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Original Article

Growth suppression of antibiotic-resistant *Salmonella typhimurium* DT104 by a non-DT104 strain in vitro

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Abstract:
Growth suppression of antibiotic-resistant *Salmonella typhimurium* DT104 by a non-DT104 strain was investigated in vitro. Chromosomal mutants of eight antibiotic-resistant DT104 strains were generated by sub-culturing on desoxycholate hydrogen sulfide lactose agar containing 25 µg/ml of nalidixic acid. Low counts of each of these mutants (designated as "minority cultures") were inoculated into 24-h cultures of a non-DT104 *S. typhimurium* strain (designated as "majority culture") to test the ability of the majority culture to suppress the multiplication of the minority culture. Multiplication of small numbers of the antibiotic-resistant DT104 strains was significantly ($P < 0.05$) prevented when the DT104s were added to 24-h brain heart infusion cultures of the non-DT104 strain. This observation has practical implications for the control of the menacing antibiotic-resistant *Salmonella typhimurium* DT104.
Key Words: *Salmonella typhimurium* DT104; non-DT104; growth suppression.

Introduction:
The present interest in bacterial competition arose out of previous reports that intestinal colonization of newly hatched chickens by a *Salmonella* strain prevented colonization by a second *Salmonella* strain inoculated 24 h later.(1,2) The inhibition was specific and 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 *Salmonella enterica* serovar Typhimurium (*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 the growth of the *S. typhimurium* Nal^r mutant was inhibited by *Salmonella* cultures but not by those of other genera (1); and that the effect was directly related to the high bacterial density.(3)

Most studies on bacterial suppression have been done with *S. typhimurium*. This serotype is a useful model since it remains the most frequently isolated serotype of *Salmonella* in human, swine, avian, and bovine salmonellosis.(4,5) Since the early 1990s, an emergent strain *S. typhimurium* DT104 notable for its multiple antibiotic resistance and wide host range has been increasingly isolated.(6-9) In addition, this strain is thought to be more virulent than the other Typhimurium strains.(10,11) Thus, there is urgent need to investigate into new ways to contain this strain. In this study, we applied the principle of bacterial competition to study the ability of stationary phase cultures of a non-DT104 strain to suppress the growth of clinical DT104s strains inoculated into it as nalidixic acid-resistant mutants. To our knowledge, no similar study with DT104 has been reported before now.

Materials and Methods:

Bacterial isolates

Eight antibiotic-resistant clinical isolates of the *S. typhimurium* DT104 from human and cattle sources were used in this study. The human strains were isolated in the United States while the cattle isolates of DT104 and the non-DT104 (*S. typhimurium* L1388) chicken strain were isolated in Japan. Both strain types were kindly provided by Dr. Kazumitsu TAMURA (formerly of the National Institute of Infectious Disease, 1-23-1, Toyama, Shinjuku, Tokyo, 102-8640, Japan). Relevant characteristics of the isolates are as shown in Table 1. Spontaneous nalidixic acid-resistant (Nal^r) mutants were produced by a method described previously.(12) Previous studies indicated that this mutation had no

effect on intestinal colonization or on in vitro or in vivo inhibition of multiplication.(1,3,13)

Culture media

All broth cultures were propagated for 24 h in 5-ml volumes of brain heart infusion broth (BHIB: BBL, U.S.A.) in a shaking (65 rpm) water-bath maintained at 37ºC. Viable bacterial counts were made by plating decimal dilutions on desoxycholate hydrogen sulfide lactose agar (DHL: Nissui Co. Ltd., Japan) containing nalidixic acid (25 µg ml^{-1}). Nalidixic acid was purchased from Wako Chemical Company, Japan.

Growth Suppression Assay

Inter-phage growth suppression of DT104 strains was tested as described previously by Nogrady et al.(14) with certain modifications. Stationary BHIB cultures of a non-DT104 strain (designated "majority culture", because higher inoculum size of it was used in the study) were inoculated with stationary BHIB culture of DT104 strains diluted in normal saline (designated "minority culture", because lower inoculum size of it was used in the study). The minority culture possessed a different antibiotic resistance marker from the experimental strain. Growth suppression ability of the investigated (majority) non-DT104 culture was tested by determining the growth of the (minority) DT104 strains 1 day after inoculation, using serial dilutions and plating onto the agar plates containing the appropriate antibiotic. Briefly, chromosomal nalidixic acid-resistant (Nal^r) mutants of DT104s were generated by sub-culturing on DHL agar containing 25 µg/ml of nalidixic acid. Each of these mutants, designated as "minority cultures", and the investigated ("majority culture") non-DT104 culture were separately grown in BHIB (37ºC, shaking at 65 rpm) for 24 h. The colony forming units (CFU) per ml of both the minority and majority cultures were determined after appropriate dilution of 0.5-ml samples in normal saline and 24-h incubation at 37ºC on DHL- 25 µg ml^{-1} Nalidixic acid and antibiotic-free DHL agar respectively. 0.5 ml of diluted ($1:10^5$) minority culture was mixed with 4.5 ml of the majority culture and re-incubated for 24 h at 37ºC with shaking. The CFU of the minority culture was determined after appropriate dilution of 0.5-ml samples of the mixed culture in normal saline and 24-h incubation at 37ºC on DHL-25 µg ml^{-1} Nalidixic acid.

Statistical Analysis

Data were analyzed by the one-way analysis of variance (ANOVA) using Smith's Statistical Package Version 2.5 (Gary Smith, August 30, 2001, Pomona College, Clairemont, California, U. S. A. Significance of results was determined at the 5 % probability level (that is, at $P = 0.05$)

Results:

Suppression of growth by 24-h BHIB broth culture of non-DT104

Multiplication of small numbers of the antibiotic-resistant DT104s was significantly prevented when the DT104s were added to 24-h brain heart infusion cultures of the non-DT104 strain.

Table 1: Sensitivity of test strains to common antibiotics

Strains	Source	Country of isolation	Minimum inhibitory concentration (MIC) in µg ml^{-1}										Resistance
			A	T	C	F	G	K	S	Su	TMP	NA	
306-98	Human	CDC (U.S.A)	>512	128	256	64	1	2	512	>512	0.5	4	ACFSSuT
T980018	Human	CDC (U.S.A)	>512	64	256	64	0.5	2	256	>512	0.5	4	ACFSSuT
T980021	Human	CDC (U.S.A)	>512	256	256	128	1	2	>512	>512	1	4	ACFSSuT
T980042	Human	CDC (U.S.A)	>512	64	256	64	1	2	128	>512	0.5	4	ACFSSuT
T980043	Human	CDC (U.S.A)	>512	128	256	64	1	2	256	>512	0.5	4	ACFSSuT
ST3	Cattle	Japan	1	2	8	8	0.5	2	128	>512	0.5	4	SSu
ST4	Cattle	Japan	>512	128	256	64	1	2	256	>512	0.5	4	ACFSSuT
ST41	Cattle	Japan	1	2	8	8	0.5	2	256	>512	0.5	4	SSu
ST L1388	Chicken	Japan	1	2	8	8	0.5	2	32	64	0.5	4	SSu

A, ampicillin; T, tetracycline; C, chloramphenicol; F, florfenicol; G, gentamicin; K, kanamycin; S, streptomycin; Su, sulfamethoxazole; TMP, trimethoprim; and NA, nalidixic acid. ST L1388, *Salmonella typhimurium* L1388. MIC was determined as described by the 1996 guidelines of National Committee for Clinical Laboratory Standards, USA.

Table 2: Growth suppression of *Salmonella typhimurium* DT104 strains by 24-h cultures of a non-DT104 strain

DT104 isolates (Minority Culture)	Bacterial CFU ml^{-1} relative to mixing (± SD)		Statistics (ANOVA)
	Before (x 10^{9})	*After (x 10^{5})*	
306-98	2.10 ± 0.54	2.00 ± 0.00	P = 0.0012
T980018	1.73 ± 0.48	16.00 ± 5.00	P = 0.0086
T980021	1.70 ± 0.26	3.00 ± 2.00	P = 0.0033
T980042	2.50 ± 1.08	6.00 ± 5.00	P = 0.0161
T980043	2.08 ± 0.64	5.00 ± 2.00	P = 0.0028
ST3	1.45 ± 0.35	1.00 ± 0.00	P = 0.0285
ST4	1.85 ± 0.25	1.00 ± 0.00	P = 0.00006
ST41	2.20 ± 0.37	2.00 ± 0.00	P = 0.0014

Discussion:

The competitive behavior of bacterial populations even within the same genera is poorly understood. It has been proposed that microbial competition is responsible for the predominance of *S. enterica* serovar Enteritidis over *S. enterica* serovar Gallinarum in Europe.(15) However, that competition is unlikely to be related directly to the one described in this study which requires high bacterial densities to be present in the gut. Although well studied, the basis of this mechanism of competition is also not understood.

When the suppression of DT104s by a non-DT104 was studied, we observed that the multiplication of small numbers of the antibiotic-resistant DT104s was significantly prevented when the DT104s were added to 24-h brain heart infusion cultures of the non-DT104 strain. This observation is not new with bacteria as reduction in the rate of bacterial multiplication in broth cultures toward the end of logarithmic phase has been documented for many years.(16-18) However, the interesting thing about our observation with DT104 is that of the ability of a high density (majority) culture of antibiotic-sensitive non-DT104 strain to suppress growth of the low density (minority) culture of multi-drug resistant DT104.

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.(2,19,20) 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. The method can also be applicable for non-isogenic mutants as shown by our study.

Although the present study did not investigate the factors responsible for the growth suppression observed in the 24-h cultures of non-DT104, results of several experiments with bacteria have indicated high cell density of the inhibiting strain(3), nutrient shortage(21), low pH resulting from fermentation of carbohydrates(22), reduced oxygen concentration(23), and presence of a diffusible but labile chemical mediator (17) as possible factors.

Conclusion:

In conclusion, the multiplication of small numbers of the antibiotic-resistant DT104s significantly prevented when the DT104s were added to 24-h brain heart infusion cultures of the non-DT104 strain deserves to be re-examined using a larger number of both DT104 and non-DT104 isolates. In addition, the specific factor responsible for the growth suppression will need to be further investigated. A favorable outcome, as observed from the results of this study, will find relevance in the design of a control measure

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