be responsive to colon carcinogens such as azoxy-methane and 1,2-dimethylhydrazine. Unfortunately, the response to these carcinogens is of small magnitude and variable. It remains to be seen whether these strains can provide a response which is reliable enough to use as an approach to studying mutagenesis in response to these colon carcinogens.

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REFERENCES


\textsuperscript{4} Unpublished observations.
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