Isolation, classification and molecular characterization of bacteriophages for *Enterobacter* species

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Out of 22 *Enterobacter* phages investigated, nine were found to be suitable for phage typing based on their different lytic spectra on 398 strains of *Enterobacter* spp. isolated from milk powder and other foods. These phages were compared on the basis of morphology, protein composition, restriction endonuclease patterns and DNA–DNA hybridization. Two phages (WS-EP19, WS-EP13) belonged to the Podoviridae family (morphotype C1), and three (WS-EP20, WS-EP26, WS-EP28) were classified as Siphoviridae (morphotype B1). The other four phages were Myoviridae of the morphological groups A1 (WS-EP57) and A2 (WS-EP32, WS-EP94, WS-EP96). SDS-PAGE revealed individual protein profiles for each phage, which corresponded to different restriction enzyme fragment patterns. DNA–DNA hybridization demonstrated the close relationship of phages WS-EP20 and WS-EP26, and of WS-EP94 and WS-EP96. In general, a good correlation was found between groupings obtained with the various methods. The nine phages could be attributed to existing enterobacterial phage species although some differences to the described type phages were observed.

Introduction

Enterobacteria represent a dominant portion of the 'recontamination flora' of dairy products made from pasteurized milk (Frazier & Westhoff, 1978; Keogh, 1971). Their presence often indicates inadequate sanitization and/or faulty construction of the processing equipment (Schmidt-Lorenz & Spillmann, 1988). Members of the genus *Enterobacter* are frequently found in dried products such as milk powder. Although they are of little pathological significance, their presence may indicate low-frequency contamination by ecologically similar enterobacterial pathogens such as *Salmonella*.

In order to correlate *Enterobacter* strains isolated from contaminated products and to identify possible sources and routes of contamination, they need to be differentiated beyond the species and biovar level. Serological discrimination of 79 different serovars among the genus *Enterobacter* has been reported by Sakazaki & Namioka (1960). However, this technique depends on the ready availability of more than 100 different antisera, and therefore seems unsuitable for routine typing of a large number of strains. Bacteriophage typing has often proved to be a practical and successful method for this

purpose (see Ackermann & DuBow, 1987). However, no phage typing system is yet available for differentiation of *Enterobacter* strains.

Only very few phages for *Enterobacter* have been studied to any extent (Grimont *et al.*, 1978; Popovici *et al.*, 1976). Neither exact particle dimensions nor data regarding their proteins or DNA are available for these phages, except that three of them belong to Bradley's morphotype C3 (Grimont *et al.*, 1978).

One aim of the present study was to isolate phages that were sufficiently specific for *Enterobacter* spp., and gave a high typability of strains. However, our main objective was to provide a detailed characterization and classification of these phages by electron microscopy, protein analysis, digestion of phage DNAs with various restriction enzymes and DNA–DNA hybridization.

Methods

Bacterial strains and culture conditions. Most of the 398 strains of *Enterobacter* spp. and most of the other enterobacteria used in this study (see Table 3) were isolated in our laboratory from milk powder and other foods (U. Schramm, unpublished results). The remaining strains were taken from the 'Weihenstephan culture collection' (WS), including the type strains of all species tested. Bacteria were cultivated on plate count (PC) agar or in PC-broth (Merck). For titration of phages, soft-agar was used (PC-broth supplemented with 0.4% agar). All strains were stored on PC-agar slants at 4 °C and transferred bimonthly. Incubation temperature was either 30 °C or 37 °C.

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Table 1. *Enterobacter* phages and corresponding propagating strains employed

Phages		Propagating strains		
Code	Source	Species	Code	Source
WS-EP19	Sewage	<i>Enterobacter cloacae</i>	W009	Minced meat
WS-EP13	Sewage	<i>Enterobacter cloacae</i>	W008	Minced meat
WS-EP20	Sewage	<i>Enterobacter cloacae</i>	W011	Milk powder
WS-EP26	Sewage	<i>Enterobacter cloacae</i>	W012	Milk powder
WS-EP28	Sewage	<i>Enterobacter cloacae</i>	W012	Milk powder
WS-EP57	ATCC	<i>Enterobacter sakazakii</i>	W212	Milk powder
WS-EP32	Sewage	<i>Enterobacter cloacae</i>	W019	Milk powder
WS-EP94	Sewage	<i>Enterobacter cloacae</i>	W096	Milk powder
WS-EP96	Sewage	<i>Enterobacter cloacae</i>	W008	Minced meat

Isolation and propagation of phages. A total of 21 phages was isolated from sewage and waste water by methods detailed previously (Loessner & Busse, 1990). Briefly, aqueous samples were centrifuged, sterile-filtered, concentrated, and spotted on lawns of indicator bacteria. Phage plaques could then be picked and amplified by the soft agar double-layer method of Adams (1959). To ensure purity of the phage preparations, isolated plaques were repeatedly picked and plated.

Phage EP57 was obtained from the ATCC (American Type Culture Collection; no. 23355B-1).

High-titre phage lysates were prepared with the double-layer technique, using carefully selected propagating strains.

Determination of host range. This was done using the reversed phage typing procedure as outlined elsewhere (Loessner, 1991). Agar plates were preinoculated with the phage suspensions to be tested, carefully flooded with bacterial broth cultures, and incubated for 16–24 h. Nine of the 22 tested phages were found to differ in their lytic spectra, and were selected for further investigation (Table 1).

Virus concentration and purification. Phage concentration was done by precipitation with PEG-8000 (Sigma), essentially according to Yamamoto *et al.* (1970). Particles were purified on a modified CsCl step-gradient as described earlier (Loessner & Busse, 1990; Sambrook *et al.*, 1989).

Electron microscopy. Purified phage particles were adsorbed to thin carbon films, placed on 400-mesh copper grids (Balzers), negatively stained with 2% (w/v) uranyl acetate, and examined in a Zeiss EM 10 electron microscope at an acceleration voltage of 80 kV. For calibration, catalase crystals (Luftig, 1967) and a standard cross grating ($d = 0.463 \mu\text{m}$; both from Balzers) were used.

SDS-PAGE profiles. In order to remove residual CsCl, phage suspensions were washed with sterile, ultrapure water (Milli-Q; Millipore), pelleted at 150 000 *g* for 90 min, and resuspended in sterile water to an optical density (OD_{260}) of 0.2–0.3. Suspensions were mixed with concentrated SDS-sample buffer and heated to 95 °C for 5 min. Ten microlitres of each were then analysed on an ultrathin, horizontal poregradient (8–18% T) gel (ExcelGel, Pharmacia) as reported previously (Zink & Loessner, 1992). Proteins were stained with Coomassie R-350 (Pharmacia), and the molecular size marker SDS-7L (Sigma) was used for calibration.

Extraction of phage DNA. The standard procedure of Sambrook *et al.* (1989) was used. Purified DNAs were resuspended in TE buffer (10 mM-Tris, 1 mM-EDTA, pH 8.0) and stored at –20 °C.

Restriction enzyme analysis. Purified phage DNAs were digested with restriction enzymes (see Table 5) according to the supplier's recom-

mendations (United States Biochemical Corp.). Generated fragments (approx. 0.7 μg total) were electrophoretically separated on 0.8% agarose gels in TAE buffer (Sambrook *et al.*, 1989). Fragment sizes were determined from co-migrating *Hind*III and *Bst*EII digests of λ DNA (Sigma).

Southern blotting and DNA–DNA hybridization. *Eco*RV-generated DNA fragments were vacuum blotted (VacuGene XL, Pharmacia) onto positively charged nylon membranes (Boehringer), covalently bound by UV irradiation (312 nm, 0.7 J cm^{–2}), and hybridized to digoxigenin-dUTP-labelled DNA from selected phages (random primed DNA labelling kit, Boehringer). Hybridization was carried out without formamide under conditions of high stringency [5 × SSC (0.75 M-NaCl, 75 mM-trisodium citrate, pH 7.0), 68 °C], as were the subsequent washes (0.1 × SSC, 0.1% SDS, 68 °C). Chemiluminescent detection of hybridization signals was done as outlined in the manufacturer's (Boehringer) protocol, except that the X-ray film exposure times could be shortened to 30–90 s.

Results and Discussion

Phage specificity and suitability for phage typing

Host ranges of the nine selected phages on their seven propagating strains are given in Table 2. Although WS-EP26 and WS-EP28 are identical with respect to these strains, they differed by lytic activity when 447 other strains of *Enterobacter* spp. and other *Enterobacteriaceae* (Table 3) were tested with the nine phages. Interestingly, some of the phages were also able to lyse strains of *Escherichia coli* (WS-EP26, WS-EP28, WS-EP57, WS-EP96) and *Serratia marcescens* (WS-EP20). This can be explained by the close relationship among the enterobacterial genera, which is reflected in the host-range of their phages (Ackermann & DuBow, 1987). These authors also state that many enterobacterial phages are polyvalent and cross intergeneric boundaries, while still retaining some specificity and preferences. Because the phages investigated here clearly show such preferences towards the genus *Enterobacter*, they should be referred to as *Enterobacter* phages.

Table 2. *Lytic spectra of Enterobacter phages on propagating strains*

Phages	<i>Enterobacter</i> strains						
	W008	W009	W011	W012	W019	W096	W212
WS-EP19	–	+	–	–	–	–	–
WS-EP13	+	–	–	–	–	–	–
WS-EP20	–	–	+	+	–	–	–
WS-EP26	–	–	–	+	–	–	–
WS-EP28	–	–	–	+	–	–	–
WS-EP57	–	–	–	–	–	–	+
WS-EP32	–	–	–	–	+	–	–
WS-EP94	–	–	–	–	–	+	–
WS-EP96	+	–	–	–	+	+	+

+, Lysis; –, no lysis.

Table 3. Phage sensitivity of 398 *Enterobacter* strains and 56 other *Enterobacteriaceae*

Species	No. of strains sensitive/no. tested
<i>Enterobacter cloacae</i>	338/362 (93.4%)
<i>Enterobacter agglomerans</i>	10/17 (58.8%)
<i>Enterobacter sakazakii</i>	8/13 (61.5%)
<i>Enterobacter aerogenes</i>	0/6
<i>Escherichia coli</i>	4/14 (28.5%)
<i>Serratia marcescens</i>	1/2 (50.0%)
<i>Serratia liquefaciens</i>	0/7
<i>Klebsiella pneumoniae</i>	0/8
<i>Klebsiella oxytoca</i>	0/10
<i>Klebsiella ozaenae</i>	0/1
<i>Citrobacter freundii</i>	0/5
<i>Salmonella arizonae</i>	0/1
<i>Salmonella enteritidis</i>	0/1
<i>Salmonella infantis</i>	0/1
<i>Salmonella tennessee</i>	0/1
<i>Erwinia amylovora</i>	0/1
<i>Erwinia carotovora</i>	0/1
<i>Erwinia herbicola</i>	0/1
<i>Erwinia stewartii</i>	0/1
<i>Hafnia alvei</i>	0/2
<i>Proteus mirabilis</i>	0/1
<i>Proteus vulgaris</i>	0/1

More than 90% of all *Enterobacter* spp. strains tested were lysed by one or more phages, with the highest typability among strains of *Enterobacter cloacae*. However, none of our phages was able to lyse any of the six tested strains of *Enterobacter aerogenes*. Host ranges of the phages were found to be quite different, which makes them useful for strain differentiation (i.e. phage typing). Sixty-six different lytic patterns (phagovars) could be observed among the 356 typable strains of *Enterobacter* spp. The frequency of phagovars varied considerably: 31 patterns could be assigned to single isolates only, while the five most frequent phagovars were observed among 34% of the typable strains. Further attempts should be made to isolate phages able to lyse strains of *E. aerogenes*, as well as to increase the number and discrimination of typable strains. However, the *Enterobacter* phages isolated and described in this study constitute a useful basis for phage typing. They are freely available upon request.

Phage morphology and taxonomic classification

As shown in Fig. 1, four different morphological types were observed among the investigated bacteriophages, and could be placed into three phage families. Their exact dimensions are given in Table 4. Phages WS-EP19 and WS-EP13 are members of the Podoviridae family, and correspond to morphotype C1 of Bradley (1967) (see

also Ackermann & Eisenstark, 1974). Phages WS-EP20, WS-EP26 and WS-EP28 were found to belong to morphological group B1 within the Siphoviridae family. The other phages are Myoviridae. However, while WS-EP57 has an isometric capsid (A1 morphotype), phages WS-EP32, WS-EP94 and WS-EP96 show slightly prolate capsids, which is characteristic for morphological group A2. The latter phages also have a clearly visible collar and long tail fibres.

Enterobacter phages may be classified using the updated scheme for morphological classification of tailed enterobacterial phages (Ackermann & DuBow, 1987). The phages characterized here may be related to existing morphological species: WS-EP19 seems very similar to phage species sd (head diameter 69 nm, tail length 19 nm, many short tail fibres). Although WS-EP13 is quite similar to WS-EP19, its slightly shorter tail corresponds more to species N4 (head diameter 70 nm, tail length 10 nm). *Enterobacter* phages WS-EP20, WS-EP26, and probably WS-EP28 (capsid diameter 65 nm, average tail length 222 nm) may be related to phage species X (capsid 63 nm, tail length 227 nm), a flagella-specific phage of *Salmonella*, *Serratia*, and *E. coli* (H.-W. Ackermann, personal communication). WS-EP57 could be related to *Citrobacter* phage FC3-9 (capsid 84 nm, tail length 115 nm), although it lacks the curly lateral tail fibres described for FC3-9 (H.-W. Ackermann, personal communication). Finally, phages WS-EP32, WS-EP94 and WS-EP96 can be regarded as members of the species T4 (head dimensions 111 × 78 nm; tail length 113 nm; 24 tail striations), the type phage of the T-even bacteriophages (Ackermann & DuBow, 1987). In fact, the protein composition of T4 is very similar to the profiles of WS-EP32, WS-EP94 and WS-EP96 (data not shown). A recent study (Maftahi, 1992) reports that 18 T-even type phages investigated fall into 6 serological and DNA homology groups. It would be interesting to see if the two homology groups described here (see below) could be assigned to any of these groups.

Protein composition of phage particles

Electrophoretic profiles of phage structural proteins have repeatedly been found to be a very useful means of phage differentiation and characterization (Coveney *et al.*, 1987; Mata *et al.*, 1986; Stewart *et al.*, 1985; Zink & Loessner, 1992), and allow for both the distinction of individual phages and the establishment of groups. SDS-PAGE profiles of the *Enterobacter* phage structural proteins are presented in Fig. 2. Protein compositions are different for each phage, although some similarities with respect to number and size of major protein bands can be seen. These clusters (e.g. WS-EP20 and WS-EP26; WS-EP94 and WS-EP96) correspond well to the results of

Fig. 1. Electron micrographs of *Enterobacter* phages of different morphotypes. Bar, 100 nm. (a) WS-EP19; (b) WS-EP20; (c) WS-EP57; (d) WS-EP32; (e) WS-EP94.

restriction enzyme analysis and DNA–DNA hybridization, as demonstrated later.

However, protein compositions should be interpreted with care, since even completely different phages may possess structural proteins of a comparable size [see Fig. 2, lane D (WS-EP26) and lane F (WS-EP57)]. On the other hand, phages of a similar morphology may show notably different protein profiles [see Fig. 2, lane A (WS-EP19) and lane B (WS-EP13)]. Therefore, protein analyses seem to be most useful for comparison of

morphologically similar phages. In addition, all visible protein bands should be compared, since the minor bands in particular enable the differentiation of closely related phages.

Restriction endonuclease patterns of phage DNAs

Table 5 summarizes the results of restriction enzyme analysis of the nine *Enterobacter* phage DNAs. All but one DNA were digested by *EcoRV* (Fig. 3). As expected

Table 4. Morphology and dimensions (in nm) of *Enterobacter* phages studied

Phage	Morpho-type	Capsid	Tail			<i>n</i> *
			Length	Diameter	Contractile	
WS-EP19	C1	70 ± 3	20 ± 2	12 ± 2	no	6
WS-EP13	C1	68 ± 3	12 ± 2	12 ± 2	no	5
WS-EP20	B1	65 ± 2	225 ± 8	13 ± 1	no	6
WS-EP26	B1	65 ± 2	220 ± 8	14 ± 1	no	8
WS-EP28	B1	68 ± 2	217 ± 8	14 ± 1	no	6
WS-EP57	A1	82 ± 3	127 ± 4	20 ± 2	yes	7
WS-EP32	A2	113 × 83 ± 3	112 ± 3	22 ± 2	yes	9
WS-EP94	A2	111 × 84 ± 3	113 ± 3	21 ± 2	yes	8
WS-EP96	A2	111 × 82 ± 3	113 ± 3	21 ± 2	yes	8

* *n*, Number of measured particles.

Fig. 2. SDS-PAGE (8–18% T) electrophoretic profiles of *Enterobacter* phage structural proteins. Lanes 7L, molecular size standard. Other lanes: A, WS-EP19; B, WS-EP13; C, WS-EP20; D, WS-EP26; E, WS-EP28; F, WS-EP57; G, WS-EP32; H, WS-EP94; I, WS-EP96.

Table 5. Restriction enzyme analysis of *Enterobacter* phage genomes

DNA from phage	No. of fragments detected after digestion with:					
	<i>Hind</i> III	<i>Bam</i> HI	<i>Eco</i> RI	<i>Eco</i> RV	<i>Kpn</i> I	<i>Sac</i> I
WS-EP19	—	—	—	19	—	—
WS-EP13	10	13	9	21	10	—
WS-EP20	—	—	—	2	≥ 35	—
WS-EP26	—	—	—	4	23	—
WS-EP28	—	12	18	17	≥ 35	—
WS-EP57	≥ 30	—	—	≥ 35	—	—
WS-EP32	—	—	—	—	—	—
WS-EP94	—	—	≥ 35	≥ 35	—	—
WS-EP96	—	—	≥ 35	≥ 35	—	—

from the results of protein analysis, no identical fragment patterns could be observed. Nevertheless, some similarities were detected (see Table 5; WS-EP20 and WS-EP26; WS-EP94 and WS-EP96). These were not host-dependent (restriction/modification systems), since the

phages in question were propagated on different strains (see Table 1). Phages WS-EP32, WS-EP94 and WS-EP96 are morphologically very similar to coliphages of the T-even type. Therefore, it may be assumed that the resistance of their DNA (especially WS-EP32) to most tested restriction enzymes is due to phage-encoded modification and/or glycosylation of bases (Warren, 1980), rather than the lack of recognition sites within their quite large genomes.

Approximate sizes of some phage genomes could be calculated from fragment lengths for phage WS-EP13 (48 kb), WS-EP26 (61 kb) and WS-EP28 (63 kb).

DNA homologies correlate well with morphology and protein profiles

Digoxigenin-labelled random primed DNAs from four phages (selected according to morphological group) were hybridized to *Eco*RV-generated fragments of all investigated phages (Fig. 4). DNA from phage WS-EP19 did

Fig. 3. Agarose gel electrophoresis patterns of *EcoRV*-generated fragments of phage DNAs. Molecular size standards: lane M, λ -*HindIII* digest; lane M', λ -*BstEII* digest. Other lanes: A, WS-EP19; B, WS-EP13; C, WS-EP20; D, WS-EP26; E, WS-EP28; F, WS-EP57; G, WS-EP32; H, WS-EP94; I, WS-EP96.

not hybridize with any other DNA, indicating a lack of homology at the DNA level, even to the morphologically similar phage WS-EP13. This was not surprising, considering their extremely different protein profiles.

All fragments of WS-EP26 were detected by labelled WS-EP20 DNA, underlining the close relationship of these two phages, which has already been shown by SDS-PAGE and electron microscopy. No signal was observed with WS-EP28, despite their similar morphology and host range. However, protein profiles and restriction enzyme analyses suggested their unrelatedness at this low level.

As expected, WS-EP57 DNA hybridized with itself only, confirming the unique characteristics of this phage.

Cross-hybridization using WS-EP94 DNA as a probe revealed extensive homology to WS-EP96, and no signal was seen with WS-EP32. As in WS-EP28, protein composition and resistance to endonucleases set WS-EP32 apart from WS-EP94 and WS-EP96, despite essentially identical morphology.

Fig. 4. Southern blots of *EcoRV* DNA fragments of *Enterobacter* phages and hybridization with digoxigenin-labelled DNAs of phages WS-EP19 (1); WS-EP20 (2); WS-EP57 (3); WS-EP94 (4). Letters correspond to the lanes in Fig. 3.

Correlations between DNA homology, structural proteins and, to a lesser extent, particle morphology were also found by a number of other workers within various bacteriophage groups (Arendt *et al.*, 1991; Braun *et al.*, 1989; Mata *et al.*, 1986; Prévot *et al.*, 1989; Stewart *et al.*, 1985). Therefore, it can be concluded that at least those genetic elements encoding major structural proteins are well conserved within each 'group' of similar phages. However, these groups or species should be defined by DNA homologies (Grimont & Grimont, 1981) or, if at all possible, protein sequences.

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