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Transduction of multiple drug resistance of *Salmonella enterica* serovar *typhimurium* DT104

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Abstract

Epidemic strain *Salmonella typhimurium* DT104 is characterized by various multiresistance patterns. At least some of the resistance genes are organized as integrons. Resistance genes of DT104 isolates can be efficiently transduced by P22-like phage ES18 and by phage PDT17 which is released by all DT104 isolates so far analyzed. Cotransduction tests demonstrate that the resistance genes, although not organized in a unique integron, are tightly clustered on the *Salmonella* chromosome. The spread of resistance genes in this strain by generalized transduction is discussed.

Keywords: *Salmonella typhimurium* DT104; Multiresistance; Generalized transduction; Integron

1. Introduction

Salmonellae continue to be a major problem for man and animals. Outbreaks of salmonellosis, mainly caused by food poisoning, occur periodically all over the world. In the 1980s the predominant strain isolated from infected individuals was *Salmonella enteritidis* PT4. The reservoir of this strain is in poultry. Since 1990 a strain of *S. enterica* serovar *typhimurium*, definite type (DT) 104, has emerged as a new epidemic strain which seems to displace PT4 in many parts of the world [1–3]. This strain originates from cattle, but it has by now found its way to many animals and to man [4]. It is characterized by multiple drug resistance of various combinations. The most frequently appearing resistance pattern comprises resistance to ampicillin, chloramphenicol, streptomycin, spectinomycin, sulfonamides and tetracycline (R-type: ACSSpSuT); the resistance genes are located chromosomally [5]. Recently it was shown that at least the genes causing resistance to ampicillin and streptomycin are parts of two separate integrons which both additionally confer resistance to sulfonamide [6,7]. However, no information could be obtained about organization and localization of the genes which determine resistance against tetracycline and chloramphenicol in DT104.

The observation of different resistance patterns

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and the appearance of additional resistances since 1994 [7] raise the question how the resistance genes become spread. Plasmid controlled transfer of chromosomal genes seems not to be common in natural strains of *Salmonella*. So far only one case of plasmid mediated transfer of a resistance gene (apramycin) in DT104 has been reported [8]. Natural competence for uptake of free DNA for transformation is not known at all in this genus. Therefore, it is obvious to consider phage controlled transduction as the means of gene transfer. We have shown that temperate phages capable of generalized transduction are common genetic elements in salmonellae [9] [10]. Hence, we investigated the possibility of transferring the resistance genes of DT104 strains to various recipients. This seemed at first glance to be a trivial task.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains are listed in Table 1.

2.2. Phages

ES18 [11]; PBA19B (Schmieger, in preparation); PDT17 (this publication). Phage titers were determined on the respective propagation strains.

2.3. Media

LB medium [12] was used for cell cultivation and phage propagation. LB agar was used for plaque assays and transduction. Antibiotics were added to 50 μg ml^{-1}.

2.4. Transduction

200 μl of an overnight culture of the recipient strain was mixed with 200 μl of the transducing phage lysate. Adsorption was allowed for 15 min at 37°C. 100-μl aliquots were added to 3 ml top agar and poured on a black membrane filter (Schleicher and Schuell, ME25/31) which was placed onto an LB agar plate. After incubation for 2 h at 37°C to permit expression of the resistance genes the membrane was transferred to an LB agar plate with the selective antibiotic. After incubation at 37°C overnight transductant colonies could easily be detected on the black background of the membrane filter and isolated for further analysis. When phage titers and/or transduction frequencies were very low, the recipient culture was concentrated 10 times by centrifugation and 100 μl was added to 1 ml phage lysate. After adsorption 3 ml top agar was added, and the procedure continued as described above. Transduction frequencies are given as the T/P ratio: transductants/plaque forming unit (pfu).

Table 1
S. typhimurium strains

Strain designation	Strain no.	Lysotype	Genotype	Resistance pattern	Source/reference
DB21		LT2	wild-type	sensitive	laboratory
His(H1)		LT2	*his*$^-$, lysogenic for phage P22	sensitive	laboratory
DT16	SZ 1980/97	DT104	wild-type, lysogenic for phage PDT104[a]	SSu	Rabsch
DT16 NG19		DT104	*thr*$^-$, lysogenic for phage PDT104[a]	SSu	this publication
DT16 NG19/3		DT104	*thr*$^-$, lysogenic for phage PBA19B[b]	SSu	this publication
DT17	2566/92	DT104	wild-type, lysogenic for phage PDT104[a]	ACSSuT	Rabsch
DT17(19B)		DT104	DT17, lysogenic for phage PBA19B[b]	ACSSuT	this publication
1205-1	1205-1	ST104	wild-type, lysogenic for phage PDT104[a]	sensitive	Tschäpe
1205-1(19B)		DT104	1205-1, lysogenic for phage PBA19B[b]	sensitive	this publication

[a]Phage PDT104 is a natural prophage of DT104 strains (Schmieger, to be published).
[b]Strains were lysogenized in our laboratory. PBA19B is isogenic with ES18 (Schmieger, to be published). Both phages are homoimmune to phage P22.

Table 2
Cotransductions of recipient DT16 NG19/3 with phage ES18

Selected marker	Resistance phenotype			Number of transductants
	A	C	T	
amp	+	+	+	180
	+	−	−	40
cam	+	+	+	144
	−	+	+	1
tet	+	+	+	70
	−	−	+	1

3. Results

3.1. Transduction of non-lysogenic and lysogenic recipient strains

S. typhimurium DT104 strain DT17 has the resistance profile ACS(Sp)SuT[1]. It was used as a donor for transduction. Strain DT16, also a DT104 lysotype, has the resistance type SSu and, therefore, it was used as recipient of the resistance genes *amp, cam,* and *tet*. The transducing phage was ES18 [11] which infects both DT104 strains. Phage ES18 was propagated on strain DT17. Possibly due to restriction barriers, phage titers were very low at the beginning. After repeated passages through DT17 particle numbers increased to a titer suitable for transduction. Strain DT16 was transduced as described in Section 2. Transductants were selected for tetracycline or chloramphenicol resistance. Both selections yielded transductants, although at very low rates. T/P ratios were only 1.2×10^{-8} for *tet* and 1×10^{-9} for *cam*. This low efficiency may be caused by lysis of potential transductants by simultaneously or later infecting non-transducing (infective) phage particles, because sequence analysis of the immunity region of our ES18 strain showed it to be an amber mutant in gene *c3* (data not shown).

In order to affirm that the few resistant colonies are real transductants a *thr*$^-$ mutant of recipient strain DT16 (= DT16 NG19) was isolated. To protect these cells against phage lysis, this mutant was subsequently lysogenized with phage PBA19B, the wild-type of ES18 (Schmieger, in preparation). The resulting strain was designated DT16 NG19/3 and used as recipient.

Transduction was repeated in the same way as described above, selecting for *cam*. Lysogenization of the recipient strain raised the transduction frequency for *cam* in a typical experiment to 2×10^{-7}. All resistant colonies assayed required threonine for growth and, hence, were true transductants.

3.2. Cotransduction

In order to investigate cotransduction of the selected resistance marker with the non-selected resistance genes, transductants from the different experiments were assayed for the additional resistance genes present in the donor strain. In most cases the resistance genes *amp, cam* and *tet* were cotransduced (resistance pattern ACT[2]; Table 2 presents the data of a representative experiment). Only 18.2% of *amp* transductants (40 out of 220) exhibited solely ampicillin resistance. No transductants were found showing the resistance pattern AC or AT. From 145 assayed *cam* transductants all but one showed the resistance pattern ACT; one transductant was only sensitive against ampicillin (pattern CT), and amongst 71 tested *tet* transductants all but one carried the three resistance genes.

Spontaneous mutation of the colonies which carried only the *amp* marker was excluded via PCR analysis assaying the presence of the *amp* integron identified in DT104 [6]. Primers 9 and 10 as indicated by [6] were used to detect the central part of the *pse-1* (*amp*) gene cassette as a fragment of 410 bp. All tested *amp* transductants as well as the donor strain DT17 yielded a PCR product of about 400 bp, whereas the recipient strain DT16 was negative. Therefore, these *amp* colonies are proven to be transductants, and the resistance genes *cam* and *tet* were

[1] Although these are not the correct designations for phenotypes, these abbreviations, which are generally used for DT104 strains, are also adopted here to describe the resistance type. For the relevant genes the following abbreviations are used, because not all resistance determining genes have been identified so far: *amp* (ampicillin resistance), *cam* (chloramphenicol), *str* (streptomycin), *sul* (sulfonamide), *tet* (tetracycline). Spectinomycin resistance was not tested and regarded in this study, because it is conferred by the same gene which determines resistance against streptomycin.

[2] Since donor and recipient strains had the resistances against strepomycin and sulfonamide in common, they are not mentioned separately in the following.

Table 3
Cotransductions of recipient strain DT16 NG19/3 with PDT17

Selected marker	Resistance phenotype			Number of transductants
	A	C	T	
amp	+	+	+	159
	+	−	−	7
cam	+	+	+	104
	−	+	+	5

separated during the transduction process from the gene *amp*.

To examine likewise the cotransduction of *str* and *sul* together with *amp, cam* and *tet*, we used DT104 isolate 1205-1 as a recipient which was sensitive against all antibiotics used in these experiments. It was lysogenized with phage PBA19B [= strain 1205-1(19B)] and transduced with ES18 propagated on DT17 to select for *cam*. Fourteen out of 16 transductants were resistant to all antibiotics, A, C, S, Su, and T. Two showed only the selected marker, *cam*. This indicates that all resistance genes, including *str* and *sul*, must be located within the packaging range of phage ES18.

This result was confirmed by a few transductants of the sensitive LT2 strain His(H1) which also were resistant against all antibiotics although only *cam* was selected.

3.3. Transduction by the phage residing in DT104 strains

We have shown that all DT104 strains harbor a prophage, PDT17, which is related to phage P22 (to be published). Due to this relationship it was expected that PDT17 is a generalized transducing phage as well. To test whether this phage is also able to transduce the resistance markers of its host strain, donor strain DT17 was lysogenized with phage PBA19B [= strain DT17(19B)]. This resulted in the loss of the prophage PDT17, because both phages obviously compete for the same attachment site, att_{P22} (to be published). This procedure rendered DT17 sensitive for its previous prophage which now could be propagated on it. With the resulting lysate PDT17:DT17 recipient strain DT16 was transduced. Since DT16 naturally also carries the prophage PDT17, it was protected against lysis. *Cam* and *amp* transductants were obtained with T/P ratios of $3–6 \times 10^{-8}$, most of them being resistant to the other antibiotics as well (Table 3).

4. Discussion

The multiresistant phenotype of the overwhelming majority of isolates of the epidemic *S. typhimurium* strain DT104 causes a serious problem for public health. The problem increases continuously, because it has been observed that the content of resistance genes extends by uptake of new resistance determinants following the introduction of new antibiotics [7]. Therefore, it is of great importance to understand the genetic organization of the resistance determinants as well as the mechanisms of their spread. Recently we have shown not only that *Salmonella* isolates harbor prophage genomes as general genetic elements, but that most of them belong to the P22-like group of lambdoid phages which are capable of generalized transduction [9,10]. Therefore, the possibility of phage mediated transduction of the resistance genes was an exciting question.

Early attempts of other groups to transduce the resistance determining genes of DT104 were unsuccessful. Also our initial experiments yielded very low numbers of transductants. Three aspects in the experimental procedure appeared to be crucial. (i) Recipient cells should be protected against the transducing phage by a homoimmune prophage. Obviously the number of surviving transductants is severely reduced by two factors: the lytic reactions of coinfecting phages and possible restriction barriers between donor and recipient. (ii) It is extremely important to allow integration of the transduced DNA fragment and expression of the resistance genes before selective stress. This was achieved by first incubating the infected cells on a membrane on antibiotic free medium and subsequent transfer of the membrane to the selective medium. This allowed quantitative evaluation of the transduction results. (iii) Recipient cells should be DT104 strains, preferably having already at least one resistance gene. LT2 derivatives and other lysotypes were very ineffective recipients.

When these precautions were observed, transduction occurred not only with the well known general-

ized transducing phage, ES18, which is closely related to P22, but also with a phage, PDT104, which resides as a prophage in all DT104 strains investigated (to be published). This implies that all DT104 strains carry in their genome a potent vehicle suitable for horizontal transfer and further spread of resistance genes. Although transduction rates may be considered very low in the experimental conditions, in nature this mode of gene transfer may be effective enough to account for the observed spread of resistances. Since DT104 originates from cattle, its predominant habitat is animal breeding stations. Under these conditions the concentration of bacterial cells which may participate in gene exchange is extremely high. Furthermore, the high infectivity of phage particles and the persistence of phages outside cells contribute to a high effectivity. Additionally, the continuous release of (transducing) phage particles from propagating lysogenic cells by spontaneous induction of prophages must be considered.

Another aim of the study was to learn something about the chromosomal organization of the resistance genes. The cotransduction of the complete set of genes conferring resistances is a very important result, as it shows that the resistance determinants are clustered within a range of less than 46 kb. This is the packaging capacity of an ES18 phage particle [13]. Sandvang and coworkers [6,7] have demonstrated that the *sul* gene is associated with the cassette for ampicillin resistance within the same integron and that another copy of *sul* is associated with the *ant* cassette for streptomycin/spectinomycin resistance (=*str*) in a separate integron. According to Ridley and Threlfall [7] these integrons are located on a chromosomal DNA fragment of about 10 kb. This explains cotransduction of *amp, sul* and *str*. The same authors argue, however, that the other very common resistance genes for chloramphenicol and tetracycline are not organized as integrons; rather they suppose them to be transposons. Whatever their organization may be, they must be located close to the two identified integrons, otherwise they would not be packaged together with them on a 46-kb fragment by the same phage particle.

It was expected that different segregation patterns of the various resistance determining elements in the cotransduction experiments would yield information about their positions relative to each other. It is the classical form of gene mapping by transduction [14]. However, against all experience, the genes showed a very unusual cotransduction behavior. In most cases, all resistance genes were cotransduced; in a few cases only the selected marker was expressed. Eighteen percent of *amp* transductants did not integrate *tet* and *cam*. This should indicate a close linkage of the latter genes and a significant distance to the selected marker. But only one out of 145 *cam*/*tet* transductants was not ampicillin resistant. These results do not allow conclusions about gene order. This should be the consequence of a very special form of chromosomal organization. One might speculate that the resistance genes, as parts of integrons or transposons or even in another form, are clustered so tightly that they are not separated by host sequences. Therefore, homologous recombination between the single genes would be impossible in the recipient cells. This very close linkage of the resistance genes, together with the observation that the genes are added successively to the strains [7], supports the idea that on the chromosome, at least on the chromosome of the multiresistant isolates of DT104, there exists a stretch of nucleotides which may act as a trap for new resistance genes. These might be similar to the 'hot spots' found within the integrons [15] acting as integration sites for new resistance cassettes into the same integron. However, a conclusive explanation requires a detailed knowledge of the molecular situation which is not yet available despite careful studies by several investigators [6,7].

Another unexpected observation was that transduction of strains other than DT104 having at least one resistance gene was either unsuccessful or extremely low. Explaining this by the lack of the supposed 'trap sequence' in the respective strains is not satisfactory: the size of an integron carrying one gene (in addition to *sul* and *qac*) may be about 3 kb [6]. All resistance genes together should not comprise much more than 12 kb. Since the phage capacity is about 46 kb and since the resistance determinants seem to be tightly clustered, there should be enough chromosomal material besides the resistance cluster which should be very similar in all strains of *S. typhimurium* to provide homology for integration.

To understand these paradoxes it is necessary to know the architecture of the resistance determining area and the nucleotide surroundings.

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