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ORIGINAL ARTICLE

Salmonella Enteritidis bacteriophage candidates for phage therapy of poultry

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Abstract

Aims: *Salmonella* is a worldwide foodborne pathogen causing acute enteric infections in humans. In the recent years, the use of bacteriophages has been suggested as a possible tool to combat this zoonotic pathogen in poultry farms. This work aims to isolate and perform comparative studies of a group of phages active against a collection of specific *Salmonella* Enteritidis strains from Portugal and England. Also, suitable phage candidates for therapy of poultry will be selected.

Methods and Results: The *Salm.* Enteritidis strains studied were shown to have a significantly high occurrence of defective (cryptic) prophages; however, no live phages were found in the strains. Bacteriophages isolated from different environments lysed all except one of the tested *Salm.* Enteritidis strains. The bacteriophages studied were divided into different groups according to their genetic homology, RFLP profiles and phenotypic features, and most of them showed no DNA homology with the bacterial hosts. The bacteriophage lytic efficacy proved to be highly dependent on the propagation host strain.

Conclusions: Despite the evidences shown in this work that the *Salm.* Enteritidis strains used did not produce viable phages, we have confirmed that some phages, when grown on particular hosts, behaved as complexes of phages. This is most likely because of the presence of inactive phage-related genomes (or their parts) in the bacterial strains which are capable of being reactivated or which can recombine with lytic phages. Furthermore, changes of the bacterial hosts used for maintenance of phages must be avoided as these can drastically modify the parameters of the phage preparations, including host range and lytic activity.

Significance and Impact of the Study: This work shows that the optimal host and growth conditions must be carefully studied and selected for the production of each bacteriophage candidate for animal therapy.

Introduction

Bacteriophages (phages) are today hypothesized as one of the possible alternatives to antibiotics in animal therapy, prophylaxis and reduction of pathogen loads in food products of animal origin. Trials with bacteriophages and farm animals have proven successful and used to kill foodborne pathogen such as: *Escherichia coli* (Smith and Huggins 1983; Smith *et al.* 1987; Huff *et al.* 2003, 2006), *Salmonella* (Higgins *et al.* 2005, 2008; O'Flynn *et al.* 2006; Atterbury *et al.* 2007), and *Campylobacter* (Loc Carrillo *et al.* 2005; Wagenaar *et al.* 2005) among others. Nevertheless, more studies are still required before phages can be considered a real approach for therapy of bacterial infections.

The use of phages for therapeutic purposes requires specific phages for the pathogenic strains of a specific

environment (Fiorentin *et al.* 2005; O'Flynn *et al.* 2006; Atterbury *et al.* 2007; Higgins *et al.* 2008). However, there are many issues that need to be addressed. Firstly, as a rule, it is not possible to isolate a single phage capable to lyse all strains of a particular bacterial species. The use of wide host range phages could be a hypothesis; however, in practice, these phages will loose their usefulness after several applications because of the emergence of phage-resistant bacterial mutants. There are also ideas of using hosts, such as safe industrial *E. coli* strains, for the production of phages but this also presents practical disadvantages as the phages capable to overcome interspecies barriers are rare and this makes it practically impossible to gather a large enough collection of such phages to compose different phage cocktails. Thus, it will be always necessary to use different pathogen strains, isolated in the course of real endemics or infection, for the isolation and production of phage products and cocktails.

Additionally, for some food pathogens, such as *Salmonella*, choices of basic hosts among the strains are required because of a wide occurrence of the restriction–modification systems. These basic hosts must be non-lysogenic, and the total collection of isolates needs to be always checked. Also, the use of basic hosts should optimally result in a good output of the phage.

Finally, there are also some general requirements for phages to be selected as therapeutic agents, as for instance: phages must be lytically active for the largest number of bacterial strains isolated from the specific areas, and they must be capable of cross lysing/killing the phage-resistant variants of each other.

Phage therapy is generally recognized as safe (Bruttin and Brussow 2005; Xie *et al.* 2005), but this assumption cannot be accepted without detailed studies, especially when considering the use of live therapeutic phages for long term purposes. So, it is once more emphasized that for a safe use of phages, the ones capable to lysogenize bacterial strains or which have DNA homologies with the chromosome of bacterial hosts are not acceptable.

Salmonella causes acute enteric infections in humans, and the main routes of transmission of this zoonotic bacteria to humans are because of the consumption of contaminated poultry products (eggs, meat) (Lee 1974; Hird *et al.* 1993; Henzler *et al.* 1994; Bryan and Doyle 1995). Poultry carcasses become contaminated with pathogens when a contaminated flock is processed on the slaughter line (Mead *et al.* 1995). Thus, the control of *Salmonella* infections at the farm and processing level is crucial, and the adoption of efficient eradication procedures can decrease the transmission of *Salmonella* to humans reducing infections and hospitalizations. This work describes the isolation, characterization and selection of suitable *Salmonella* phages for phage therapy.

Materials and methods

Bacterial strains

Thirty-one *Salmonella* Enteritidis strains isolated from contaminated food products in Portugal and from poultry farms in England were used for phage isolation and phage lytic spectrum assessment. Also, seven additional strains from the Regensburg strain collection (Germany) were used in the blot hybridization studies. All strains used (Table 1) were grown at 37°C on LB broth or LB agar (Miller 1972).

Collection bacteriophages

Several *Salmonella* bacteriophages were provided by the laboratory of bacteriophage genetics (State Research Institute for Industrial Microorganisms, Moscow, Russia), and phage Felix01 was from the Profos AG collection (Regensburg, Germany) (Table 2).

Verification of bacterial strains for presence of intact temperate phages

Semi-solid (0·6%) LB agar with 50 μl of fresh suspension of the tested strains was brought on the Petri Dishes with a layer of normal agarized (1·2%) LB medium. The drops of the same bacteria (after treatment with $CHCl_3$ and successive centrifugations to remove bacterial debris and $CHCl_3$) were placed on the lawns. After overnight incubation, the presence of phage lysis zones or other features of possible lysis (e.g. bacteriocins) were evaluated in the drops.

Isolation of new bacteriophages

An enrichment procedure was used for bacteriophage isolation. Briefly, the enrichment culture flasks (0·5 l) contained 50 ml of 5× concentrated LB nutrient medium, 200 ml of aqueous sample and a mixture of all non-lysogenic *S.* Enteritidis strains. The bacterial mixture was prepared by growth of the *Salm.* Enteritidis strains, overnight and at 37°C, on agarized LB medium Petri dishes (15 g of agar per litre of medium). Afterwards, two loops of each different strain were placed into Eppendorf tubes with 1 ml of 0·9% NaCl, and the tubes were vortexed 1–2 min. Five Hundred microlitres of each of the prepared strain suspensions were mixed in a glass tube. The mixture (0·5 ml) was used to infect culture flasks containing different samples, and the flasks were incubated overnight at 37°C on shaker (*c.* 250–400 rev min^{-1}). After incubation, 1·5 ml samples were taken from each of the flasks into Eppendorf tubes, vortexed with the addition of

Table 1 Characteristics of the *Salmonella* Enteritidis used

Strain	Isolation year	Source	Country	Phage type	Antibiotic resistance							
					N	CAZ	CIP	CN	NA	AMP	S	TE
922F	2003	INRJ	PT		S	S	S	S	S	S	S	S
392	1981	INRJ	PT		S	S	S	S	S	S	S	I
Ex1	2004	UB	UK	6	S	S	S	S	S	S	S	I
550	2003	INRJ	PT		S	S	S	S	S	S	S	I
777	2003	INRJ	PT		S	S	S	S	S	S	S	I
626	1984	INRJ	PT		S	S	S	S	S	S	S	I
629 A	2003	INRJ	PT		S	S	S	S	S	S	S	I
9510-85	Unknown	UB	UK	8	S	S	S	S	S	S	S	R
S 1400	1998	UB	UK	4	S	S	S	S	S	S	S	R
Ex2	2004	UB	UK	6	S	S	S	S	S	S	S	R
La5	Unknown	UB	UK	4	S	S	S	S	S	S	S	R
547	2003	INRJ	PT		S	S	S	S	S	S	S	R
855	2004	INRJ	PT		S	S	S	S	S	S	S	R
269	2003	INRJ	PT		S	S	S	S	S	S	S	R
932	1984	INRJ	PT		S	S	S	S	S	S	S	R
905	1984	INRJ	PT		S	S	S	S	S	S	S	R
1067	1990	INRJ	PT		S	S	S	S	S	S	S	R
1649	1992	INRJ	PT		S	S	S	S	S	S	S	R
851	1984	INRJ	PT		S	S	S	S	S	S	S	R
821	2003	INRJ	PT		S	S	S	S	S	S	S	R
126	1981	INRJ	PT		S	S	S	S	S	S	I	R
1188	1996	INRJ	PT		S	S	S	S	S	R	S	R
C6b	2004	UB	UK	4	S	S	S	S	S	R	S	R
869	2004	INRJ	PT		S	S	S	S	R	S	S	I
856	2003	INRJ	PT		S	S	S	S	R	S	S	I
629 B	2003	INRJ	PT		S	S	S	S	R	S	S	I
696	2002	INRJ	PT		S	S	S	S	R	S	S	I
475	2004	INRJ	PT		S	S	S	S	R	S	S	R
546	2003	INRJ	PT		S	S	S	S	R	S	S	R
583	2003	INRJ	PT		S	S	S	S	R	R	S	I
657	2003	INRJ	PT		S	S	S	S	R	R	S	R

INRJ, *Salm*. Enteritidis provided by Instituto Nacional Ricardo Jorge; UB, *Salm*. Enteritidis provided by the Bristol University; S, susceptible; I, intermediate; R, resistant; N, neomycin (30 μg); CAZ, ceftazadime (30 μg); CIP, ciprofloxacin (5 μg); CN, gentamicin (10 μg); NA, nalidixic acid (30 μg); AMP, ampicillin (10 μg); S, streptomycin (10 μg); TE, tetracyclin (30 μg).

Table 2 Bacteriophages isolated and collection bacteriophages used

Phage	Isolation/source
Felix01	Profos AG, Regensburg, Germany
φB1 SE5, φB2 SE5, φ22, φ62, φ44, φ79, φ105, φ147, φ162, φ164, φ171, φ177	Phage collection of the State Research Institute for Industrial Microorganisms, Moscow, Russia
φ**68**, φ81	Faeces from Poultry farm 1, Braga, Portugal
φ**169**, φ111	Faeces from Poultry farm 2, Braga, Portugal
φ**31**(φ31m, φ31tu), φ**38**, φ**39**, φ**135** (φ135lar, φ135sm), φ45	Raw sewage, wastewater treatment plant, Braga, Portugal
φ**2** (φ2a, φ2b)	Zoo pond, Moscow, Russia
φ**12** (φ12ht)	Nests from a Poultry farm, Santiago de Compostela, Spain
φ**2**/**2**, φ**4**/**1**(φ4/1a, φ4/1b), RBS cl, RBS tu, φS151, φ**A1**, φ**N5**, φN4	Wastewater treatment plant, Regensburg, Germany

30 μl of $CHCl_3$, centrifuged 2–3 min (12 000 rev min^{-1}) to sediment bacterial cells, and debris and drops were placed on the plates with lawns of the tested bacterial strains. After overnight incubation at 37°C, material was taken from the lysis spots and was plated on separate plates with the same bacteria performing dilutions up to

single plaques could be seen. Phages with reproducible plaque type features were reisolated from a single plaque and plated for confluent lysis (Adams 1959) on several Petri dishes. After overnight incubation, the phage was gathered, processed as usual vortexing, centrifugation at 6000 ***g***, 30 min at 4°C, $CHCl_3$ treatment and kept in refrigerator.

The new isolated bacteriophages were designated: RBS cl, RBS tu, phi 4/1a, phi 4/1b, phi 2/2, phiN4, phiN5, phi A1, phiB1 SE5, phiB2 SE5 (Table 2).

Purification and concentration of phages in CsCl preformed gradients

Purification and concentration of phages were accomplished as described by Sambrook and Russell (2001). Phages sensitive to CsCl treatment were concentrated by precipitation with polyethylene glycol (PEG)/NaCl (Yamamoto *et al.* 1970) with the subsequent sedimentation, resuspension in phage buffer SM (Sambrook and Russell 2001) and at least two additional cycles of washing by sedimentation and resuspension. Such preparations were treated with exonuclease (10 mcg l^{-1}, 1 h at 37°C) to disrupt bacterial DNA and centrifuged 3 h at 40 000 ***g*** to sediment the phage. Finally, phage pellet was resuspended in SM buffer.

Isolation of phage DNA, treatment of DNA with endonucleases, electrophoresis, Southern hybridization

These procedures were performed as described in Sambrook and Russell (2001).

Antibiotic susceptibility test

The susceptibility of the 31 *Salm.* Enteritidis isolates to eight antimicrobial agents was determined by the disc diffusion method on modified Mueller Hinton II agar according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The antimicrobial agents and the respective concentrations (in micrograms) used are shown in Table 1. The results were interpreted according to established criteria by the EUCAST (http://eucast.www137.server1.mensemedia.net/)

Results

This works aim at grouping the isolated phages as a first step for their classification and at studying the homology between the DNA, the different phages and bacterial hosts genomes to exclude all phages capable of enhancing recombination promoting inclusion into the bacterial genome genes, which could code pathogenic products. These are compulsory experiments that need to be performed to verify phage safety and to select suitable candidates for therapeutic purposes.

Isolation of phages

Verification of production of intact temperate phages by the bacterial target hosts

To isolate phages, it was necessary to exclude from the enrichment experiments evident lysogens, otherwise the host's temperate phage(s) would interfere with the interpretation of the results. All 25 Portuguese strains were checked, and the different strains did not reveal live temperate phages. Thus, for isolation of new phages, all these strains were mixed and used together in the enrichment experiments. It was necessary to ensure that the strains used in the isolation procedure were different. For that all hosts, isolated from either contaminated foods or animals (poultry), were tested for their susceptibility towards eight antibiotics. Based on the antibiotic susceptibility patterns, the isolates were grouped into nine distinct clusters (Table 1). Furthermore, the UK isolates have been previously characterized (Cooper *et al.* 1994; Cogan *et al.* 2004), and therefore their phage type was included in Table 1.

Selection of phages after enrichment procedure

The bacteriophages used in this work were isolated from different environments and countries (Table 2). All phages with different plaque types were repeatedly plated to assure their phenotype stability and phage plaque parameters such as size, turbid/clear appearance, edge morphology, presence of halo around plaques, growth of phage-resistant bacterial colonies in the lysis spot and estimation of their number were studied (data not shown). Phages with the best plaque parameters (clear appearance and the smallest number of phage-resistant colonies) were chosen for detailed studies (see bold in Table 2).

Interestingly, the strains used in this work were shown to be highly sensitive to most of the phages. In fact, the only strain studied that was resistant to all selected phages was strain 392.

General comparison of single selected phages on the basis of their primary features: plaque phenotypes and the growth on different strains in collection

In this work, a mixture of all nonlysogenic hosts were used to isolate new phages, to evaluate the effect of the phage propagation host on the host range, to differentiate potentially genotypically different phages and include them in further studies.

Optimal hosts for each potentially different phage were thus selected, in the course of comparison of phage growth on different hosts, and the strain which supported

the phage growth in the best way (larger plaque size, higher transparency, small number of resistant variants) was accepted as an optimal host for that particular phage (data not shown). Also, in comparison with the quality of phage growth on different hosts, optimal bacterial strains capable to support the growth of as many as possible phages in the collection were chosen. An additional feature which was used to select and differentiate phages was their plaque appearance. According to the results of this work, the best strains for differentiation of the phages used were Ex2 and 9510 (both from the University of Bristol collection).

Differentiation of the selected phages with RFLP profiles

To have comparable results of the phage RFLPs, most of the restriction experiments were performed with *Eco*RV endonuclease. To simplify the interpretation of results, they were grouped in several independent experiments.

The DNA digestions of several *Salmonella* phages were performed (Figs 1, 2 and 3). The DNA of six digested phages revealed nonidentical RFLP profiles and phage φ31m showed to be resistant to *Eco*RV (Fig. 1). However, there were some evident similarities between these six phages and their genome sizes, calculated as sums of the molecular weights of the restrictional fragments, which varied between 32 and 38 kb. Thus, it can be suggested that the six *Eco*RV digested phages, namely φ135lar, φ135sm, φ38, φ169, φ68 and φ39, may belong to the same phage group (group 1) while φ31m belongs to a different group.

Phages φ2 and φ4/1 produced morphologically different plaques on their hosts. Therefore, digestions of the DNA of the two specific variants of each phage (φ2a, φ2b, φ4/1a and φ4/1b) were performed (Fig. 2a). The digestion profile differed considerably from the ones obtained after *Eco*RV digestion of the six phages belonging to phage group 1 (compare Figs 1 and 2a). Therefore, φ2 and φ4/1 phages are unrelated from the phages of group 1 and may be considered as presenting two additional phage groups, groups 2 and 3, respectively. Both variants of each group, *a* and *b* respectively, have a similar restriction profile showing a great number of bands. Despite the morphological plaque dissimilarity between the two phage variants (*a* and *b*) of each phages φ2 and φ4/1, their RFLP profiles cannot be differentiated (Fig. 2a).

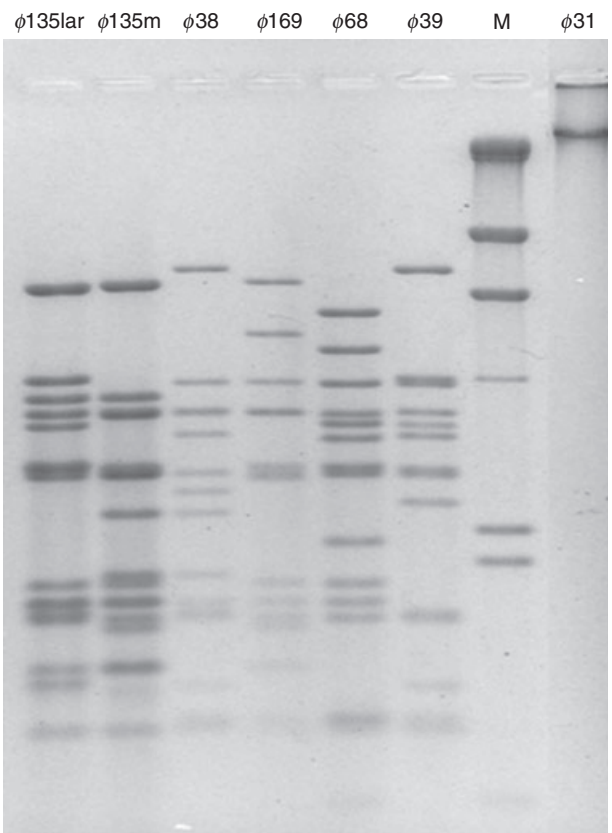


Figure 1 Digestion of the DNA of different *Salmonella* phages with endonuclease *Eco*RV. Lane M – λ *Hin*dIII molecular weight marker.

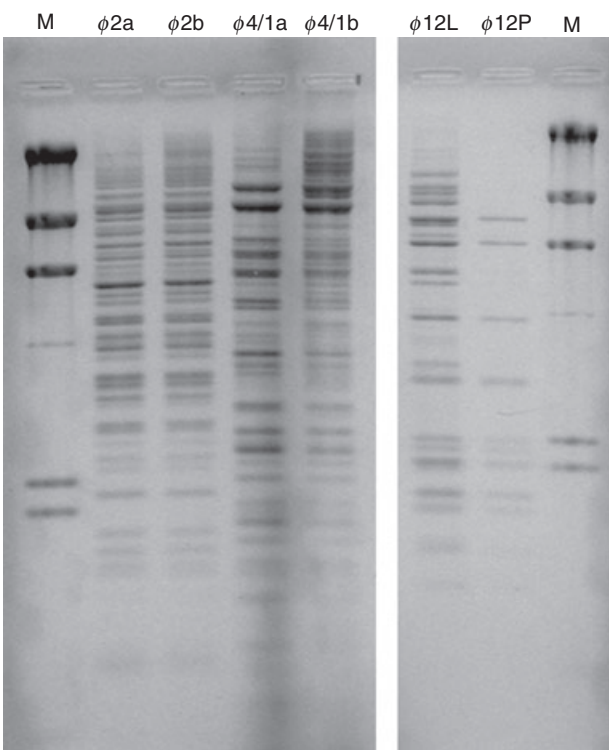


Figure 2 *Eco*RV digestion of the DNA of different *Salmonella* phages. Lanes M – λ *Hin*dIII molecular weight marker.

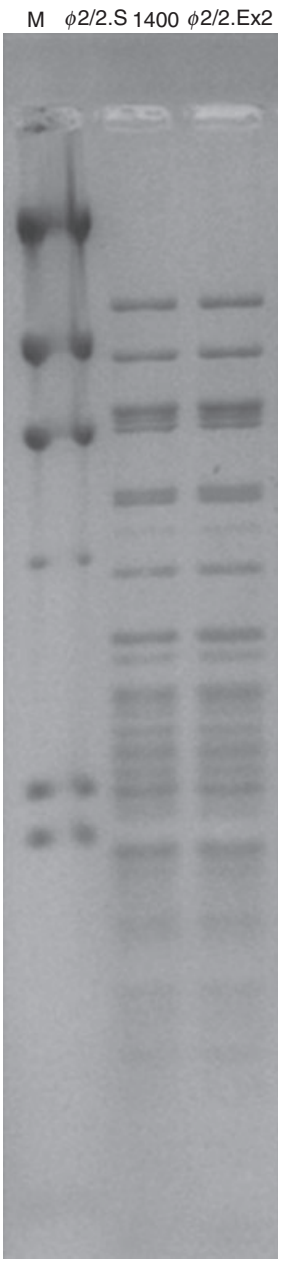


Figure 3 *Hin*dIII digestion of *Salmonella* phage $\varphi 2/2$ DNA grown on *Salmonella* Enteritidis strains 1400 and Ex2. Lane M – λ *Hin*dIII molecular weight marker.

In this work, a high titre of phage $\varphi 12$ preparation was purified, concentrated by treating with PEG (see Materials and methods) and afterwards treated with DNase. After DNase removal by sedimentation, the DNA was isolated either directly from the lysate ($\varphi 12$L) or from the single band obtained after CsCl centrifugation ($\varphi 12$P). As possible to observe (Fig. 2b), there was a great difference in the RFPL profiles of both DNAs. Most of the restriction fragments found in the DNA of phage preparation $\varphi 12$ in the lysate disappeared after CsCl centrifugation (Fig. 2b). Also, phage $\varphi 12$ is different from the other phages and therefore belongs to a different group (group 4).

Phage $\varphi 2/2$ DNA digestion with *Hin*dIII was performed after the growth of this phage on two distinct hosts (1400 and Ex2) (Fig. 3). Phage $\varphi 2/2$ has the biggest genome among the studied phages which was *c.* 92–94 kb as calculated by summing the sizes of the restrictional fragments. Phage $\varphi 2/2$ grown in both strains, resulted in equivalent digestions without additional nonequimolar fragments. Thus, this phage is different from the other ones found belonging to groups 1–4 and therefore belong to a new group (group 5).

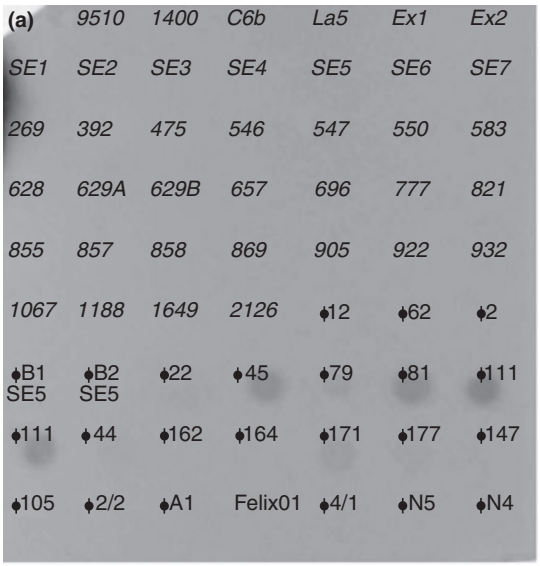
Besides the DNA of $\varphi 31$m, the DNAs of three other phages $\varphi 31$tu, φN5 and φA1 were also shown to be resistant to both *Eco*RV and *Eco*RI (data not shown). Thus, these phages may provisionally be considered as different from the other groups of phages. However, they cannot be grouped together without further characterization.

Estimation of DNA homology between the selected phages and between phages and their target bacterial strains

Homology studies of three different phages were performed. The phages used were phages $\varphi 169$ (group 1), $\varphi 4/1$ (group 3) and $\varphi 31$tu (so far unassigned group), respectively. From the studied phages, the DNAs of $\varphi 169$ and $\varphi 31$tu did not show homology with bacterial DNA in the dot blots (data not shown). The dot blot-hybridization of P^{32} labelled $\varphi 169$ DNA (as zond) with bacterial and other *Salmonella* phages DNA was performed (Fig. 4). According to results obtained, phage $\varphi 169$ revealed DNA homology with nine other phages as observed by the presence of plaques in the spots of $\varphi 68$, $\varphi 39$, $\varphi 135$, $\varphi 38$, $\varphi 2$, $\varphi 45$, $\varphi 79$, $\varphi 81$ and $\varphi 111$ but no relation with other phages.

DNA hybridization of P^{32} labelled DNA of phage $\varphi 31$tu showed no homology with bacterial DNAs, but revealed a good homology with phage φN5 and a trace of homology with phage $\varphi 4/1$. Furthermore, there was no homology with any other phages (data not presented).

The hybridization of P^{32} labelled DNA of phage $\varphi 4/1$ with DNAs of other phages revealed homology with phages φRBSc1 and φA1 (Fig. 5). It was also observed a weak DNA homology of $\varphi 4/1$ with DNAs of several of the tested bacterial strains (data not shown). This may suggest that phage $\varphi 4/1$ has a higher general input for defective prophages that are capable to be packed in the phage particles than all other studied phages. Thus, phage $\varphi 4/1$ may be considered as a virulent mutant of a temperate phage, and it has DNA homology with some



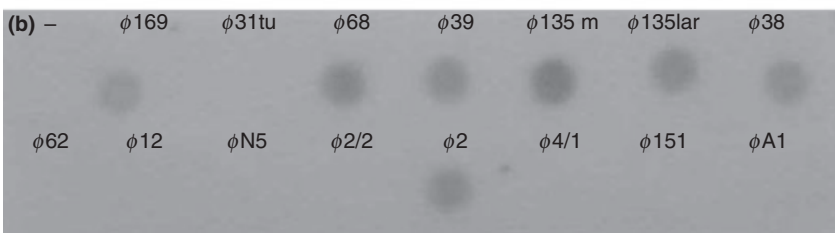


Figure 4 Blot hybridization of phage DNA. (a) φ169 phage DNA labelled of P^{32} (as zond) with DNAs of other phages (marked with *solid black*) and *Salmonella* strains DNAs (marked with *italic*). (b) Blot hybridization of φ169 phage DNA labelled of P^{32} (as zond) with DNAs of other phages immobilized on nylon membrane.

defective prophage(s), possibly with phage P22 of lambda group that are common in some of the *Salmonella* strains of the studied collection.

The RFLP profile studies together with the study of DNA hybridizations between phage DNAs permitted the distribution of all isolated phages in different unrelated groups. Although RFLP profiles were not performed with phages φ111, φ45, φ79 and φ81, the DNA homology studies allocate them among the group 1 of phages, together with phages: φ135lar; φ135sm; φ38; φ169; φ68 and φ39. The other unrelated phage groups are composed by the following phages: group 2 consists of phage φ2 (variants *a* and *b*); group 3 consists of φ4/1, φRBSc1 and φA1; group 4 consists of φ12;, group 5 of φ2/2 and a new group was created based on the DNA homology studies which comprises phages φ31tu and φN5 (group 6).

Overall, of these different phage groups, only phages belonging to group 3 (φ4/1) should not be used for phage therapy because of the homology with a number of bacterial strains and the hypothesis of having an input for defective prophages.

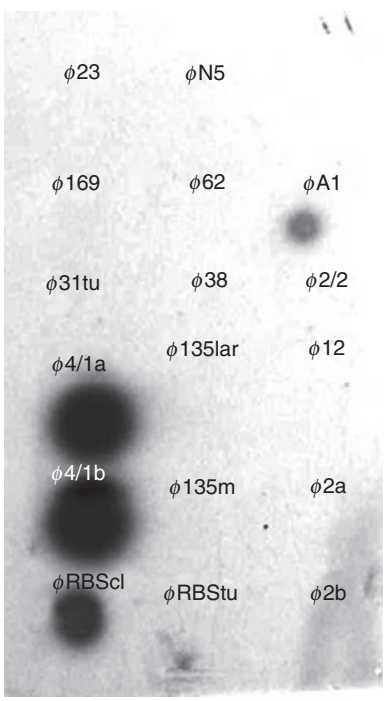


Figure 5 P^{32}-labelled DNA hybridization of phage φ4/1 with other phage's DNA.

Dependence of growth of the selected phages from their last host

Considering this possibility, the host range of phages φ68 (group 1) and φ2 (group 2) was compared (see Table 3).

Table 3 Lytic spectra of phages φ2, φ68 and Felix 01 (F01) grown in different hosts

	(Phage name).(Host strain used for growth)								
Strain	φ2.905	φ2.629B	φ2.9510	φ68.932	φ68.9510	φ68.Ex2	F01.821	F01.696	F01.Ex2
922F	CL	CL	CL_r	CL	CL_b	CL_b	CL_r	CL_r	mix1
392	no	no	no	no	no	no	CL_r	tu_r	tu
Ex1	CL	tu	no	no	tu	tu	no	no	mix
550	CL	tu	no	no	tu	tu	tu	tu	mix
777	CL	tu	no	tu	tu	tu	CL_r	CL_r	tu
626	CL	CL_{db}	CL_r	CL	CL	CL	CL_r	CL	tu
629 A	CL	CL_b	CL_r	CL	CL	CL	CL_r	CL_r	tu
9510-85	CL	CL_h	CL_r	CL	tu_h	tu_h	CL_r	tu	tu
S 1400	CL	CL	CL_r	CL	CL	CL	tu	no	mix
Ex2	CL	CL	CL_r	mix2	$CL_{r,h}$	$CL_{r,h}$	tu_r	tu_r	tu
La5	CL	CL	CL_r	CL	CL	CL	mix	CL_r	tu
547	CL	tu	no	no	tu	tu	tu	no	tu
855	CL	tu	no	tu	tu	tu	CL_r	CL	mix
269	CL	$CL_{b,lar}$	tu_r	CL	CL	CL	tu	CL_r	tu
932	CL	CL_h	CL_r	CL	CL_h	CL_h	tu	tu	tu
905	CL	$CL_{db,r}$	CL_r	mix2	CL_h	CL_h	tu	CL	mix
1067	CL	CL	CL_r	CL	CL_b	CL_b	CL	tu	mix1
1649	CL	CL_b	CL_r	CL	CL	CL	CL_r	CL_r	tu
851	CL	CL_{db}	CL_r	CL	CL	CL_h	CL_r	CL	mix
821	CL	CL_b	CL_r	CL	CL_h	CL_h	CL	CL	mix
126	no	tu	no	no	tu	tr	CL_r	tu	tu
1188	tu	tu	no	no	tu	tu	tu	no	no
C6b	CL	CL	CL_r	CL	CL	CL	tu	no	mix1
869	CL_b	CL_{db}	CL_r	CL_b	CL_h	CL_h	CL_r	CL	mix
856	CL	CL_{db}	CL_r	CL	CL_h	CL_h	CL	CL	mix
629 B	CL	CL	CL_r	CL	CL_h	CL_h	CL	CL	mix
696	CL	CL_r	CL_r	CL	–	–	CL_r	CL_r	$CL_{db,r}$
475	mix2	$CL_{b,lar}$	CL	mix	CL_b	CL_b	CL_r	CL_r	mix
546	CL	CL_{db}	CL_r	mix2	$CL_{r,h}$	$CL_{r,h}$	CL_r	CL	mix
583	tu	tu	no	no	tu	tu	tu_r	CL_r	tu
657	tu	tu	no	no	tu	tu	tu	tu	tu

CL, clear; no, no growth; r, presence of phage resistant colonies; tu, turbid; h, halo around the plaque; b, border of plaque; db, double border of plaque; mix, mixture of different types of clear and turbid plaques; mix1, mixture of turbid plaques; mix2, mixture of clear plaques; tr, traces of growth; –, no data; lar, large colony.

To differentiate the origins of phage preparations, generally accepted designations were used. So, the stock of phage 'P' grown on bacterial strain 'S1' will be designated as 'P.S1'. Among the phages studied in this work, phage φ2 may have special interest. Table 2 presents the results of the phage φ2 growth characteristics which were propagated on different strains (905, 629B and 9510) and then tested on the set of all bacterial strains. As can be observed, all three variants of φ2 preparations had slightly different effects on the set of bacteria used. The best results for phage φ2 were obtained when grown on strain 905 where a clear plaque was observed in a higher number of strains (Table 3).

The dependence of host range from the last host in which φ68 was grown was also performed (Table 3). The differences obtained in this study were not as impressive as the ones obtained with the growth of φ2 in distinct strains; however, it can be observed that phage φ68's preferred hosts are equally strain Ex2 and 9510 (φ68.Ex2 and φ68.9510).

Phage φ68 grown using strain 932 failed to kill eight specific strains (see Table 3) that were susceptible to the phage grown on *S.* Enteritidis strains Ex2 and 9510.

Also, the effect of host dependence of phage Felix01 on three distinct hosts (821, 696 and Ex2) was compared (Table 3). Felix01 grown on strains 821 and Ex2 was capable of forming plaques on almost all strains tested. Nevertheless, the lytic efficiency of Felix01 is clearly compromised when grown on different host susceptible strains. For instance, Felix01 growth on strain 696 made the phage no longer capable of infecting five isolates that the same phage grown on strain 821 succeeded to attack (Table 3).

The lytic spectra of phages grown in different hosts also allowed to distinguish the bacterial isolates used, because the phage spot patterns on the bacterial lawns of the nine clusters, grouped according to their resistance patterns, varied greatly. A closer analysis of the lytic spectra data shows that all 31 tested hosts are different (Table 3).

Overall, this work shows that to choose the right hosts for obtaining industrial quantities of phages, it is highly necessary to perform preliminary studies of restriction–modification relations among the phages and their target bacterial strains.

Discussion

Phages are ubiquitous in nature and can be good candidates for use in animal therapy. This work shows that *S.* Enteritidis phages are readily isolated and worldwide distributed and can be efficient in killing bacterial isolates from different countries.

In this work, for phage isolation, a mixture of all nonlysogenic hosts was used and therefore it was assumed that single phage particles would find the most appropriate host in the first or subsequent cycles of growth. This strategy was adopted because the use of a single bacterial strain for enrichment not only may be too time consuming but can also lead to the loss of rare phages present in the sample because of the use of a 'wrong' first host. However, using all bacterial strains in a mixture makes difficult the identification of which bacterial strains were the last hosts for each phage. Consequently, some phages isolated from the initial plantings may present the same genotypes but may result in a variation in their phenotype because of the different host-induced modifications. Using comparisons of phage host ranges, performing growth on the same hosts or on several specially selected common hosts makes possible to differentiate potentially genotypically different phages and include them in further studies.

The phages studied here proved to belong to several different groups. The first group of phages gathers several closely related phages having genome sizes in the range of 32–38 kbp, sharing DNA homology and several common restrictional fragments (Fig. 1). DNA isolated from preparation of phage φ2, the second phage group, (Fig. 2a) had some homology with the DNAs of phages belonging to the first group. Nevertheless, it was possible that this preparation included an additional DNA-containing component.

The third phage group included phage φ4/1 with a broad host range, having traces of homology with bacterial DNA. This last feature does not allow the use of such phages for the purpose of phage therapy.

Considering that these two phages, φ2 and φ4/1, and their respective variants which were obtained from the same bacterial host, the difference in the number and location of these additional bands (Fig. 2a) exclude their origination from bacterial DNA. The only possible explanation for this situation is that phages such as φ2 and φ4/1 can specifically induce different defective prophages in the same bacterial host. Based on the DNA homology experiments, phages φA1 and also φRBSc1 have homology to phage φ4/1, and therefore these phages were grouped with this phage.

The fourth phage group includes phage φ12 which has no DNA homology with other phages. It also may represent a phage complex (or mixture of two phages). Furthermore, this phage proved to be useful for the differentiation of bacterial strains. An interesting special feature of φ12 was its great sensitivity to purification with CsCl because most of the restriction fragments, which were typically present in the total complex, disappeared in φ12L. It can be concluded that the infecting phage φ12 activates the development of a defective prophage whose DNA is being packed into the phage capsid. However, the defective prophage was not stable and disrupted in the course of CsCl treatment.

The fifth phage group comprises only phage φ2/2. This phage has a large genome with nonequimolar restrictional fragments, a broad host range and no DNA homology with all other phages. The work described here suggests that the infection of both strains with phage φ2/2 does not lead to activation of cryptic phage(s), or in the case that cryptic phage(s) are activated, they were not able to survive the CsCl gradient centrifugation.

From this study, it is evident that some of the strains used contain genes which could be packed into the phage capsids. These results confirm also previous studies performed with *Salmonella* sp. (Figueroa-Bossi *et al.* 1997; Schicklmaier *et al.* 1998; Schmieger 1999; Rabsch *et al.* 2002; Figueroa-Bossi and Bossi 2004; Mikasová *et al.* 2005; Kropinski *et al.* 2007).

The broad host range phage φ31tu showed DNA homology with φN5, and therefore these phages belong to group 6. Both phages were shown to have DNA resistant to digestion with *Eco*RI, *Eco*RV and *Hin*dIII.

The restriction–modification effects are very frequent in *Salmonella* sp. Thus, in this study, it was necessary to work with plenty of unstudied natural bacterial isolates and different newly isolated phages to know the possible effects of phage movements throughout the different hosts and their consequences in sense of their influence on phage activity through possible DNA modifications. The second important reason to check the 'effect of the last host' is because *Salmonella*

genomes are known to carry plenty of different live and/or defective (cryptic) prophages (Figueroa-Bossi *et al.* 1997; Schicklmaier *et al.* 1998; Schmieger 1999; Rabsch *et al.* 2002; Figueroa-Bossi and Bossi 2004; Mikasová *et al.* 2005; Kropinski *et al.* 2007). Therefore, recombination studies of infection with such phages or transactivation of such phage by any mechanism can result in a change of the genetic composition of the phage preparation and consequent influence on its final antibacterial activity. Furthermore, the host range of the different selected bacteriophages (and their possible relation in the phage grouping attribution) may be interesting for the use of veterinary phage therapy.

Interestingly, in this work, it was observed a change of the lysis spots into absolutely different ones because of growth of phages in different hosts, which consequently altered their lytic spectrum of activity. To exemplify the importance of the last host and the effect of phage growth on the phage plaque, a short example will be illustrated. So, for instance, a droplet of phage φ2.629B placed on the lawn of strain *Salmonella* 9510 formed a highly transparent spot of lysis with a halo around the plaque while a droplet of φ2.9510 placed on strain 9510 formed a plaque with a number of resistant colonies. The material from this spot, now called φ2.629B.9510, when plated (after chloroform treatment) on the same fresh lawn of strain 9510, resulted in an absolutely different lysis spot that was extremely turbid and filled with phage-resistant cells (data not shown). One possible explanation for the transformation of, for instance, highly transparent spots with few resistant colonies, into very turbid spots filled with phage-resistant cells, as observed in the phage φ2 growth experiments, might be the trans-activation effect of a defective phage (or bacteriocin) present in this case in the strain of 629B when the strains was infected. This defective helper phage will most likely kill bacterial mutants of strain 9510 that were resistant to φ2; however, this helper phage seemed to disappear as it had no capability to replicate by itself. This phenomenon explains the high transparency of the lysis spot and presence of a small number of resistant colonies. The material taken from the spot formed on strain 9510 with phage φ2.629B does not contain the active killer substance/additional defective phage that was activated in strain 629B, and the *Salmonella* 9510 strain did not have such transactivated parts in its genome. So, this work shows that the plaque differences obtained because of the last host where phage φ2 was grown may depend on the transactivation of any cryptic phage and the consequent package of additional DNAs in the phage capsids and also on a modification activity of the last host. Nevertheless, these results clearly depend on the last bacterial growth host of phage φ2. Consequently, in the final phage lysate, it is most likely that there are occurring two different killing activities. According to published results, bacteriophages of *S.* Enteritidis belong to several groups: P27-like (phage ST64B), P2-like (Fels-2, SopEphi, and PSP3), lambdoid (Gifsy-1, Gifsy-2, and Fels-1), P22-like (epsilon34, ES18, P22, ST104 and ST64T), T7-like (SP6) and three outliers (epsilon15, KS7 and Felix O1) (Kropinski *et al.* 2007). Thus, the most frequent prophage components in *Salmonella* are different derivatives of P22 or lambda-like phages. The second phage in a mixed lysate of φ2 cannot be multiplied by itself but it can kill bacterial mutants resistant to φ2 (low number of phage-resistant colonies to φ2 preparation). Thus, it is possible that the cryptic phage genome belongs to a virulent phage, because the capability to kill bacteria just with adsorption of tail structure is typical for genuine virulent phages (as an example killing activity of T2 ghosts) (Herriott and Barlow 1957) or action of tail-like R2 bacteriocin of *Pseudomonas aeruginosa* (Nakayama *et al.* 2008). The use of such phenomenon to increase the activity of phage mixtures can be promising and advantageous in an industrial scale production: contributing to a more economic way (as less phages will be necessary to obtain), saving other phages for future use and the possibility to use phages giving a high proportion of phage-resistant cells.

In conclusion, this work shows that the bacterial hosts need to be specially selected for each phage. Despite evidence shown that the used bacterial strains did not produce viable phages, we have confirmed that some phages, when grown on particular hosts, behave as complexes of phages. Thus, any change of the bacterial host used for maintenance of phages can drastically change the parameters of the phage preparations, including host range and lytic activity. This fact suggests that the use of phages as prophylactic agents against *Salm.* Enteritidis should be avoided to limit the massive disseminations of phages into natural habitats. Most importantly, the use of phages should be restricted to therapeutic purposes.

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