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Salmonella Toxin Synthesis Is Unrelated to the Presence of Temperate Bacteriophages

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Several strains of *Salmonella* were examined for an association between the capacity to produce *Salmonella* toxin and the presence of bacteriophages. Based on the data obtained from this study, we concluded that genetic information responsible for *Salmonella* toxin synthesis was not inherited by lysogenic conversion.

The specific type of genetic information that codes for the synthesis of *Salmonella* toxin (9, 11, 15, 16) is unknown. Bacterial genetic information may be expressed by bacteriophage conversion, plasmids, or chromosomal DNA. Since several genera of bacteria can be lysogenic for bacteriophages that carry genes for various types of toxins (2, 5, 6, 18, 20), we have attempted to determine whether *Salmonella* bacteriophages (18) carry the genetic information for the synthesis of *Salmonella* toxin. Based on the results in this report, we have eliminated lysogenic conversion as a possible means for expressing the toxin gene(s).

Salmonella typhimurium strain Q1 was supplied by P. Gemski, Walter Reed Army Medical Center. The suppliers of the other *Salmonella* strains are listed in a previous publication (14). A filtrate and sonic extract of each strain were prepared as described previously (9).

The method of Hershey et al. (8) was used to detect and quantitate the presence of mitomycin C (MTC)-induced bacteriophage in cell-free culture filtrates of several strains of *Salmonella*. Bacteriophage, in 15-μl droplets, was stained with 2% uranyl acetate and examined by electron microscopy by previously described methods (1).

A modification of the CHO floating cell assay devised by Nozawa et al. (13) was used in this study (9, 14). Quantitation of *Salmonella* toxin antigen was accomplished by the use of the ELISA, employing specifically purified cholera antitoxin (9, 14), since *Salmonella* toxin has antigenic determinants similar to those of cholera toxin (15–17).

Bacteriophage was induced from lysogenic strains of *Salmonella* and new lysogens were constructed by infecting *Salmonella* strains that appeared to be free of bacteriophages. Bacteriophages were MTC-induced from donor *Salmonella* strains SR11, M206, SL1027, and 986. *Salmonella* strains 2000 and Q1 mixed with a soft agar overlay were used as "phage-free" host lawns onto which drops of a cell-free phage preparation of each donor were placed. After an overnight incubation at 37°C, portions of turbid plaques were harvested with a sterile wire loop and streaked onto Casamino Acids yeast extract (CYE) agar plates. The inoculated plates were incubated overnight at 37°C. Two CYE agar plates, one with a host strain of *Salmonella* in soft agar overlay and the other without, were placed on grid templates containing 24 square areas. A total of 24 selected isolated colonies from CYE agar streak plates were each inoculated with sterile toothpicks onto a corresponding square area of the plain CYE agar plates followed by inoculation of the CYE agar plates containing host strains of *Salmonella*. The plates were incubated at 37°C for 24 h before examination. Clearing around the colonies on plates containing the host lawn indicated phage lysis, and, therefore, the formation of a new lysogen. The duplicate culture grown on the plain CYE agar plate was used as the donor for two repeat experiments. Five newly constructed lysogens were grown in CYE broth for 24 h at 37°C, and the filtrates and sonic extracts of each lysogen were assayed by the ELISA.

As an indicator of growth, a 5-ml sample of broth cultures from each *Salmonella* strain was measured for turbidity with a Klett-Summerson colorimeter with a number 54 filter. Duplicate 1,000-ml screw cap side-arm flasks, each containing 100 ml of CYE broth, were labeled in the following way: uninoculated control, uninoculated control (MTC), Q1, Q1 (MTC), TML-R66, and TML-R66 (MTC). Growth conditions were as previously described (14).

Data from a phage donor host survey demonstrated that MTC does not induce bacteriophages from all donor *Salmonella* strains. Of the 20 *Salmonella* strains utilized in this study, 55% of the MTC-induced *Salmonella* donors released bacteriophage capable of causing lysis on 40% of

the host strains. These MTC-resistant donor strains may harbor defective bacteriophages that were incapable of infecting other *Salmonella* strains. Another possibility may be that the bacteriophages associated with these MTC-resistant strains had a host range that excluded all of the host strains used in this study. Also, the possibility exists that these MTC-resistant strains did not harbor bacteriophage at all. Finally, the apparent MTC resistance exhibited by these strains could have been a function of MTC concentration.

Electron microscopy was used to further characterize the temperate bacteriophages associated with selected *Salmonella* strains. The most common morphological type of bacteriophage associated with the donor strains had a hexagonal-shaped head with an extremely short tail. Based on Bradley's morphological classification of bacteriophages, it appeared to be *Salmonella* phage P22 (3).

An attempt was made to determine whether there was a correlation between the presence of temperate bacteriophages and the level of intracellular toxin synthesized in *Salmonella* strains. Our data (Table 1) indicated no correlation between the concentration of intracellular *Salmonella* toxin, as determined by the CHO floating cell assay, and presence of inducible bacteriophages. MTC induction of host strains was investigated to determine if these strains were genuinely phage free. Both *Salmonella* strains Q1 and 2000 exhibited cell lysis and release of *Salmonella* toxin in the presence of MTC. When filtrates of these MTC-treated *Salmonella* strains were spotted onto indicator lawns of host *Salmonella* strains, no area of lysis or phage plaques were observed. These results suggested that either the filtrates were phage free or contained defective bacteriophage.

We attempted to determine whether *Salmonella* bacteriophages induced by MTC contained the genetic information for toxin synthesis, since other bacteria are known to be lysogenic for bacteriophages that carry the gene(s) for toxin synthesis (5, 6, 20). Host *Salmonella* strains 2000 and Q1 which appeared to be free of bacteriophages were subsequently infected with bacteriophages from selected *Salmonella* donor strains. *Salmonella* toxin levels (Table 2) in the filtrates of the new lysogens were similar to the host strains. The ratio of intracellular toxin to extracellular toxin (sonic extract/filtrate ratios) for the new lysogens was basically the same as the ratios for two host strains. These data imply that bacteriophage probably are not involved with the synthesis of *Salmonella* toxin since the hosts are capable of producing as much *Salmonella* toxin as the new lysogens.

To further investigate the nature of the MTC effect in *Salmonella* strain Q1, identical optical density growth-curve experiments with *Salmonella* strains Q1 and TML-R66 were conducted. A sudden drop in optical density occurred with both strains 3 h after the addition of MTC, indicating bacteriophage induction. Culture filtrates were prepared from samples taken 9 and 24 h after the initial inoculation of both *Salmonella* strains Q1 and TML-R66. Only the MTC-induced *Salmonella* strain TML-R66 demonstrated phage plaques on host lawns, whereas both strains TML-R66 and Q1 revealed the presence of bacteriophage as determined by electron microscopy. The Q1 bacteriophage appeared to be defective in both its morphology and in its ability to infect other *Salmonella* cells.

Based on the data obtained from this study, we have concluded that genetic information responsible for *Salmonella* toxin synthesis was not inherited by lysogenic conversion. Presently, we are investigating the roles of plasmid and chromosomal DNA in the synthesis of *Salmonella* toxin. This possibility is most likely, since *Sal-*

TABLE 1. Survey of *Salmonella* strains for presence of bacteriophage and *Salmonella* toxin production

Salmonella strains[a]	Klett units	CHO floating cell assay[b]	Presence of inducible bacteriophage[c]
LT7	209	20.7	+
2000	296	20.5	−
8994	290	19.3	−
W118-2	260	18.7	+
M206	281	18.6	+
10016	282	18.3	−
986	260	17.1	+
9630	265	17.0	−
SR11	307	11.4	+
8832	274	11.3	−
9SR2	228	11.2	+
6229	313	10.5	−
SL1027	251	9.5	+
TML-R66	270	8.6	+
3774	305	8.4	−
2816	286	8.4	+
RIA	308	7.9	+
Thax-1	244	7.6	+
9186	296	6.1	−
Uninoculated control		1.08	−

[a] Approximately 4×10^4 bacteria from an overnight culture of each strain was inoculated into 25 ml of fresh CYE broth. The cultures were allowed to incubate at 37°C for 16 h.

[b] *Salmonella* cell sonic extracts were used in the CHO floating cell assay. Values are expressed in nanograms per milliliter of cholera toxin equivalent values.

[c] Bacteriophage lysis of sensitive host *Salmonella* strains.

TABLE 2. Detection of *Salmonella* toxin in filtrates and sonic extracts of newly constructed *Salmonella* lysogens and their parent strains

Salmonella strains[a]	Toxin-producing capacity (ng/ml)[b]		Sonic extract/filtrate ratio
	Sonic extracts	Filtrates	
Phage Donors			
SR11	12.6	2.0	6.3
M206	12.4	2.3	5.4
986	4.9	3.3	1.5
SL1027	6.3	1.0	6.3
Phage hosts			
2000	11.4	0.9	12.7
Q1	12.4	0.5	24.8
New lysogens			
2000 (SR11)	12.3	0.5	24.6
2000 (M206)	11.3	0.3	22.6
Q1 (M206)	5.3	0.5	10.6
Q1 (986)	8.9	0.4	22.3
Q1 (SL1027)	7.3	0.7	10.4

[a] Approximately 8.5×10^5 bacteria from an overnight culture of each parent strain and newly constructed lysogen was inoculated into 10 ml of fresh CYE broth. The cultures were allowed to incubate at 37°C for 16 h.

[b] The ELISA was used to obtain the cholera toxin equivalent values. The cholera toxin equivalent value for the uninoculated control was subtracted, and the values are listed above.

monella toxin is very similar to the known chromosomal and plasmid heat-labile enterotoxins of *Vibrio cholerae* (10, 19) and *Escherichia coli* (4, 7, 12), respectively. If we are successful in our endeavor to enhance *Salmonella* toxin synthesis by genetic means, then larger quantities will be used for extended purification, biological, and biochemical studies.

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