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Frequency of Generalized Transducing Phages in Natural Isolates of the *Salmonella typhimurium* Complex

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From 85 natural isolates of the *Salmonella typhimurium* complex, including the *Salmonella* reference collection A (P. Beltran, S. A. Plock, N. H. Smith, T. S. Whittam, D. C. Old, and R. K. Selander, J. Gen. Microbiol. 137:601–606, 1991), 65 strains (76.5%) released 71 different temperate phages. Forty-three (93.5%) of 46 tested phages were able to transduce the chromosomal markers *his*$^+$ and *trp*$^+$ and the cloning vector pBR325.

Generalized transduction was discovered in *Salmonella typhimurium* more than 40 years ago by Zinder and Lederberg (18), who showed that genetic material of strain LT22 could be transferred to recipient cells of strain LT2 by means of a temperate bacteriophage, PLT-22 (now called P22). In 1955, it was realized that *Escherichia coli* phage P1 was also able to transfer the genetic material of host cells (8), and since that time, generalized transducing phages have become invaluable tools for fine mapping of genes.

Recently, there has been increasing interest in horizontal gene transfer. In the early years of microbial genetics, the mechanisms of the various gene transfer systems were studied. Nowadays, a prevalent question is to what extent gene transfer occurs in natural environments. A better understanding of the quantitative contribution of horizontal transfer to genetic exchange is of interest for evolution as well as for more pragmatic considerations, such as how antibiotic resistance genes can be spread so fast over wide ranges of taxonomically distant organisms. Horizontal gene transfer in nature also has a strong impact on risk assessment in connection with the release of genetically engineered microorganisms.

Interestingly, most studies on horizontal gene transfer in nature are dedicated to conjugative systems or, to a lesser extent, transformation, but almost no attention has been paid to phage-mediated transduction. At the 4th Symposium on Bacterial Genetics and Ecology, held in Wageningen in 1993, there were 46 contributions concerning horizontal gene transfer (oral presentations and posters), 78% of them dealing with conjugation and 18% with transformation, and only 2 contributions were devoted to bacteriophages. One reason for this may be that only a few generalized transducing phages are known to the broad scientific community, suggesting that generalized transduction is a rare event in nature and may therefore be neglected as a serious factor in gene exchange.

One difficulty in estimating the occurrence of generalized transduction arises from the fact that under normal circumstances this process can only be carried out effectively by temperate bacteriophages. The search for temperate bacteriophages is hampered by the need for a sensitive indicator strain; a lysogenic strain which liberates an unknown phage is generally not suited as an indicator for this phage because it is protected by the resident prophage.

Although it is known that some virulent phages are able to encapsulate bacterial DNA and to transduce it to new host cells because they are packaging DNA by the headful mechanism, these are pathological situations which may not play any role in nature. For instance, it has been shown that mutants carrying multiple mutations affecting the hydroxymethylcytosine pathway of virulent phage T4 and hence protecting bacterial donor DNA from degradation are able to perform generalized transduction (16). Also, *E. coli* virulent phage T1 is able to encapsulate and to transduce bacterial DNA, provided that the recipient cells are protected from lytic action by using amber mutants of T1 (5).

It is also possible to transduce effectively with clear-plaque mutants of the known generalized transducing phages, such as P22 (12) and P1 (7). However, they are still temperate phages and are excluded by prophages from lytic infection. Therefore, reasonable transduction results with clear-plaque mutants can only be obtained by using lysogenic recipients.

Here, we present a study on the frequency of temperate phages in general, with special emphasis on phages capable of transduction, in a selected group of natural isolates of the *Salmonella typhimurium* complex, the *Salmonella* reference collection A (SARA collection; 1), together with 14 strains from various hospitals in the area of Munich, kindly provided by Vera Preac-Mursic, Max von Pettenkofer Institute, University of Munich. The latter strains are designated throughout this paper as *S. typhimurium* Pk.

Assay for release of temperate bacteriophages on standard laboratory indicator strains. All strains of the SARA collection (strains 1 to 72 except for 36, which has been lost) and the 14 additional *S. typhimurium* isolates from Munich hospitals (strains 73 to 86) were grown, starting with a single colony, in LB (11) to the stationary phase. No prophage-inducing agents were added, and no inducing procedures were applied. Overnight cultures were centrifuged, and supernatants were assayed for free phages by dropping 10-μl aliquots on LB plates which were seeded by top agar with the following indicator strains: *S. typhimurium* DB21 (wild-type LT2, carries cryptic prophage *Fels1*); *S. typhimurium* Q (free of cryptic prophages *Fels1* and *Fels2* [2]); *S. typhimurium* LB5000 (smooth) and LB5010 (rough), defective in all three restriction systems of *S. typhimurium* LT2 (3); *E. coli* K-12 strain C600 (r^-m^+); and *E. coli* C. The plates were incubated overnight at 37°C. When lysis was observed, it varied from a few single (turbid) plaques in the dropping area to total turbid lysis. This highly variable number of phages, especially on indicator strains DB21 and Q, may reflect the activity of the restriction systems hsd_{LT}, hsd_{SA}, and hsd_{SB} rather than the release rate of prophages.

In some cases, at the stage of the spot test, the presence of several morphologically different plaques could already be ob-

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served. Since all cultures assayed for phages started from single colonies, all phages detected should be released from the growing cells after spontaneous induction of prophages and hence could not be virulent phages. This was confirmed by the observation that all plaques were turbid. However, some phages form clearer plaques on some indicator strains than on others, indicating that in these hosts, the ratio between lytic and lysogenic responses is shifted in favor of the lytic response. If lysogenization occurred in a low percentage of the infected cells, the plaque would appear more or less clear. Some phages show a considerable tendency to accumulate clear-plaque mutants during propagation.

Phages were purified from the lysis areas by removing an agar plug with a Pasteur pipette, suspending the phages in LB, and plating dilutions on the respective indicator strain. Single plaques were used to propagate lysates on the respective indicator strains. When different plaque types could be distinguished, a representative of each type was propagated.

Forty-four of 85 strains tested liberated temperate phages which could be propagated on at least one of the above-listed standard indicator strains. SARA strains 25, 29, 37, and 69 released up to four different phages, which could be easily distinguished by plaque morphology. In this way, a total of 50 different phages were isolated. A careful search for more subtle differences in the plaque morphology or comparison of the restriction patterns of DNAs from phages released by the same host would certainly reveal a greater number of distinguishable isolates.

All phages were named by the prefix PS followed by the number of the host in the SARA collection. When more than one phage type was released by a host, they were distinguished by adding letters to the phage name (e.g., PS37A).

Since most of the supernatants released phages that were active on more than one of the standard strains, all phages were assayed after purification on all standard indicator strains to see whether we were dealing with different phages or whether one phage type was able to grow on several hosts. Table 1 shows that a variety of *Salmonella* host range patterns could be identified. One phage (PS69B) was able to grow on some *S. typhimurium* strains and on *E. coli* K-12 strain C600 and *E. coli* C.

Surprisingly, there are some phages which plate on DB21 but not on LB5000, although the only relevant difference between these strains should be that LB5000 is defective in the three restriction systems of LT2. We conclude that during the construction of LB5000, which included mutagenization by nitrosoguanidine, additional changes have occurred which might affect adsorption or other essential steps in phage propagation.

Assay for bacteriophages on strains of the SARA collection. As Table 2 shows, there was a striking gap in the list of the serovars *Salmonella paratyphi* B and *Salmonella muenchen*, since many strains did not release phages detectable on the standard laboratory indicators. Considering the high proportion of lysogenic strains in the other serovars, it seemed unlikely that the majority of strains of the latter groups should lack prophages. One reason could be that some of the strains release only small numbers of phages, which are inactivated by the restriction systems of the standard indicator strains; another reason may be the lack of suitable receptor sites. To extend the series of possible indicator strains, supernatants of all hitherto phageless SARA and Pk strains were assayed on all *Salmonella* strains of the SARA collection (+PK strains). As shown in Table 2, this permitted detection of spontaneously released temperate phages in another 21 strains. This means that from 85 isolates of the *S. typhimurium* complex, a total of 65 strains (76.5%) released at least one type of temperate phage. Altogether, 71 temperate phages were isolated.

TABLE 1. Host range of the *Salmonella* phages

PS phages	Growth on indicator strains					
	S. typhimurium				*E. coli* C600 and C	SARA strains
	DB21	Q	LB5000	LB5010		
5, 7, 19, 30, 39, 40, 41, 43, 46, 76	+	−	−	−	−	−
10, 22, 35, 44	+	+	−	−	−	−
1, 3, 4, 12, 17, 25B, 29A/B, **31, 32, 33, 34, 37A, 37D, 38, 45, 69A, 78**	+	+	+	−	−	−
81, 82, 84, 85	+	+	+	+	−	−
69B	+	−	+	−	+	−
11	+	−	+	+	−	−
21, 25A, 26, **28, 37C,** 42	+	−	+	−	−	−
79	−	−	+	−	−	−
49, **74,** 83	−	−	+	+	−	−
37B	−	+	+	−	−	−
6, 14, 15, 18, 27, 47, 50, 51, 52, 53, 54, 55, 57, 59, 61, 63, 65, 66, 68, 70, 71	−	−	−	−	−	+

Immunity. We tested whether the new phages are homoimmune or heteroimmune to our reference phage P22. Prophage P22 has an immunity system which is much more complicated than that of other phages, e.g., lambda. In addition to *immC*, which is analogous to the lambda immunity system with the repressor C (product of gene *cI*) as the main component, P22 also has *immI* (for detailed information, see reference 10). The gene *ant* in this operon codes for an antirepressor. The antirepressor of the infecting phage is activated after infection of a host cell and inactivates the repressor molecules of a homoimmune prophage, such as phage ES18, which has no *immI* system (11a). Therefore, propagation of the homoimmune phage P22 on the ES18 lysogenic host is possible. In the prophage state of P22, antirepressor synthesis is prevented by the

TABLE 2. Spontaneous release of temperate phages

Strain and no. of different serovars in SARA collection (strain nos.)	No. of strains releasing phages	
	On standard indicator strains	On SARA strains only
S. typhimurium, 21 (1–21)	11	4
S. saintpaul, 8 (22–29)	5	1
S. heidelberg, 10 (30–40)	10	0
S. paratyphi B, 22 (41–62)	7	10
S. muenchen, 10 (63–72)	1	6
S. typhimurium Pk, 14 (73–86)	10	0
Total, 85[a]	44	21

[a] Total, 65 strains (76.5%).

TABLE 3. Test for homo- and heteroimmunity with phage P22

Growth on indicator strain		Interpretation (PS phages)
DB21(P22) (*sieA sieB*)	DB21(ES18)	
−	−	Homoimmune, no *immI* system (25B, 30, 31, 32, 35, 69, 82)
−	+	Homoimmune, with *immI* (1, 4, 22, 37A, 38)
+	+	Heteroimmune (3, 5, 7, 10, 11, 12, 17, 19, 21, 25A, 26, 28, 29, 33, 34, 37C, 37D, 39, 40–46, 69A, 69B, 76, 78, 81, 82, 84, 85)

repressor activity of the product of gene *mnt*, which also belongs to the *immI* operon. The gene product Mnt of a P22 prophage also prevents transcription of the *ant* gene of an incoming P22. Therefore, plating on P22 and ES18 lysogenic hosts should yield information about homo- or heteroimmunity and about the absence or presence of an antirepressor.

However, in addition to this dual immunity system, P22 (but not ES18) has two systems for superinfection exclusion (*sieA* and *sieB*), which may affect infection and propagation of other phages (14). Therefore, these have to be inactivated when testing for immunity.

We plated all phages able to grow on DB21 on DB21 derivatives which were lysogenic for mutant P22 *sieA sieB* or for phage ES18, respectively. Conclusions were drawn according to the different plating patterns, and the results are shown in Table 3. Interestingly, different phages released from the same host exhibit different immunity patterns (e.g., PS25A and B and PS37A and C and D).

Generalized transduction. Our main interest was to test how many of this astonishingly high number of new phages were able to encapsulate and to transfer the genetic material of their respective bacterial hosts, i.e., to perform generalized transduction. This assay was only performed with those phages which could be propagated on the wild-type indicator strain DB21, because only auxotrophic derivatives of the LT2 line of *S. typhimurium* were available. Transduction was examined with the mutant HisHB22(P22), harboring a deletion in the histidine operon and lysogenic for wild-type phage P22. To screen quickly a large number of different phages for transduction ability, we modified a method developed for detection of high-transducing mutants (13). Samples of the phage lysates to be tested were dropped on a layer of DB21 distributed with soft agar on minimal medium. After incubation overnight, lysis areas became visible. Each lysis area represented an individual lysate of the respective phage, containing a high number of plaque-forming phages and possibly a minor fraction of phage particles carrying different sections of the DB21 chromosome. DB21 indicator (donor) cells were then killed by treating the plates with evaporated chloroform. A concentrated overnight culture of the auxotrophic mutant strain was sprayed over the plates, and incubation of the plates was continued for 2 days. During this time, colonies of wild-type transductants were able to grow only on and around the lysis areas of transducing phages (Fig. 1).

A total of 46 different phages could be assayed. Forty-one of them (89%) were able to transduce the *his*$^+$ marker. Since the integration sites on the host chromosome for the various new phages are unknown, we could not exclude the possibility that at least some of them, like *E. coli* phage lambda, perform specialized transduction because of incorrect prophage excision. Therefore, transduction of strain TrpB8(P22), a Trp$^-$ mutant (13) and also lysogenic for P22, to wild type was also examined. All phages which were able to transduce the *his*$^+$ marker also transferred the *trp*$^+$ marker. Additionally, transduction of the cloning vector, pBR325, was tested by growing lysates of the transducing phages on DB21(pBR325), infecting the plasmid-free strain HisHB22(P22), and selecting for ampicillin resistance. All *his*$^+$- and *trp*$^+$-transducing phages were also able to encapsulate and transfer this plasmid. Therefore, all phages tested were generalized transducing phages. Two phages (PS74 and PS75) from which high-titered lysates could only be obtained from the rough mutant LB5010 were shown to transduce pBR325 from an LB5010(pBR325) donor to the plasmid-free LB5010 as a recipient. Therefore, the total number of transducing phages increased to 43, i.e., 93.5%. All

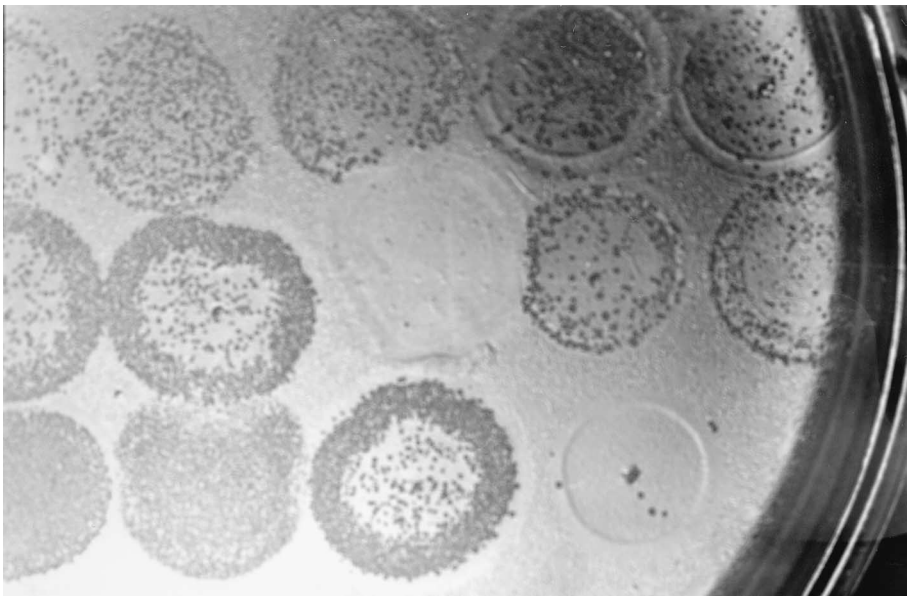

FIG. 1. Sector of a petri dish treated by the spraying method as described in the text. Lysis areas of phages which are able to transduce are covered with wild-type (*his*$^+$) transductants arising from the *his* mutant recipient cells sprayed over the plate. No wild-type colonies can be observed in the lysis areas of phages incapable of generalized transduction.

phages able to transduce are indicated in Table 1 by boldfaced letters. It should be noted that transduction of the P22 lysogenic recipient strains was possible not only with phages which are homoimmune with P22, but also with heteroimmune phages, which represent the majority of the phages (Table 3).

For some of these phages, the transduction frequencies for the various marker genes were determined exactly. The ratio of transductants to PFU ranged between 10^{-6} and 10^{-8} for the two chromosomal markers.

Stability of lysates. We have repeatedly assayed the plaque-forming and transducing capacities of the phages. Under normal storage conditions (LB medium, 4°C), the plaque-forming ability of all phages was stable over a period of 1 year. The transducing capacity of about two-thirds of all transducing lysates was retested after 3 months. No loss of transducing ability was observed. In that sense, these phages are very similar to P22, which in our hands is very stable.

The results reported indicate that in nature, the potential for phage-mediated gene transfer, at least in the *Salmonella* group, is much higher than expected. Although the host range of phages appears to be more restricted than that of conjugative plasmids, there are various examples of phages which infect different species or even genera, e.g., actinophages ΦC31 (15) and R4 (4) and the *E. coli* phage mu (6). Phage-mediated gene transfers, e.g., by phage P22, between different species (17) or genera, e.g., *E. coli* (9), have also been reported. Having this in mind, and considering that phage-encapsulated DNA is protected against nucleases and many organic solvents, that phages are not sensitive to antibiotics and many other deleterious chemicals, and, finally, that many phages survive for many years without loss of biological activity, much more attention should be paid to generalized transduction as a means of gene exchange during evolution and in the discussion about the release of genetically engineered microorganisms.

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