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Exploiting the Role of TolC in Pathogenicity: Identification of a Bacteriophage for Eradication of *Salmonella* Serovars from Poultry▽

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Using a screening procedure, three bacteriophages, ST27, ST29, and ST35, were identified with selective activity for *Salmonella enterica* serovar Typhimurium (SL1344) but not SL1344 *tolC*::*aph*. Overproduction of TolC led to a lower efficiency of plating (EOP), further suggesting that TolC was the target receptor. Activity against other serovars of *Salmonella* was observed but not against other species of *Enterobacteriaceae*. This study provides proof of principle that bacteriophages can be active against the outer membrane protein of tripartite resistance-nodulation-division (RND) efflux pumps and so could be used to reduce the numbers of *Salmonella* cells in animals reared for food production.

Measures to reduce numbers of pathogens in food animals without antibiotic use are attractive (21). Current *Salmonella* vaccines that are commercially available are directed toward specific serovars and have little cross-protection to other serovars or serogroups. In addition, some vaccines do not produce a strong immune response and problems arise with fecal excretion of salmonellae by poultry postvaccination (5).

Bacteriophages could provide an alternative to antibiotics for eradication of specific bacterial species that colonize food-producing animals (13, 18). Advantages of a bacteriophage therapeutic strategy include specificity to the target pathogen, no toxicity to the host, reduced cost compared with that of conventional antimicrobials, single dose, and no concern of bacteriophage-resistant pathogens being a risk to human health. In some cases, reduced virulence of bacteriophage-resistant mutants has been seen (3).

The use of bacteriophages to prevent salmonella colonization of poultry has been explored by various workers (1, 2, 7, 12, 16). Overall, the studies have shown that the use of bacteriophages in reducing the colonization of poultry, and other farm animals, by *S. enterica* is feasible but may need to be in conjunction with bacteriophage lytic enzymes, vaccines, or a mixed-phage preparation to achieve a long-lasting reduction in bacterial carriage.

So far, bacteriophages active against *Salmonella* have been obtained by screening naturally occurring bacteriophages, with no knowledge of the target bacterial receptor. TolC of *S. enterica* serovar Typhimurium is the outer membrane protein component of the multidrug-resistant (MDR) efflux pump AcrAB-TolC. This pump confers innate multidrug resistance and when overproduced confers resistance to clinically relevant antibiotics (17). TolC is also important for *S.* Typhimurium to colonize and infect the host (6, 9, 15). Therefore, not only should bacteriophages that target TolC eradicate these organisms from poultry, but bacteriophage-resistant bacteria are likely to be avirulent. Furthermore, TolC is conserved across all serovars of *Salmonella* and a bacteriophage specific for TolC is likely to offer cross-serovar protection.

The aim of this study was to obtain proof of principle for a simple procedure to identify a bacteriophage which targets a specific protein, TolC. Such a phage would be highly amenable for use in the eradication of *Salmonella* in animals used for food production.

S. enterica serovar Typhimurium SL1344 was used as the wild-type strain (20) along with a TolC-deficient isogenic mutant, SL1344 *tolC*::*aph* (L108), which has been described previously (9). A TolC-overexpressing strain, L729, was also constructed from SL1344 by ligating the entire *tolC* gene into the arabinose-inducible vector pBAD (Invitrogen, United Kingdom). Electroporation was used to introduce the pBAD-TolC construct into *S.* Typhimurium SL1344, and recovery was performed on LB agar containing 100 μg/ml ampicillin. The pBAD-TolC construct in SL1344 was then grown with various concentrations of arabinose (Sigma, United Kingdom) to establish the concentration of arabinose required to obtain optimum *tolC* expression.

To verify and quantify *tolC* expression, bacteria were grown in minimal media and RNA was isolated as described by Bailey et al. (4). Reverse transcription-PCR (RT-PCR) amplification of *tolC* and PCR product quantification by denaturing high-performance liquid chromatography (HPLC) analysis were as described previously (10). For strains containing pBAD-TolC, arabinose was added to the media at concentrations ranging from 1% to 0.00001%. Data are presented as means ± standard deviation from results of the independent RT-PCR amplifications. Mutant and parental strains were compared by Student's *t* test; a P value of <0.05 was considered significant. Expression of TolC was also observed by SDS-PAGE and Western blotting using an anti-TolC antibody as described by Touzé et al. (19).

All *Salmonella* bacteriophages used in this study were obtained from the Health Protection Agency (Colindale, London, United Kingdom) (Table 1). The activity of the bacteriophages was determined by the overlay method as described previously by Atterbury et al. (2). Briefly, a bacteriophage suspension containing approximately 10^9 PFU/ml was applied

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TABLE 1. Differential activity of three bacteriophages depending upon production of TolC

Strain	Genotype or phenotype	Bacteriophage (PFU/ml)			Fold change in *tolC* expression/ TolC expression relative to SL1344[a]
		ST27	ST29	ST35	
L354	SL1344	10^6	10^5	10^6	1/1
L729	SL1344/pBAD *tolC*	10^5	10^2	10^4	1.7/11.5
L108	*tolC*::*aph*	0	0	0	ND/ND
L773	*tolC*::*aph*/pWKS30 *tolC*	10^5	10^4	10^6	1.07

[a] The level of *tolC* was measured by RT-PCR and denaturing HPLC analysis. The level of TolC was measured by Western blotting. ND, not detected.

TABLE 2. Susceptibility of 10 *Salmonella* serovars and members of the *Enterobacteriaceae* to *S.* Typhimurium bacteriophage ST27, ST29, and ST35

Organism	% TolC similarity to SL1344	Susceptibility[a] to bacteriophage:		
		ST27	ST29	ST35
Salmonella serovars				
Haifa	100	S a	S b	S b
Kedougou	NDA[b]	S a	S b	S a
Virchow	100	S b	S b	R
Heidelberg	99	S a	S b	R
Enteritidis	99	R	S b	S a
Saintpaul	99	R	S a	R
Dublin	99	R	S a	S a
Mbandaka	NDA	S a	S a	R
Montevideo	NDA	R	S a	R
Newport	99	R	S a	S a
Enterobacteriaceae				
Escherichia coli	89	R	R	R
Citrobacter freundii	89	R	R	R
Enterobacter cloacae	86	R	R	R
Klebsiella pneumoniae	85	R	R	R
Serratia marcescens	72	R	R	R
Hafnia spp.	67	R	R	R
Providencia stuartii	67	R	R	R
Morganella morganii	64	R	R	R

[a] R, resistant; S a, susceptible with less than semiconfluent lysis; S b, susceptible with semiconfluent lysis.
[b] NDA, no data available.

as 10-μl spots to pre-prepared bacterial lawns of *S.* Typhimurium at the mid-exponential phase of growth and allowed to adsorb into the top layer of agar. The plates were then incubated for 24 h at 37°C under aerobic conditions. The host range of phages with differential activity, ST27, ST29, and ST35, were assessed on the basis of their ability to form plaques on a range of *Salmonella* serovars and a selection of other *Enterobacteriaceae* (Table 2).

RT-PCR data showed that an arabinose concentration of 0.00004% produced optimal *tolC* expression in L729 (SL1344 pBAD-TolC), which resulted in a 1.7-fold increase in the *tolC* transcript over that in SL1344 (data not shown). Western blotting showed that L729 produced 10-fold more TolC protein than the wild-type strain SL1344 (Fig. 1).

Thirty bacteriophages active against *S.* Typhimurium were investigated for differential activity against wild-type S. Typhimurium (SL1344) and its isogenic mutant SL1344 *tolC*::*aph*, where the *tolC* gene has been inactivated by the insertion of the *aph* gene (9). Both SL1344 and SL1344 *tolC*::*aph* were resistant to 13 *S.* Typhimurium bacteriophages. Of the 17 remaining bacteriophages, three had no activity against SL1344 *tolC*::*aph* but produced semiconfluent lysis with SL1344 (data not shown). Three bacteriophages, ST27, ST29, and ST35, were investigated further to determine their efficiencies of plating (EOP) against SL1344, SL1344 *tolC*::*aph*, SL1344 pWKS30*tolC* (*tolC* complemented in *trans* [9]), and SL1344 pBAD-TolC. This was achieved by serially diluting a phage stock suspension, containing 10^9 PFU/ml, 1:10 to give a sequential series of suspensions ranging from 10^9 to 10^1 PFU/ml. The suspensions were then used in the plaque assay, as described previously. Inactivation of *tolC* conferred resistance to the three bacteriophages, and susceptibility was conferred when *tolC* was complemented in *trans.* Overproduction of TolC led to a lower EOP than was seen for SL1344, further suggesting that this protein was the target receptor (Table 1). This conclusion arises from the hypothesis that if the putative receptor (TolC) is an attachment point, then more TolC receptors, due to overexpression, will lead to lower efficiencies of plating (EOP) because one is effectively increasing the phage concentration at the surface, which can lead to the possibility of lysis at lower titers.

Due to the similarity of the TolC amino acid sequences between serovars of *Salmonella* and other *Enterobacteriaceae*, we determined the activity of the three salmonella bacteriophages ST27, ST29, and ST35 to 10 different serovars of *Salmonella enterica* and nine different species of *Enterobacteriaceae* (Table 2). Of the three bacteriophages used, ST29 showed activity for all of the 10 serovars. Bacteriophages ST27 and ST35 demonstrated activity against 5 of the 10 serovars. None

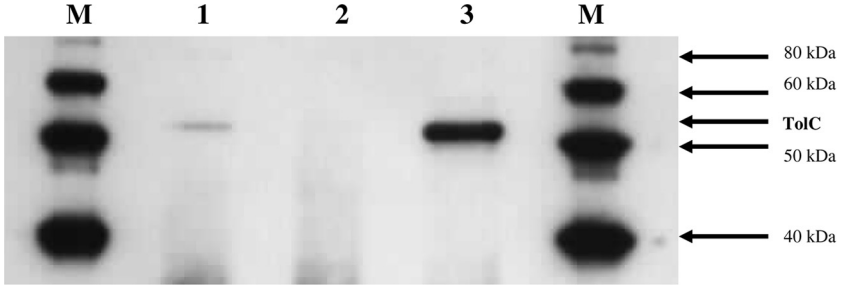

FIG. 1. Western blot to show TolC expression in SL1344, L108, and L729. Lanes: M, protein standard; 1, SL1344; 2, L108 (*tolC*::*aph*); 3, L729 (pBAD-TolC).

of the other species of *Enterobacteriaceae* tested were susceptible to any of the three bacteriophages, indicating species selectivity.

The AcrAB-TolC efflux pump is important in the colonization of chickens by *S.* Tyhimurium (6, 9). Buckley et al. (9) also showed that *S.* Typhimurium in which *tolC* had been disrupted could not adhere to, invade, or survive in mouse macrophages and human embryonic intestinal cells. To reduce the colonization of poultry (and other animals) by *S. enterica* using bacteriophage treatment, a bacteriophage specific for TolC would be advantageous. German and Misra (11) showed that the TolC protein of *Escherichia coli* serves as a cell surface receptor for the TLS bacteriophage, and so it was predicted that a bacteriophage that binds to TolC of *S.* Typhimurium would be found. Assays performed in this study using the TLS bacteriophage showed that *S.* Typhimurium was resistant to this bacteriophage (data not shown). In the present study, three bacteriophages were identified as potential candidates and the titers of these bacteriophages against a TolC-deficient mutant and a TolC-overexpressing mutant further suggested that the receptor was TolC. Of the three bacteriophages, ST29 exhibited no serovar specificity, as activity was observed against all serovars tested. This is presumably due to the high percent sequence similarity (>98%), as calculated by the BLAST search alogirithm, of the SL1344 TolC amino acid sequences between serovars (e.g., *S. enterica* serovar Enteritidis, *S. enterica* serovar Virchow). No activity was observed against other *Enterobacteriaceae*, which may be due to the decrease in percent sequence similarities of the TolC proteins between members of the *Enterobacteriaceae*, which range from 64% to 89%.

There are circumstances in which the evolution of bacteriophage-resistant bacteria could be of positive benefit, and we hypothesize that an example of this is resistance to a TolC-targeted bacteriophage. It is proposed that the bacteriophage-resistant bacterium would either be TolC deficient or have a mutant TolC and so may colonize poultry poorly. Resistance to a bacteriophage can also reduce the fitness of bacteria per se (8, 14); this could impair the ability of bacteria to compete with bacteriophage-sensitive bacteria. With respect to a TolC-targeted bacteriophage, the evolution of resistance to the bacteriophage would create mutant bacteria no longer able to cause disease, and as long as the bacteriophage is present as a selective agent, any resistant attenuated mutants should replace pathogenic strains.

The aim of this pilot study was to identify a bacteriophage which targeted the TolC protein of *S.* Typhimurium. Having done this, experimentation is now required to fully characterize the three bacteriophages and perform experiments to determine the dose to prevent colonization of *S. enterica* in poultry. Data described herein also provide proof of principle; bacteriophages active against the outer membrane protein components of other RND efflux pumps, such as CmeC of *Campylobacter jejuni*, could also be a useful tool to eradicate this species from poultry.

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