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Generalized transduction of serotype 1/2 and serotype 4b strains of *Listeria monocytogenes*

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Summary

This is the first report of generalized transduction in the Gram-positive, food-borne pathogen *Listeria monocytogenes*. Bacteriophages were isolated from the environment and from lysogens, or were obtained from other laboratories. Of the 59 bacteriophages tested, 34 proved to be capable of transduction. We exploited the ability of *L. monocytogenes* to grow at room temperature and isolated bacteriophages that were incapable of growth at 37°C. Transductions at this temperature therefore eliminated transductant killing and lysogeny, as did inclusion of citrate and the use of a low multiplicity of infection. Transducing bacteriophages were found for each of the well-characterized *L. monocytogenes* strains: EGD, 10403, Mack (serotype1/2a), L028 (serotype 1/2c), Scott A (serotype 4b) and strains from the Jalisco and Halifax, Nova Scotia outbreaks (serotype 4b). P35 (ϕLMUP35) is a particularly useful generalized transducing bacteriophage with a wide host range (75% of all serotype 1/2 strains tested). Its disadvantages are that it is small and transduction is relatively infrequent. U153(ϕCUSI153/95) is larger than P35 and transduction frequency increased 100-fold, but it has a very narrow host range. We demonstrated interstrain transduction and used transduction to test linkage between transposon insertions and mutant phenotypes in a variety of strains.

Introduction

Listeria monocytogenes is a Gram-positive bacterium that causes the rare but deadly food-borne disease listeriosis. The bacterium is ubiquitous in nature being found in soil and is carried in the gut of up to 10% of individuals. The bacterium is an opportunist pathogen and has developed the ability to proliferate over a wide range of temperatures (2.5–45°C), pHs (pH 5.2–9.6) and salinities, and in aerobic and anaerobic conditions. The ability to tolerate such adverse conditions explains why many food preservative regimes, e.g. refrigeration, pickling, salting and vacuum packing, are ineffective. However, pasteurization effectively destroys the bacterium (Bahk and Marth, 1990).

Infection with *L. monocytogenes* is usually asymptomatic or may result in mild, influenza-like symptoms. Following a primary infection, an individual's cell-mediated immunity system is primed to clear rapidly any subsequent infections. When an individual's cell-mediated immune system is compromised because of pregnancy, extreme age or immune-compromising diseases or treatments, the patient may develop a bacteraemia leading to septicaemia, meningitis, meningo-encephalitis and, in pregnant patients, abortion. In its full-blown form, the disease can lead to 30–40% mortality rates in adults, neonates and foetuses. Listeriosis can occur sporadically or as distinct outbreaks, and there have been a number of large outbreaks involving several hundreds of individuals. The causative strains are most commonly of the 4b serotype, although strains of the 1/2 serotype have also been isolated from patients (Schuchat *et al*., 1991). The *L. monocytogenes* pathogenicity cycle has been well characterized at the cell biological level (Cossart and Lecuit, 1998).

Listeriosis is not a major disease as regards humans. It is really a zoonose and is more commonly associated with animal infections, particularly sheep. There is evidence that one of the causes of listeriosis outbreaks in farm animals is the preparation of poor-quality silage. Pickling herbaceous material using lactic acid bacteria generates silage. If the pH does not drop below pH 5.0, because of insufficient sugar content, then listeria can proliferate. This listeria-infected silage, when fed to farm animals, has been shown to be the cause of listeriosis (Weidmann *et al*., 1994).

The study of *L. monocytogenes* is of interest for two reasons: (i) it has the potential as a live vaccine for the specific delivery of antigens to the cell-mediated immune system (Pan *et al*., 1995), and (ii) it also serves as a useful model system for the study of intracellular pathogenesis. There are a number of obligate intracellular pathogens, which are significant causes of human misery, that are not experimentally tractable. Examples include *Mycobacterium leprae,* the causative agent of Hanson's disease, and *Chlamydia trachomatis*, the causative agent of the

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sexually transmitted infection, lymphogranuloma venereum, and blindness caused by conjunctival scarring from trachoma. However, *L. monocytogenes* is easily cultured in broth and there are a number of simple tissue culture systems that allow rigorous analysis of different aspects of the pathogenesis process. There is also an excellent murine infection model.

L. monocytogenes has a small genome (3.15 megabases) and a physical restriction map has been prepared for the serotype 1/2c strain LO28 (Michel and Cossart, 1992). The bacterium is susceptible to a wide range of bacterial genetic techniques. Transposons Tn*916*, Tn*917* and Tn*1545* have been shown to insert into the *L. monocytogenes* genome. Protoplast transformation and electroporation have proved to be possible, but both processes are very inefficient. A wide variety of plasmid vectors developed for use in *Bacillus* spp., *Staphylococcus* spp. and lactic acid bacteria has been proven to be maintained in *L. monocytogenes*. There is no report of large-scale genetic exchange, such as conjugation, between listeria strains, nor has bacteriophage-mediated genetic exchange transduction been reported.

Generalized transduction is a powerful tool in the analysis of bacterial functions and is often used for fine structure gene mapping. Another use is demonstrating that specific transposon insertions have or have not caused a specific mutation. This is very important when using the drug resistance determinant of the transposon to clone a gene. There are a number of transposons, including the Tn*916* family, that often generate multiple insertions. Transduction allows the identification of the transposon insertion that caused the phenotype of interest by co-segregation of the phenotype and the transposon drug resistance determinant. A very powerful technique, made possible by generalized transducing bacteriophages, is the transposon-tagging of point mutations (Kaiser, 1984). The mutated gene can then be cloned, using the selectable marker in the transposon, and so genes with a non-selectable phenotype can be isolated efficiently. Transposon-tagged genes and transducing bacteriophages can also be used in strain construction, localized mutagenesis, identification of allelic mutations and fine-structure gene mapping.

The first report of the isolation of a bacteriophage capable of infecting *Listeria* (then known as *Listerella*) *monocytogenes* was that of Schultz (1945). Since that time many bacteriophages have been isolated for bacteriophage subtyping of *Listeria* spp. strains, and a number of such bacteriophage-typing schemes have been used successfully (Bille and Rocourt, 1996).

To aid in the identification of useful generalized transducing bacteriophages of *Listeria monocytogenes*, we were able to exploit the ability of the bacterium to grow over a wide temperature range. One of the causes of failure to identify transduction is the killing of transductants (a product of a transducing particle rather than a bacteriophage interaction with the cell) by bacteriophages released from infected bacteria. We were able to isolate bacteriophages that grew at room temperature but could not grow at 37°C, thus transductants were not subject to transductant killing when the out-growth step was carried out at 37°C. Another advantage of this approach is that the chance of lysogeny is lessened. This is important if the transductant is to be used in further crosses as it will be resistant to the transducing bacteriophage used to create it and might be resistant to other transducing bacteriophages through hetero-immunity. Therefore, all newly isolated bacteriophages were isolated at room temperature prior to testing for transduction ability.

Bacteriophages were isolated by three different methods: (i) from environmental samples contaminated with *Listeria* spp., (ii) from *Listeria monocytogenes* lysogens, and (iii) from listeriophage collections around the world. Ideally, to demonstrate that transduction had occurred, crosses were set up such that any transductant product was of a genotype that had not existed before in the laboratory. This paper reports the first examples of generalized transduction of *Listeria monocytogenes* serotype 1/2 and 4b and preliminary characterization of the bacteriophages.

Results and discussion

Isolation and characterization of bacteriophages

Martin J. Loessner of the Technical University of Munich, Germany, supplied us with bacteriophages from an extensive collection that had been assembled, in part, for bacteriophage-subtyping *L. monocytogenes* strains. The serotype 1/2a-infecting bacteriophages were A620, A511, A502, A118 and A006 (Table 1). The serotype 4b-infecting bacteriophages were A500, A640, B021, PSA, 90666, 90861, 93253 and 910716 (Table 1). Bacteriophages were isolated from lysogens in the large culture collections of *L. monocytogenes* of Daniel A. Portnoy and of Carl Batt of Cornell University; the latter supplied by Martin Weidmann. Bacteriophages were named after the strain from which they were isolated with the prefix ϕ (Table 1).

Bacteriophages were isolated from environmental samples likely or known to contain *L. monocytogenes*. Bacteriophages isolated from sewage effluent were given the prefix ϕEF. Silage isolates were named with reference to the silage sample number they were isolated from and given the prefix ϕLMUP (*L. monocytogenes* University of Pennsylvania) (Table 1). As discussed in the *Introduction*, all the environmental and lysogen bacteriophages were cultured at room temperature in the expectation that bacteriophages that were incapable of growth at 37°C would be isolated.

Table 1. Bacteriophages.

Name	Synonyms	Origin	Hosts[a]	Transduction	Size	Temperature[b] (°C)	Citrate[c]	Reference
Broad host range bacteriophages								
A511		Sewage	All 1/2 and 4b serotype strains tested	No	116 kb[3]	?		Loessner and Busse (1990)
ϕLMUP121		Silage	All 1/2 and 4b serotype strains tested	No	?	?		This report.
ϕLMUP213N1		Silage	All 1/2 and 4b serotype strains tested	No	?	?		This report.
ϕLMUP4b4		Silage	All 1/2 and 4b serotype strains tested	No	?	?	?	This report
ϕLMUP4b4N1		Silage	All 1/2 and 4b serotype strains tested	No	?	?		This report
ϕLMUP4b11		Silage	All 1/2 and 4b serotype strains tested	No	?	?		This report
Serotype 1/2 infective bacteriophages								
A620		Lysogen	10403[1] EGD Mack, NCTC7973	Yes	38.3 kb[2]	30		Loessner *et al.* (1994a)
A502		Sewage	10403[1] Mack EGD NCTC7973	Yes	39 kb[3]	30		Loessner and Busse (1990)
A118		Lysogen	10403[1] Mack[4]	Yes	?	30[5]		Loessner (1991)
A006		Lysogen	10403[1] Mack[4]	Yes	34 kb[3]	30		Loessner (1991)
ϕEF1[9]		Sewage	10403[6]	Yes	?	30[5]	S	This report
ϕEF2		Sewage	10403[6]	Yes	?	30[5]	S	This report
ϕEF3		Sewage	10403[6]	Yes	?	30[5]	S	This report
ϕEF4		Sewage	10403[6]	Yes	?	30[5]	S	This report
ϕLMUP111[9]		Silage	10403[6]	Yes	37.9 kb[2]	30[5]	S	This report
ϕLMUP31a[9]		Silage	10403[6]	No	?	?	S	This report
ϕLMUP31b[9]		Silage	10403[6]	No	?	?	S	This report
ϕLMUP32a[9]		Silage	10403[6]	Yes	?	30[5]	S	This report
ϕLMUP32b		Silage	10403 Mack EGD L028[7]	Yes	?	RT	S	This report
ϕLMUP33[9]		Silage	10403[6]	No	?	?	S	This report
ϕLMUP35[9]	P35	Silage	10403 Mack EGD L028 NCTC7379[8]	Yes	?	RT	S	This report
ϕLMUP332[9]		Silage	10403[6]	Yes	?	30	S	This report
ϕLMUP41		Silage	10403[6]	No	?	?	S	This report
ϕLMUP42		Silage	10403[6]	No	?	?	S	This report
ϕLMUP61a		Silage	10403[6]	No	?	?	S	This report
ϕLMUP61b		Silage	10403[6]	No	?	?	S	This report
ϕLMUP62		Silage	10403[6]	No	?	?	S	This report
ϕ10403		Lysogen	Mack	Yes	?	RT		This report
ϕATCC43251		Lysogen	Mack	No	?	?	S	This report

Table 1. Continued.

Name	Synonyms	Origin	Hosts[a]	Transduction	Size	Temperature[b] (°C)	Citrate[c]	Reference
ϕCU-EN1/96		Lysogen	10403[6]	Yes	?	?	S	This report
ϕCU-F31/93		Lysogen	Mack	No	?	?	S	This report
ϕCU-F34/93		Lysogen	Mack EGD NCTC7379	Yes	?	30[5]		This report
ϕCU-SI133/93		Lysogen	Mack NCTC7379	Yes	?	30		This report
ϕCU-SI153/95	U153	Lysogen	10403 Mack NCTC7379	Yes	40.8 kb[2]	30		This report
ϕCU-SI162/94		Lysogen	Mack NCTC7379	Yes	?	RT	S	This report
ϕCU-SI165/94		Lysogen	10403 Mack	Yes	?	?		This report
ϕEGDSm^R		Lysogen	Mack NCTC7379	Yes	?	30[5]	S	This report
ϕEGD857		Lysogen	10403 Mack	Yes	?	?		This report
ϕLL124		Lysogen	Mack	Yes	?	RT		This report
Serotype 4b infective bacteriophages								
A500	ATCC 23074-B1 243	?	1089 F2379 F2381 ATCC23074	Yes	38[3]	37		Loessner and Busse (1990) Loessner *et al*. (1994b)
A640[9]		Lysogen	4bs F2175 ATCC23074	Yes	?	30		Loessner *et al*. (1994a)
B021[9]		Lysogen	F2379 F2381 ATCC23074	Yes	?	30		Loessner *et al*. (1994a)
PSA		Lysogen	4bs 1089 F2379 F2381 2191 ATCC23074	No	?	?	S	Loessner *et al*. (1994a)
90666		Lysogen	1089 F2379 F2381 ATCC23074	Yes	?	37		Estela *et al*. (1992)
90861		Lysogen	4bs 1089 2121 2191 2193 2214 F2175 F2379 F2381 ATCC23074	No	?	?		Estela *et al*. (1992)
93253		Lysogen	4bs 1089 2121 2191 2193 2214 F2175 F2379 F2381 ATCC23074	No	?	?	S	Estela *et al*. (1992)
910716		Lysogen	4bs 1089 2121 2191 2193 2214 F2379 F2381 ATCC23074	No	?	?	S	Estela *et al*. (1992)
ϕLMUP4b6		Silage	4bs	Yes	?	37		This report
ϕLMUP236O1[9]		Silage	2121 21912193 2214 F2379 F2381 ATCC23074	No	?	?	S	This report
ϕLMUP4b6N1		Silage	4bs C52	Yes	?	37		This report
ϕLMUP4b6N2		Silage	4bs 2193 F2379	Yes	?	37		This report
ϕDD957		Lysogen	4bs 2191 2193 F2379 F2381 ATCC23074	No	?	?	S	This report
ϕDD1056		Lysogen	4bs 2191 2193 F2379 F2381 ATCC23074	No	?	?	S	This report

Table 1. Continued.

Name	Synonyms	Origin	Hosts[a]	Transduction	Size	Temperature[b] (°C)	Citrate[c]	Reference
φDD1308		Lysogen	4bs 2191 2193 F2379 ATCC23074	No	?	?	S	This report
φDD2359		Lysogen	C52 F2379 F2381 ATCC23074	Yes	?	37		This report
φDD6290		Lysogen	4bs 2191 2193 F2379 F2381 ATCC23074	No	?	?	S	This report
φLCDC 82-130		Lysogen	1089 F2379 F2381 ATCC23074	Yes	?	37		This report
φLCDC 81-861		Lysogen	1089 C52 F2379 F2381 ATCC23074	Yes	?	37		This report
φL99-1		Lysogen	4bs Scott A ATCC23074	Yes	?	30		This report

a. Host range tested: serotype 1/2 specific bacteriophages 10403, Mack, EGD and L028; serotype 4b specific bacteriophages 4bs, 1089, 2121, 2191, 2193, 2214, F2175, F2379, F2381, Scott A and ATCC23074, unless otherwise stated. Serotype 4b transducing bacteriophages were also tested for growth on C52.
b. Tested at room temperature (RT), 30°C and 37°C. Highest temperature that gave plaques.
c. Sensitive (S), unable to form plaques in the presence of 10 mM sodium citrate.
[1] Poor plaque definition; [2] determined this report; [3] Loessner *et al.* (1994b); [4] EGD, L028 and NCTC7973 not tested; [5] fewer, smaller plaques; [6] Mack, EGD, L028 and NCTC7973 not tested; [7] NCTC7973 not tested; [8] 75% of all serotype 1/2 strains tested (M. J. Loessner, personal communication); [9] sensitive to chloroform. Remove intact cells by filter sterilization; ?, not tested.

Plaques were identified using *L. monocytogenes* strains of serotype 1/2a and serotype 4b. The primary isolation serotype 1/2a strains were derivatives of 10403 and Mack. The latter strain proved to be a very useful strain as plaques that were often tiny, cloudy or invisible on 10403 strains were always clearer. A number of bacteriophages were isolated from lysogens that grew on Mack but could not grow on 10403S (φ10403, φATCC43251, φCU-F31/93, φCU-F34/93, φCU-SI133/93, φCU-SI162/94, φEGDSm^R and φLL124). These include a bacteriophage that was isolated from 10403, φ10403, and one that came from an EGD strain, φEGDSm^R. The latter bacteriophage is different from bacteriophage φEGD857, which was isolated from the EGD strain DP-L857 and which could grow on 10403 derivatives. A bacteriophage with the same host range as φEGD857 was isolated from another EGD derivative, DP-L189 (Table 2). Bacteriophages with the same host range as φEGDSm^R were isolated from DP-L857 and DP-L1595. It is clear that the two genetically well-characterized serotype 1/2a strains, EGD and 10403, contain at least one prophage. Indeed, the genes for a bacteriophage autolysin and a holin have been isolated from an EGD prophage (McLaughlin and Foster, 1998).

After isolation, the bacteriophages were tested for host range, growth at different temperatures, ability to grow in the presence of 10 mM citrate, sensitivity to chloroform and ability to transduce their host. The majority of bacteriophages isolated would only grow on one serotype, however some grew on all *L. monocytogenes* strains tested, i.e. new silage isolates φLMUP121, φLMUP213N1, φLMUP4b4, φLMUP4b4N1 and φLMUP4b11 and Loessner isolate A511. The φLMUP121 generated clear plaques. and A511 is known to be virulent.

Of bacteriophages that infected serotype 1/2a strains, including A511 and φLMUP121, 14 were isolated from silage, four from sewage effluent, 12 from lysogens and five were obtained from Dr Loessner (Table 1). Of these 35 bacteriophages, a number were probably represented more than once. Of bacteriophages that infected serotype 4b strains, including φLMUP213N1, φLMUP4b4, φLMUP4b4N1 and φLMUP4b11, 15 were isolated from silage (only eight are shown in Table 1, as seven had an identical host range and came from the same sample as φLMUP213N1), none from sewage effluent, eight from lysogens and eight from Dr Loessner (Table 1). Of these 31 bacteriophages, it is probable that some were duplicates.

Test for transduction

In each case, the test for transduction was the ability to transfer a drug resistance marker of a transposon from one strain to another. The putative transductants were

Table 2. *Listeria monocytogenes* isolates.

Strain	Synonym	Serotype	Origin	Reference
10403	DP-L921	1/2a	Isolated from wild rabbit droppings	E. Hall (personal communication)
10403S	DP-L184	1/2a	Streptomycin resistant 10403	Bishop and Hinrichs (1987)
EGD	NCTC12427 DP-L189 DP-L857 DP-L1595	1/2a	Human infection, early 1960s	Blanden *et al*. (1966)
Mack	DP-L861 SLCC5764	1/2a	From Trudeau Institute, possible derivative of NCTC7973	H. Hof (personal communication)
NCTC7973	ATCC35152 DP-L3332	1/2a	Guinea-pig mesenteric lymph node 1924.	Stephens *et al*. (1991)
L028	DP-L1952	1/2c	'Spanish clinical origin' – faeces of healthy pregnant woman	Pérez-Díaz *et al*. (1982) P. Cossart (personal communication)
1089	WSLC1761	4b	Propagating strain of international *Listeria monocytogenes* phage typing series	Rocourt *et al*. (1985) M. J. Loessner (personal communication)
2191		4b	Nova Scotia outbreak isolate surface antigen$^-$	S. Kathariou (personal communication)
2193		4b	Nova Scotia outbreak isolate	S. Kathariou (personal communication)
2214		4b	Nova Scotia outbreak isolate surface antigen$^-$	S. Kathariou (personal communication)
4bs	NCTC10527 ATCC13932	4b	Spinal fluid of child with meningitis in 1967 – Seeliger 1071/53	Zheng and Kathariou (1994) ATCC catalogue
ATCC23074	WSLC1042	4b	Stanford University AT-25	ATCC catalogue
C52		4b	Unknown	I. S. Roberts (personal communication)
F2121		4b	Los Angeles Jalisco soft cheese outbreak isolate surface antigen$^-$	S. Kathariou (personal communication)
F2175		4b	Los Angeles Jalisco soft cheese outbreak isolate	S. Kathariou (personal communication)
F2379	ATCC43257	4b	Los Angeles Jalisco soft cheese outbreak isolate	S. Kathariou (personal communication)
F2381		4b	Los Angeles Jalisco soft cheese outbreak isolate	Zheng and Kathariou (1994)
F4642	Scott A	4b	Massachusetts outbreak isolate	Stephens *et al*. (1991)
Lysogens				
ATCC43251	DP-L2957	1/2a	Derived from NCTC7379	ATCC catalogue
CU-EN1/96	DP-L3242	?	Environmental	M. Wiedmann (personal communication)
CU-F31/93	DP-L3249	?	Meat	M. Wiedmann (personal communication)
CU-F34/93	DP-L3250	?	Meat	M. Wiedmann (personal communication)
CU-SI133/93	DP-L3265	?	Silage	M. Wiedmann (personal communication)
CU-SI153/95	DP-L3266	?	Silage	M. Wiedmann (personal communication)
CU-SI162/94	DP-L3269	?	Silage	M. Wiedmann (personal communication)
CU-SI165/94	DP-L3271	?	Silage	M. Wiedmann (personal communication)
LL124	DP-L2288	4b	Blood of a baby in 1986 during Swiss listeriosis epidemic	J. Bille (personal communication)
DD957	DP-L3237	?	Soft cheese	M. Wiedmann (personal communication)
DD1056	DP-L3238	?	No information	M. Wiedmann (personal communication)
DD1308	DP-L3239	?	From cheese associated with meningitis	M. Wiedmann (personal communication)
	L2190a			McLaughlin *et al*. (1990) Bille and Rocourt (1996)
DD2359	DP-L3240	?	Ice cream	M. Wiedmann (personal communication)
DD6290	DP-L3241	?	No information	M. Wiedmann (personal communication)
LCDC 82-130	DP-L2254	?	No information	M. Wiedmann (personal communication)
LCDC 81-861	DP-L2255	?	No information	M. Wiedmann (personal communication)
L99/1	DP-L3259	?	Meat	M. Wiedmann (personal communication)

then screened for inheritance of the transposon-induced phenotype change. In the case of the bacteriophage that could grow on 10403S, the donor was DP-L1764, a Tn*917*-LTV3-induced adenine auxotroph (*ade*::Tn*917*-LTV3), and the recipient was an engineered haemolysin deletion, DP-L2161 (*hly*$^{\Delta}$) (Table 3). Transduction would therefore create a strain that did not exist before, i.e. a haemolysin-deficient adenine auxotroph that was erythromycin and lincomycin resistant.

Unfortunately, we did not have any appropriately marked recipients for the serotype 4b strains, nor for the bacteriophage that grew on Mack but not 10403S, and

Table 3. *Listeria monocytogenes* mutants.

Name	Genotype	Phenotype	Reference
10403S derivatives			
DP-L212	*hly*::Tn*1545* Str^R	Sm^R Haemolysin$^-$ Tc^R Em^R Km^R	D. A. Portnoy (personal communication)
DP-L973	*hly*::Tn*917*-LTV3 Str^R	Sm^R Haemolysin$^-$	Sun *et al.* (1990)
DP-L1075	*prfA*::Tn*917*-LTV3 Str^R	Sm^R Haemolysin$^-$ Lecithinase$^-$ Em^R	D. A. Portnoy (personal communication)
DP-L1764	*ade*::Tn*917*-LTV3 Str^R	Sm^R Adenine$^-$ Em^R	Marquis *et al.* (1993)
DP-L1775	*aro*::Tn*917*-LTV3 Str^R Tyrosine $^-$ Em^R	Sm^R Phenylalanine$^-$ Tryptophan$^-$	Marquis *et al.* (1993)
DP-L1777	*phe*::Tn*917*-LTV3 Str^R	Sm^R Phenylalanine$^-$ Em^R	Marquis *et al.* (1993)
DP-L1786	*gly*::Tn*917*-LTV3 Str^R	Sm^R Glycine$^-$ Em^R	Marquis *et al.* (1993)
DP-L1809	*thr*::Tn*917*-LTV3 Str^R	Sm^R Threonine$^-$ Em^R	Marquis *et al.* (1993)
DP-L1822	*pro*::Tn*917*-LTV3 Str^R	Sm^R Proline$^-$ Em^R	Marquis *et al.* (1993)
DP-L1839	*nia*::Tn*917*-LTV3 Str^R	Sm^R Niacin$^-$ Em^R	Marquis *et al.* (1993)
DP-L1851	*ura*::Tn*917*-LTV3 Str^R	Sm^R Uracil$^-$ Em^R	Marquis *et al.* (1993)
DP-L2161	*hly*^{i} Str^R	Sm^R Haemolysin$^-$	Jones and Portnoy (1994)
Mack derivative			
DP-L1415	*hly*::Tn*917*-LTV3	Haemolysin$^-$ Em^R	D. A. Portnoy (personal communication)
EGD derivatives			
EGDSm^R	Str^R	Sm^R	Gaillard *et al.* (1991)
BUG5	*inlAB*::Tn*1545* Str^R Sm^R Tc^R Em^R Km^R	Unable to infect epithelial cells	Gaillard *et al.* (1991)
L028 derivative			
Bof415 (LO28(*hlyA*::Tn*917*))	*hly*::Tn*917*-LTV3	Haemolysin$^-$ Em^R	Cossart *et al.* (1989)
4bs derivatives			
4b1	Str^R	Sm^R	Zheng and Kathariou (1994)
FlaY	Str^R *fla*-Tn*916*ΔE	Sm^R non-motile Em^R	S. Kathariou (personal communication)
F2381 derivatives			
2381 l	Str^R	Sm^R	Zheng and Kathariou (1994)
DK1	Str^R *zzz*::Tn*916*ΔE	Sm^R Em^R	S. Kathariou (personal communication)

Em^R, erythromycin resistant; Km^R, kanamycin resistant; Sm^R, streptomycin resistant; and Tc^R, tetracycline resistant.

so we had to be very careful to assure that the apparent transductants were not just donor contaminants. The transduction test for the bacteriophages that grew on Mack used DP-L1415 (*hly*::Tn*917*-LTV3) (Table 3) as a donor and DP-L861 (wild type) (Table 2) as a recipient. The donor serotype 4b strains were supplied by Sophia Kathariou of the University of Hawaii, and consisted of Tn*916*ΔE insertions. Tn*916* was modified by replacement of the tetracycline resistance determinant with an erythromycin resistance determinant to create Tn*916*ΔE (Rubens and Heggen, 1988). The transposants had either a non-motile phenotype (FlaY) or no phenotype beyond erythromycin resistance (DK1) (Table 3).

The transduction test was the same in all cases. The recipient cells were mixed with the bacteriophage lysate at a low (0.1–1.0) multiplicity of infection (moi – number of plaque-forming units per number of cells). The mixture was incubated at room temperature and then exposed to a selection plate. The selection plate contained citrate and was incubated at 37°C. The combination of citrate, low moi and 37°C minimized transductant killing and lysogen formation for those bacteriophages incapable of growth at 37°C.

Serotype 1/2 transducing bacteriophages

None of the broad host range listeriophages were capable of generalized transduction. For those bacteriophages capable of infecting 10403S, 17 out of 25 were capable of transduction (Table 1). The frequency of transduction [number of transductants per 10^7 plaque-forming units (pfu)] varied from 1–35 (ϕLMUP111, ϕLMUP32a, ϕLMUP32b, ϕLMUP35, ϕLMUP322, A620, A502, A118, A006, ϕEF1, ϕEF2, ϕEF3, ϕEF4 and ϕCU-EN1/96) to 100–300 (ϕCU-SI153/95, ϕCU-SI165/94 and ϕEGD857). Four bacteriophages, ϕLUMP35 (P35), A620, A502 and ϕCU-SI153/95 (U153), were chosen for their different characteristics, i.e. host range, transduction frequency, temperature sensitivity and capacity, and subjected to further characterization. P35 was of particular interest as it had a wide host range, i.e. it grew on 75% of all serotype 1/2 *L. monocytogenes* tested (Table 1).

Sizes of bacteriophages

ϕLMUP111, A620 and U153 were cultured on a large scale, and the virions purified on caesium chloride step

gradients. The sizes of the genomic DNAs were obtained by sizing restriction fragments. The sizes were 37.9 kb for ϕLMUP111, 38.3 kb for A620 and 40.8 kb for U153. P35 is a novel bacteriophage of the family Siphoviridae, order Caudovirales (Professor Hans-Wolf Ackermann, personal communication), especially considering its host range, and has been deposited in the Felix d'Herelle Reference Center for Bacterial Viruses at the University of Laval, Quebec, Canada under the accession number HER 247. P35 plaques were large and had cloudy centres. However, bacteria isolated from within the plaque were P35 sensitive, and we were unable to isolate a P35 lysogen. U153 is a member of the very common *Listeria* bacteriophage species 2671 of the family Siphoviridae, order Caudovirales (Professor Hans-Wolf Ackermann, personal communication).

Having the size of the bacteriophage DNA capacity and the size of the *L. monocytogenes* genome, we can work out the true number of transducing particles in a bacteriophage lysate. For A620, the average number of transductants per 10^7 pfu was 11.08 ± 9.0 (13 samples). The size of the bacteriophage DNA was 38.3 kb, which is 1/82.7th of the *L. monocytogenes* genome. Therefore, the ratio of transducing particles to virions in an A620 lysate is $\approx 10^{-4}$. The same ratio was true of ϕLMUP111 and A502, which have the same genome size as A620 (Table 1) and produced the same number of transductants per 10^7 pfu. For U153 the average number of transductants per 10^7 pfu was 283 ± 260 (6 samples). The size of the bacteriophage DNA was 40.8 kb, which is 1/77.2th of the *L. monocytogenes* genome. Therefore, the ratio of transducing particles to virions in a U153 lysate was 4.56×10^{-2} (4.6%). We do not know the genome sizes of ϕEGD857 or ϕCU-SI165/94, but they gave similar transduction frequencies to U153, unfortunately these two bacteriophages have the same limited host range as U153 (Table 1).

A number of interesting mutants of *L. monocytogenes* have been isolated using the 26 kb transposon Tn*1545* (Gaillard *et al.*, 1991). Considering the small size of some of the bacteriophages, the possibility arose that they might not be able to transduce with markers caused by this large transposon. To test this we used DP-L212 (*hly*::Tn*1545* Str^R) as donor and DP-L184 (Str^R) as recipient (Tables 2 and 3), using P35, A620 and U153 as potential transducing agents. A620 and P35 failed to generate transductants. U153 did generate Hly^- transductants, but they were a minor (10–25%) component of the total population. The simplest interpretation of these results is that DP-L212 contains more than one copy of Tn*1545,* only one of which is inserted in the *hly* gene. The frequency of Tn *1545* transduction was 10-fold lower than for markers, including Tn*917*-LTV3 (15.5 kb). U153 has a genome size of 40.8 kb, whereas A620 has a genome size of 38.3 kb.

Transduction is generalized

There remained the possibility that the transducing bacteriophages were only capable of specialized transduction of the *ade* gene, so the ability to transduce with other markers was tested. DP-L1775 (*aro*::Tn*917*-LTV3), DP-L1777 (*phe*::Tn*917*-LTV3), DP-L1786 (*gly*::Tn*917*-LTV3), DP-L1809 (*thr*::Tn*917*-LTV3), DP-L1822 (*pro*::Tn*917*-LTV3), DP-L1839 (*nia*::Tn*917*-LTV3) and DP-L1851 (*ura*::Tn*917*-LTV3) (Table 3) were used as donors for P35, and DP-L2161 was used as recipient. Transductants were found in all cases at the same frequency as for the *ade* marker, and all transductants were non-haemolytic and bore the appropriate auxotrophic marker. A620 and U153 were also grown on donors DP-L1786 (*gly*::Tn*917*-LTV3), DP-L1809 (*thr*::Tn*917*-LTV3) and DP-L1822 (*pro*::Tn*917*-LTV3) and both transduced DP-L2161 with the same frequency as when grown on DP-L1764 (*ade*::Tn*917*-LTV3). A large number of U153 transductants were tested for bacteriophage sensitivity and in all cases no lysogens were found, showing the effectiveness of the high temperature, citrate plus low moi regime.

Transduction can occur in serotype 1/2 strains other than 10403

We wished to see if transduction of EGD derivatives was possible. The only appropriately marked strain was BUG5, which contains a Tn*1545* induced *inlAB* mutation. We could not use A620 or P35 as they could not pick up Tn*1545* marked genes and U153 could not grow on EGD. We therefore tested A502 for its ability to carry Tn*1545*. We used BUG5 as a donor and DP-L857 and DP-L1595 as recipients. We could ensure that any resultant transductants were not spontaneous mutants because BUG5 is $Str^{R,}$ whereas DP-L857 and DP-L1595 are Str^S. From both crosses we isolated two potential transductants. All four colonies were Str^S, Em^R, Tc^R, and able to infect fibroblasts (L-cells) but unable to infect Henle (epithelial) cells, i.e. Inl^-. Therefore, the Tn*1545* was linked to the *inlAB* mutation, as expected from the results of Gaillard *et al.* (1991), and A502 was capable of transfer of Tn*1545* and transduction of EGD. The genome size of A502 has been reported to be 39 kb (Loessner and Busse, 1990), which is not much larger than that of A602. The frequency of transduction with the Tn*1545*-marked gene was 10-fold less than that with the Tn*917*-LTV3-marked gene.

Having demonstrated that A502 could mediate transduction of EGD strains in addition to 10403S, we also tested whether P35, A602 and U153 could mediate transduction of other strains. The donors and recipients were: Mack, DP-L1415 (*hly*::Tn*917*-LTV3) and DP-L861 (wt); and L028, Bof415 (*hly*::Tn*917*-LTV3) and DP-L1952

(wt). The P35 Mack/Mack and L028/L028 transduction frequencies were reduced 10-fold compared with those of the 10403/10403 crosses. Transductions of Mack to Mack by A602 and U153 were also reduced 10-fold compared with 10403/10403 transductions.

Genes can be exchanged between strains

Having shown that transductions were possible within some strains, we then asked if interstrain transductions were possible. P35 was grown on DP-L1777 (*phe*::Tn*917*-LTV3) and transduction was attempted with DP-L861 (Mack wt), DP-L857 (EGD wt) and DP-L1952 (L028 wt) as recipients. The 10403/Mack cross was successful with a twofold higher frequency of transductants than the equivalent 10403/10403 cross. The 10403/EGD cross was also successful, but with a fourfold decrease in transduction frequency compared with the equivalent 10403/10403 cross. Half of the resultant transductants were Phe^+, which implies that the Tn*917*-LTV3 had jumped. The 10403/L028 cross yielded the same number of transductants as the equivalent 10403/10403 cross and again half were Phe^+. The apparent increase in transposition in the interstrain crosses with L028 and EGD presumably reflects chromosome sequence differences between these strains and 10403. We never saw any evidence of Tn*917*-LTV3 transposition in any of the 10403/10403 crosses using any of the bacteriophages.

A620 was grown on DP-L1764 (*ade*::Tn*917*-LTV3) and transduction was attempted with DP-L861 (Mack wt) and DP-L857 (EGD wt). A620 was also grown on DP-L973 (*hly*::Tn*917*-LTV3) and DP-L1075 (*prfA*::Tn*917*-LTV3) and transduction was attempted with DP-L3332 (NCTC7973 wt). Successful interstrain transduction was achieved with the 10403/Mack and 10403/NCTC7973 crosses at the same frequency as the intrastrain transduction. The 10403/EGD transduction was reduced more than 10-fold and 10% of the transductants did not carry the Tn*917*-LTV3 induced marker. Again this implies secondary transposition.

U153 was grown on DP-L1764 (*ade*::Tn*917*-LTV3), DP-L1786 (*gly*::Tn*917*-LTV3), DP-L1809 (*thr*::Tn*917*-LTV3) and the Mack derivative DP-L1415 (*hly*::Tn*917*-LTV3) and tested for ability to transduce DP-L3332 (NCTC7973 wt) with the transposon marker. In all cases, interstrain transduction was possible, but the frequency was reduced 10-fold compared with the 10403/10403 crosses and so were the same as the Mack/Mack crosses.

Mack transducing bacteriophages

A number of bacteriophages were isolated from lysogens that grew on Mack but could not infect 10403 and EGD or their derivatives (ϕ10403, ϕATCC43251, ϕCU-F31/93, ϕCU-F34/93, ϕCU-SI133/93, ϕCU-SI162/94, ϕEGDSm^R and ϕLL124). There were other bacteriophages that were isolated from different stocks of the same strain, but these were ignored as possible duplicates. DP-L1415 (*hly*::Tn*917*-LTV3) (Table 3) was used as a donor and DP-L861 (wild type) as a recipient and each of the bacteriophages tested for transduction ability. ϕATCC43251 and ϕCU-F31/93 failed to yield transductants. All the others yielded between two and 51 transductants per 10^7 pfu used. All of the putative transductants proved to be true transductants.

Serotype 4b transducing bacteriophages

The freshly isolated and previously characterized bacteriophages that grew on serotype 4b strains were found in each case to grow either on 4 bs derivatives or F2381 derivatives. Some grew on both. This therefore allowed all of the bacteriophages to be tested for ability to carry out transduction. Either FlaY (Str^R *fla*-Tn*916*ΔE), the nonmotile 4 bs derivative, was used as donor and 4b1 (Str^R) as recipient, or DK1 (Str^R *zzz*::Tn*916*ΔE), the F2381 derivative with no phenotype associated with the transposon, as donor and 2381L (Str^R) as recipient. Transductants were obtained with 11 bacteriophages, not one of which was a broad host range bacteriophage. Six of the transducing bacteriophages infected F2381: ϕLCDC82-130, ϕLCDC 81-861, ϕDD2359, B021, A500 and 90666, and five infected 4 bs: ϕLMUP4b6, ϕLMUP4b6N1, ϕLMUP4b6N2, ϕL99-1 and A640. Of particular interest was bacteriophage ϕL99-1, because this bacteriophage was capable of infection of Scott A, which is probably the most well-characterized serotype 4b *L. monocytogenes* strain. Bacteriophage A500 is an interesting bacteriophage. It was deposited in the ATCC from Stanford many years ago and it is a matter of record that the first *Listeria* sp. bacteriophage was isolated in Stanford (Schultz, 1945). The transduction frequencies were of the order of 10 per 10^7 pfu for ϕLCDC82-130, ϕLCDC 81-861, ϕDD2359, A500, 90666, ϕLMUP4b6, ϕLMUP4b6N1, ϕLMUP4b6N2 and A640, but were 10-fold lower for B021 and ϕL99-1. The genome size of A500 is the only one known at 38 kb. Therefore, the ratio of transducing particles to virions is the same as for A620, i.e. $\approx 10^{-4}$.

Conclusions

Generalized transduction is a common property of a wide range of listeriophages. Unfortunately, with the exception of the novel bacteriophage, P35, broad host range and transduction ability has so far proved to be incompatible. There are bacteriophages capable of very high frequencies of transduction of the serotype 1/2a strain, e.g. U153. However, unfortunately, these seem to have the narrowest of host ranges. It has proved possible to identify

bacteriophages capable of transducing all of the *L. monocytogenes* strains tested. The strategy of isolating bacteriophages that only replicate at room temperature and the use of citrate and low moi proved very successful. Low frequency transducing bacteriophages were identified and lysogenic transductants were not found.

Transduction was used in a large number of cases to demonstrate unequivocally whether a particular transposon insertion had or had not caused a mutant phenotype (see above, and unpublished results). In some cases, we found evidence that some serotype 1/2a and 4b strains contained multiple inserts of Tn*916* and Tn*1545*, and we were able to isolate transductants that contained the single insertion that caused a mutant phenotype (see above for DP-L212, and unpublished results). We have recently used U153 to transposon-tag (Kaiser, 1984) a pathogenicity mutation. U153 was used to transduce the point mutant with a pool of Tn*917* insertions. A transductant in which the wild-type pathogenicity phenotype was recovered proved to have a transposon inserted within $\approx$ 11 kb of the mutation (Jonathan Telfer, unpublished).

All of the bacteriophages discussed in this paper that we have isolated are available to the scientific community, and we will be happy to send them on request. If anyone has used any *L. monocytogenes* serotype 1/2 or 4b strains that have not been tested here for transducing bacteriophage sensitivity, they are welcome to send them to our laboratory and we will test them and send out any useful bacteriophage(s).

Experimental procedures

Bacteria and bacteriophage strains and media

The *L. monocytogenes* strains used are listed in Tables 2 and 3. The bacteriophages used are listed in Table 1. *L. monocytogenes* strains were cultured in brain–heart infusion (BHI) broth (Difco) or on BHI broth solidified with 1.5% agar. *L. monocytogenes* lawns were prepared in 3 ml of LB top agar (0.7% agar) on a LB plate (1.5% agar) supplemented with 10 mM magnesium sulphate and 10 mM calcium chloride. LB medium is Luria–Bertani medium (Maniatis *et al*., 1982).

Our minimal medium was based on that of Premaratne *et al*. (1991), following Marquis *et al*. (1993). Unfortunately, this medium would not support colony formation if solidified. We modified the Premaratne medium by reducing the phosphate concentration 10-fold and buffering instead with 100 mM MOPS. This modified medium would support colony formation and patch growth after velvet pad replication. L3M (*Listeria monocytogenes* 10403 minimal medium) contained 100 mM MOPS pH 7.4, 4.82 mM KH_2PO_4, 11.55 mM Na_2HPO_4, 1.7 mM $MgSO_4$, 55 mM glucose, 4.11 mM glutamine, 0.67 mM methionine, 0.635 mM cysteine, 0.47 mM arginine, 0.76 mM isoleucine, 0.76 mM leucine and 0.85 mM valine, 24 pM thioctic acid (lipoic acid), 2.96 μM thiamine, 1.33 μM riboflavin and 2.05 μM biotin. The MOPS was autoclaved as a 10$\times$ solution, the phosphate salts were autoclaved as a 100$\times$ solution, the $MgSO_4$ was autoclaved as a 100$\times$ solution, the glucose was autoclaved as a 50$\times$ solution, the amino acids were filter sterilized as a 100$\times$ solution, the thioctic acid was filter sterilized as a 10 000$\times$ solution and the rest of the vitamins were filter sterilized as a 500$\times$ solution.

Blood plates consisted of 12 ml of LB agar, (1.5%) containing 5% of defibrinated sheep's blood, in a $\approx$ 9 cm diameter Petri plate. This very thin plate allowed ready visualization of the weak zone of haemolysis around haemolytic 10403 derivatives after velvet replication of patches.

Bacteriophage isolation

Bacteriophages were isolated from lysogens by culturing the *L. monocytogenes* strains at room temperature and then placing drops of filtered culture supernatant onto lawns of susceptible *L. monocytogenes* strains incubated at room temperature and looking for plaques. The cultures were filtered to remove intact bacteria. We avoided the use of chloroform as some bacteriophages are sensitive to the solvent (Table 1, note 9). Effluent from Finham sewage treatment plant, Coventry, UK, was collected, filtered, spotted onto susceptible *L. monocytogenes* lawns, incubated at room temperature and plaques picked. Bacteriophages were isolated from silage known to contain *Listeria* spp. supplied by Martin Weidmann of Cornell University. The silage was washed with TM buffer, the filtered washings spotted onto lawns of susceptible *L. monocytogenes,* incubated at room temperature and resultant plaques picked.

Tissue culture

Plaque assays on L2 (mouse fibroblast) and Henle 407 (human epithelial) cell lines were performed as in Sun *et al*. (1990) and Marquis *et al*. (1995) respectively.

Bacteriophage manipulation

All bacteriophages were cultured in lawns of *L. monocytogenes* on LB agar plus 10 mM $MgSO_4$ and 10 mM $CaCl_2$ incubated for 48 h at room temperature. Lysates were harvested in sterile TM buffer (8 mM $MgSO_4$ plus 10 mM Tris HCl pH 8.0) and cleared of bacteria using filter sterilization (chloroform-sensitive bacteriophage) or the addition of chloroform. The bacteriophage concentration of the lysate was determined as plaque-forming units (pfu) ml^{-1}.

Transduction of Listeria monocytogenes

Bacteriophage (10^7 and 10^8 pfu) grown on the appropriate donor strain was mixed with 10^8 of mid-exponential phase recipient cells, and the mixture incubated at room temperature for 40 min. The selection of transductants depended on the nature of the drug resistance marker used.

For tetracycline resistance, i.e. Tn*916* or Tn*1545,* 3 ml of molten BHI top agar (BHI plus 0.75% agar) plus 10 mM sodium citrate pH 7.5 (N.B. citrate has no effect on *L. monocytogenes* growth or drug sensitivity) was added to the mixture and poured onto a BHI plate containing 10 mM sodium citrate pH 7.5 and 12.5 μg ml^{-1} tetracycline. Incubation at

37°C for 2–3 days was necessary before transductant colonies appeared. The transductants were picked onto 12.5 $\mu g\,ml^{-1}$ tetracycline BHI citrate plates.

For erythromycin resistance, i.e. Tn*917*, *Tn916*ΔE or Tn*1545,* 3 ml of molten BHI top agar plus 10 mM sodium citrate pH 7.5 and 1.33 $\mu g\,ml^{-1}$ erythromycin was mixed with the cells and bacteriophages, and the mixture poured onto a BHI plate containing 10 mM sodium citrate pH 7.5 and no antibiotic. The plate was incubated at 37°C for 2 h before another 3 ml of molten BHI top agar plus 10 mM sodium citrate pH 7.5 and 13.33 $\mu g\,ml^{-1}$ erythromycin was poured on top. The plate was incubated at 37°C until colonies formed. The transductants were picked onto 1 $\mu g\,ml^{-1}$ erythromycin BHI citrate plates. The reason for the double overlay procedure is that the erythromycin resistance determinant is inducible, and this ensures induction before inactivation of the ribosomes (Philip Youngman, personal communication). There is a possibility that chromosomal erythromycin-resistant mutants may be selected by this procedure. One way this was avoided was to add 0.333 $mg\,ml^{-1}$ lincomycin to the final overlay. Alternatively, we picked the erythromycin-resistant colonies onto 25 $\mu g\,ml^{-1}$ lincomycin BHI citrate plates to ensure the colonies were transductants and not mutants.

The problem with using tetracycline selection was that there were always lower yields of transductants than when erythromycin selection was used. We were able to directly compare the selections when transducing with a Tn*1545* insert, as this transposon encodes both resistances.

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