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Transduction of antibiotic resistance markers among *Actinobacillus actinomycetemcomitans* strains by temperate bacteriophages Aaϕ23

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Abstract. *Actinobacillus actinomycetemcomitans* (*Aa*) strain ST1 carries the tetracycline (Tc) resistance transposon Tn*916* and the AaϕST1 prophage, which is closely related to temperate bacteriophage Aaϕ23. High titre phage preparations were obtained from this strain by mitomycin C induction and were used to transduce the Tc^R determinant to the Tc^S recipient strains ZIB1001 and ZIB1015 (MIC 2 μg Tc/ml). Tc^R transductants (MIC $\geq$ 32 μg Tc/ml) were detected at frequencies of 3×10^{-6} to 5×10^{-8} per pfu. All Tc^R transductants examined contained the entire Tn*916* inserted at several different locations within the *Aa* genome. They appear to have resulted from generalized transduction. In addition both bacteriophages, Aaϕ23 and AaϕST1, were capable of transducing the chloramphenicol (Cm) resistance marker of plasmid pKT210 (transduction frequencies of 2×10^{-5} to 3×10^{-7} per pfu) to the recipient strain ZIB1001 (MIC 8 μg Cm/ml). Eleven Cm^R ZIB1001 transductants (MIC $\geq$ 100 μg Cm/ml) studied carried a plasmid indistinguishable from pKT210 by restriction analyses. In view of the high prevalence of this phage family, and the increasing use of tetracycline in periodontitis therapy, these findings may have clinical importance.

Key words. *Actinobacillus actinomycetemcomitans*; temperate bacteriophage; transduction; antibiotic resistance; periodontitis.

The gram-negative oral bacterium *Actinobacillus actinomycetemcomitans* (*Aa*) has been implicated as a causative agent of several forms of periodontal diseases. On the other hand it is also found in healthy individuals as well as in nondiseased oral sites of periodontitis patients [1, 2]. *Aa* possesses a variety of putative virulence factors including the ability to invade epithelial cells, to produce a membrane-bound leukotoxin which specifically kills human polymorphonuclear leukocytes and macrophages, a potent endotoxin and other surface components inducing cytokine release and tissue destruction (for review see [3]). Since *Aa* is difficult to eradicate reliably from periodontal lesions by conventional therapy alone, adjunctive antibiotic use is widespread [4, 5].

In several other bacterial species bacteriophages contribute to the virulence potential of their host, e.g. in *Corynebacterium diphtheriae* and *Vibrio cholerae* [6, 7]. Among *Aa* isolates the presence of phages is a common feature. Three morphologically distinct phages were demonstrated in periodontal pockets of a few patients [8]. Temperate bacteriophages were detected in more than 50% of the *Aa* isolates sampled from subgingival plaques of periodontitis patients [9]. A comparison of

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five of these temperate bacteriophages isolated in three European countries revealed a very close genetic relationship [10]. Phage Aaϕ23 represents the type strain of this phage family. It has an icosahedral head of 65 nm, a contractile tail of 110 nm and a DNA genome of about 44 kb. Its relations to the similar, inducible phage ϕAa isolated in North America [11] have not been determined. There was a high prevalence (44%) of temperate bacteriophages among *Aa* isolates from periodontally healthy oral cavities most of which were related to phage Aaϕ23 [12]. So far there are no epidemiological or clinical indications that this phage alone would significantly increase the virulence of the *Aa* host [13, 14].

However, bacteriophages may not only modulate the virulence of their host, they may compromise antibiotic therapy by transferring genes encoding drug resistance to susceptible bacteria [6]. So far transduction has not been described in *Aa*. As Aaϕ23-related bacteriophages are so frequent among *Aa* isolates from different geographic locations as well as from diverse clinical conditions, we examined the transduction of antibiotic resistance genes located either on the bacterial chromosome or on a plasmid by Aaϕ23 phages. The results identify Aaϕ23 as a generalized transducing phage.

Materials and methods

Bacterial and plasmid strains. The donor and recipient *Aa* strains and plasmids used in this study are listed in table 1.

Media and growth conditions. *Aa* strains were grown in Todd Hewitt broth (THB; BBL Becton Dickinson, Münchenstein, Switzerland) at 37 °C in air and 10% CO_2. Transductants were selected on Columbia blood agar plates containing tetracycline (Tc; 8 μg/ml) and chloramphenicol (Cm; 25 μg/ml), respectively, and subsequently grown at 37 °C in air and 10% CO_2 in THB supplemented with the appropriate antibiotic.

Minimum inhibitory concentration. Final concentrations of tetracycline tested ranged from 0.1 to 32 μg/ml, final concentrations of chloramphenicol from 0.1 to 100 μg/ml. Cultures of *Aa* strains containing 5×10^4 colony forming units (cfu) were spotted onto Columbia blood agar plates containing the antibiotic, and then incubated at 37 °C in air and 10% CO_2. After 48 h and 72 h, the plates were inspected for growth in the spotted area.

***A. actinomycetemcomitans* transformation.** The *Aa* recipient strains were washed twice in half-volumes of ice-cold electroporation buffer (15% glycerol, 272 mM sucrose, 2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4) as described in [15]. This concentrated bacterial suspension was incubated on ice for 15 min prior to high-voltage pulse. Plasmid DNA of pAM120 containing Tn*916* [16] as well as of the pRSF1010 derivative plasmid pKT210 [17] was obtained by the plasmid purification kit (Qiagen AG, Basel, Switzerland). High-voltage pulses were delivered with the BTX ECM (electro cell manipulator) 600 at 2500 V and 187 Ω. Following electroporation, bacteria were immediately transferred into 1 ml THB without antibiotic, and the culture was incubated at 37 °C in air and 10% CO_2 for 1 h. Subsequently, the transformed cultures were plated on Columbia blood agar plates containing the appropriate selective antibiotic.

Table 1. Bacteria and plasmids.

Bacteria and plasmids	Characteristics	Reference
A. actinomycetemcomitans *strains*		
ST1 (::Tn*916*, AaϕST1)	SPA-defective mutant of strain Y4; Tn*916*, Tc^R donor strain; natural lysogen of phage AaϕST1	[18]
ZIB1001, ZIB1015	recipient strains; Tc^S, Cm^S; indicator strains for phages Aaϕ23 and AaϕST1	[10]
ZIB1023 (Aaϕ23)	natural lysogen of phage Aaϕ23	[10]
ZIB1001(pKT210, Aaϕ23)	donor strain in plasmid transduction	this paper
ZIB1001(pKT210, AaϕST1)	donor strain in plasmid transduction	this paper
Plasmids		
pKT210	pRSF1010-derivative; 11.8 kb; Cm^R	[17]
pAM120	pBR322-derivative carrying Tn*916*	[16]

Lysogenization of ZIB1001(pKT210) with phages Aaϕ23 and AaϕST1, respectively. Freshly filtered lysates containing phage Aaϕ23 or AaϕST1 were prepared from the original lysogens ZIB1023 [10] and ST1 [18], respectively. The lysogenization procedure has been previously reported [14].

Transducing lysates. High titre phage lysates AaϕST1 and Aaϕ23 were obtained from mitomycin C-induced (1 μg/ml) donor strains ST1, ZIB1001(pKT210, AaϕST1) or ZIB1001(pKT210, Aaϕ23), respectively, followed by PEG precipitation as described earlier [10]. All lysates were filter-sterilized (0.2 μm FP 030/3, Schleicher & Schuell, Riehen, Switzerland) before the transduction assay. A loop of each lysate was tested for the absence of viable cells by incubating on Columbia blood agar plates.

Transduction assay. Log-phase cultures of recipient strains were mixed with freshly prepared phage lysates at multiplicity of infection (m.o.i.) ranging from approximately 0.01 to 2.5 in the presence of DNaseI (1 μg/ml). The mixture was incubated at 37 °C in air and 10% CO_2 for 30 min. Then 100 μl were plated on selective plates containing the appropriate antibiotic and incubated at 37 °C in air and 10% CO_2. After 3 days the plates were examined for antibiotic-resistant transductants. The experiments were repeated two to four times.

Screening for prophage. The presence of prophage was tested in two ways. Transductants were screened for phage release by the overlay plate technique as previously described [9]. Additionally, the transductants were tested for the presence of AaϕST1 or Aaϕ23 DNA in chromosomal Southern blot hybridizations.

DNA isolation and hybridization. Chromosomal DNA of transduced *Aa* strains was obtained with a genomic DNA purification kit (Qiagen AG). Digestion with restriction endonucleases (Boehringer Mannheim, Rotkreuz, Switzerland), separation of the fragments by agarose gel electrophoresis and the alkaline transfer [19] to nylon membranes (Biodyne A, Pall, Wohlen, Switzerland) were performed according to [12]. The probes used in Southern hybridizations were prepared as follows: polymerase chain reaction (PCR) products were directly labelled and used in hybridization procedures. pAM120 containing Tn*916* was digested with the restriction enzyme *Asn*I (Boehringer Mannheim) and electrophoretically separated on a 0.7% agarose gel. As expected from the DNA sequence of Tn*916* [20], *Asn*I digestion produced bands of 2.2 kb (containing most of the *tet*(M) gene), 3.7 kb (left portion of Tn*916*) and 12 kb (right portion of Tn*916*). DNA fragments were recovered using the QiaexII kit (Qiagen AG). Labelling using the ECL gene detection system and hybridization procedures followed methods given by the manufacturer (Amersham, Rahn, Zürich, Switzerland). As size markers the 1-kb DNA ladder (Life Technologies AG, Basel, Switzerland) was used.

Primers and PCR amplification. PCR primers were designed with help of the computer program OLIGO 4.0-s [21] using the Tn*916* sequence of Flannagan et al. and Clewell et al. [20, 22]. Primer positions on Tn*916* and their sequences are shown in figure 1. Primers were custom-synthesized and HPLC-purified (MWG-Biotech, Synthesis Lab, Ebersberg, Germany). The reaction mix (50 μl volume) contained 1 ng of template DNA, 0.2 mM dNTP, 0.2 μM primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5–8 mM $MgCl_2$, and 2 U Taq polymerase (Boehringer Mannheim). The PCR amplifications were performed in a Thermocycler 2400 (Perkin Elmer, Rotkreuz, Switzerland). The temperature profile was set to 95 °C for 2 min; 25 cycles at 95 °C for 30 sec, 57 °C for 30 sec and 70 °C for 30 sec; and a final extension step at 70 °C for 3 min. Amplification products were separated on 1% agarose gels containing 0.5 μg/ml ethidiumbromide, and analysed by a gel documentation system (Bio-Rad Laboratories, Glattbrugg, Switzerland).

Results

Transduction of a chromosomal Tc^R-marker by temperate bacteriophage AaϕST1. The *Aa* strain ST1 harbours the conjugative transposon Tn*916* including the Tc^R determinant *tet*(M) and is also lysogenic for the Aaϕ23-related temperate phage AaϕST1. From this strain high titre phage lysates (5×10^9 to 5×10^{11} pfu/ml) were produced and filter-sterilized to remove intact cells and thus to prevent a conjugational event in subsequent transfer experiments. The Tc^S recipient strains ZIB1001 and ZIB1015 were incubated with AaϕST1 lysates at an m.o.i. of 0.25 and 2.5. During the transduction assay DNaseI was added to reduce the transfer of naked DNA by transformation to a level about 100 times below detection limits. Tc^R ZIB1001 as well as Tc^R ZIB1015 transductants were obtained at frequencies of 3×10^{-6} to 5×10^{-8} per pfu. The minimal inhibitory concentration (MIC) of tetracycline increased from 2 μg Tc/ml before to $\geq$32 μg Tc/ml after transduction. This indicates that the Tc^R determinant was transduced to the Tc^S recipient strains. The presence of the *tet*(M) gene in the transductants was verified by PCR amplification using primers 1 and 2 specific for the *tet*(M) gene (fig. 1). All 13 transductants tested revealed a PCR product of about 480 bp (data not shown). A product of the same size was amplified from plasmid pAM120 and strain ST1 harbouring Tn*916*, but neither from its parental strain Y4 nor from the recipient strains ZIB1001 and ZIB1015. Additionally, the presence of *tet*(M) was confirmed by Southern blot hybridizations of *Hin*dII-digested chromosomal DNAs probed with the *tet*(M)-specific PCR product (called a in fig. 1) obtained from strain ST1 (data not shown). All trans-

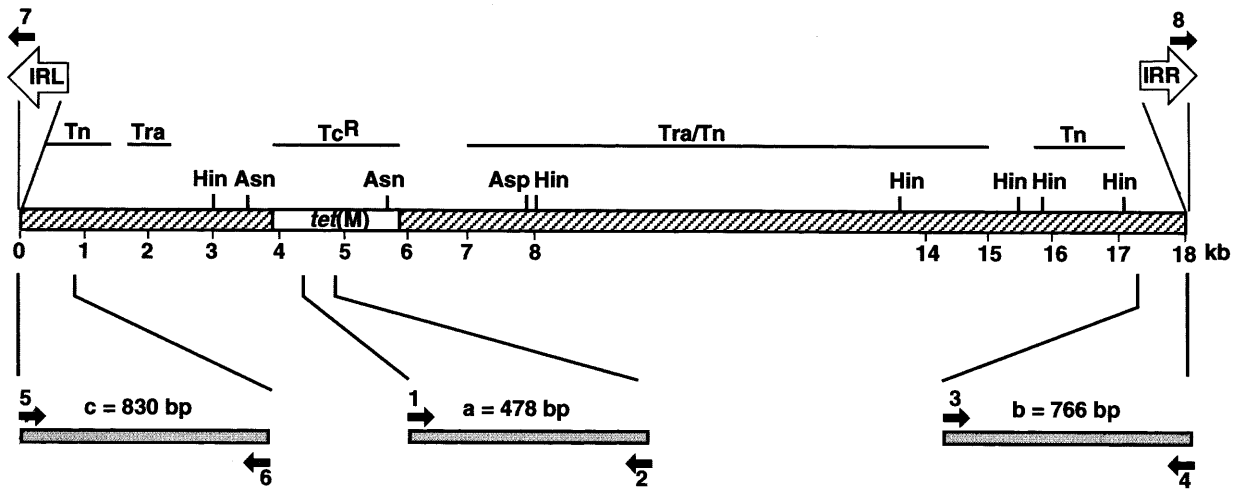

Figure 1. Map of Tn*916*. Shown are the positions of the 26-bp inverted repeats at the left (IRL) and the right (IRR) end, of the *tet*(M) gene coding the tetracycline resistance (Tc^R), of the regions for transfer (Tra), transposition (Tn) or both (Tra/Tn) [23], of restriction enzyme sites for *Hin*dII (Hin), *Asp*700 (Asp), and *Asn*I (Asn), of PCR primers 1–8 and PCR products a, b, c used in this study. Sequence and position of the PCR primers are: primer 1: 5′ TTT ACA GTC CGT CAC ATT CCA ACC A 3′ (position 4417–4441), primer 2: 5′ TGA AAA TCC GCA CCC TCT ACT ACA A 3′ (position 4894–4870), primer 3: 5′ GTC TTC ATT TTG GAT TCT CAC TTC A 3′ (position 17266–17290), primer 4: 5′ AAA TAG CAT AAA AAT CTA GTT ATC CGC 3′ (position 18031–18005), primer 5: 5′ AAA CAA AGT ATA AAT TTC TAA TTA TCT TT 3′ (position 1–29), primer 6: 5′ AGA CCG TAC TAA CAG AAG AAC AG 3′ (position 830–808), primer 7: 5′ GCG GAT AAC TAG ATT TTT ATG CTA 3′ (position 18005–18023), primer 8: 5′ AAA GAT AAT TAG AAA TTT ATA CTT TGT TT 3′ (position 29–1). The map corresponds to the inverted sequence of Tn*916* (EMBL accession no. EF09422) [20].

ductants carried *tet*(M) sequences on a *Hin*dII fragment of about 5 kb which was expected according to the restriction map of Tn*916* (fig. 1).

The screening for phage release and for the presence of prophages revealed that 10 of 13 transductants were lysogenic, i.e. released phage and showed an Aaϕ23-like prophage hybridization pattern (data not shown). This relatively high rate of lysogenization was probably due to double infection based on the high m.o.i. of 2.5 used in some of the transduction experiments. The observation of unchanged Aaϕ23-like prophage hybridization patterns in the transductants' DNA excludes the possibility that Tn*916* had transposed to the phage genome before transduction.

Was the entire Tn*916* transduced? The 18 kb conjugative transposon Tn*916* contains in addition to *tet*(M) transfer and transposition functions and 26 bp inverted repeats at both ends (see fig. 1). To determine whether the whole Tn*916* was transduced, *Asp*700-digested chromosomal DNA of all 13 transductants was hybridized with the 3.7 kb *Asn*I fragment representing the left portion of Tn*916* as well as with the 12 kb *Asn*I fragment representing the right portion of Tn*916*. The results indicated that the entire Tn*916* was transduced from the donor to the recipient *Aa* strains (data not shown). In order to confirm this conclusion PCR primers specific for the left (primers 5 and 6) and right (primers 3 and 4) ends of Tn*916*, respectively (fig. 1), were used to amplify the termini. All 13 transductants tested yielded a PCR product of about 830 bp specific for the left and a PCR product of about 760 bp specific for the right end of Tn*916* (data not shown). The controls Y4, ZIB1001 and ZIB1015 showed no PCR product.

Are there different integration sites for Tn*916*? The fact that all transductants contained the *tet*(M) gene and both ends of Tn*916* supports the notion that Tn*916* integrated as a unit. In order to assess variations in the integration site(s), Southern blots of *Hin*dII-digested chromosomal DNA of five transductants were hybridized using the PCR product (called b in fig. 1) obtained from *Aa* strain ST1 representing the right end of Tn*916* as the probe. Different hybridizing signals were observed (fig. 2), suggesting several different insertion locations for Tn*916* after transduction. Additionally, two transductants (lanes 1 and 5) had two hybridizing bands, indicating that Tn*916* either integrated twice or transposed within these transductants.

Plasmid transduction. In order to test for phage Aaϕ23-mediated plasmid transduction, the broad host range plasmid pKT210 carrying a Cm^R-determinant was transferred to *Aa* donor strain ZIB1001 by electroporation transformation. Cm^R transformants were obtained at 1×10^2 cfu/μg plasmid DNA. Cm^R *Aa* transformants were then lysogenized either by phages Aaϕ23 or AaϕST1, respectively. Phage lysates obtained from strains ZIB1001(pKT210, Aaϕ23) and ZIB1001 (pKT210, AaϕST1) were used in the plasmid transduction assays at m.o.i. ranging from 0.01 to 1. The Cm^R marker was transduced to the Cm^S recipient strain ZIB1001 by both bacteriophages, Aaϕ23 and AaϕST1, at frequencies of 2×10^{-5} to 3×10^{-7} transductants per

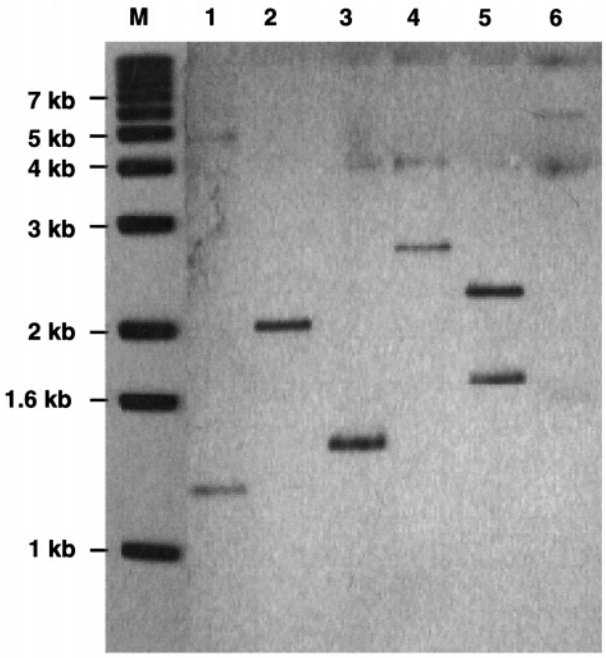

Figure 2. Different integration sites of Tn*916*. Southern blot hybridization of *Hin*dII-digested chromosomal DNA of Tc^R transductants hybridized with PCR product b (right end of Tn*916*). Lanes 1–5: transductants; lane 6: ST1; M: 1 kb DNA ladder as size marker.

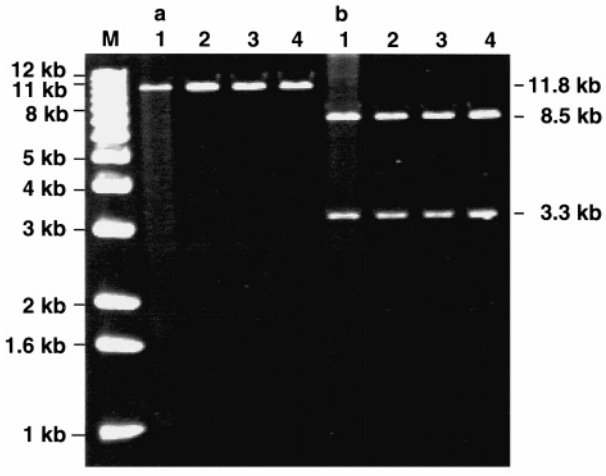

Figure 3. Restriction analyses of pKT210 and of plasmids isolated from Cm^R transductants digested with (*a*) *Eco*RI and (*b*) with *Pst*I. Lane 1: pKT210 (from *E. coli* C600); lanes 2–4: plasmids from Cm^R transductants; M: 1-kb DNA ladder as size marker.

pfu. The MIC for chloramphenicol increased from 8 μg Cm/ml before to $\geq$100 μg Cm/ml after the transduction. Further, transduction frequencies were consistently up to 100-fold lower at high m.o.i., which might be due to the presence of non-infectious killing particles in the transducing lysates. The same observation was made for transduction of the Tc^R marker.

Plasmid analysis of 11 Cm^R transductants indicated that all transductants acquired a plasmid of about 12 kb which showed the same *Eco*RI and *Pst*I restriction patterns as the original plasmid pKT210 (fig. 3). No Cm^R plasmid transductants could be detected when ZIB1015 was used as the receptor strain. This is most likely due to the earlier described restriction modification system active in ZIB1015 [10].

Generalized or specialized transduction? In order to determine whether the Tc^R marker was transferred by generalized or specialized transduction, two sets of experiments were carried out. First, since specialized transducing particles contain covalently linked phage and host DNA sequences, this DNA can be maintained as a prophage in transductants and may lead to a high frequency of transduction in subsequent transfer experiments. Therefore, a high titre phage lysate was made from a Tc^R transductant ZIB1001 lysogenic for AaϕST1 and was then used in a second transduction experiment. The frequency of transduction of the Tc^R marker (7×10^{-7} to 3×10^{-8} transductants per pfu) was in the same range as in the previous experiments, indicating that the Aaϕ23-like bacteriophages do not seem to promote specialized transduction.

Second, the origin of DNA sequences flanking the Tn*916* was determined. If Tc^R had been transferred by generalized transduction, the Tn*916* flanking sequences were expected to be of chromosomal origin, while phage DNA sequences would be proof for specialized transduction. Therefore, the chromosomal DNA of one Tc^R transductant was digested with *Eco*RI (Tn*916* contains no *Eco*RI restriction site) and re-ligated. The Tn*916* flanking regions were amplified with primers 7 and 8 (fig. 1), resulting in a PCR product which was then used as the probe in a Southern blot hybridization of *Aa* chromosomal DNA as well as of phage Aaϕ23 DNA (fig. 4). The PCR product revealed homologies to chromosomal DNA but showed no bands hybridizing to phage DNA. This result indicated that the temperate Aaϕ23 phage family are generalized transducing phages.

Discussion

We presented the first evidence of drug resistance transfer among *Aa* strains by phage-mediated transduction. Transduction is an effective genetic transfer system through which donor chromosomal DNA segments or plasmids can be delivered to recipient strains contributing to horizontal gene transfer in nature. Transduction has been studied in several bacterial genera and species,

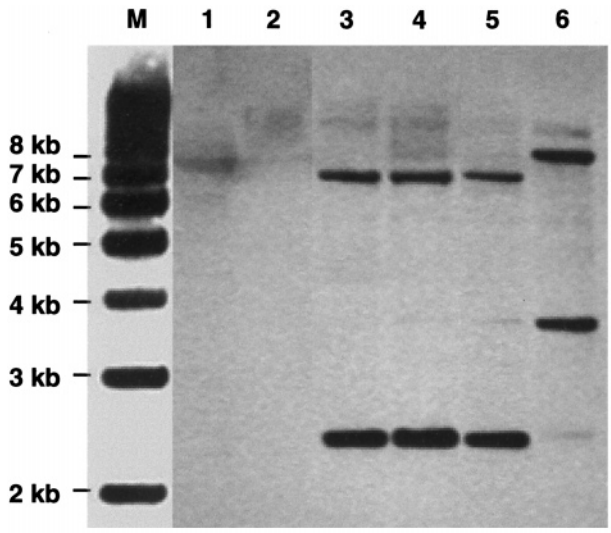

Figure 4. Southern blot hybridization of *Asp*700-digested phage and chromosomal *Aa* DNA hybridized to the PCR-product obtained with primers 7 and 8. Lane 1: AaϕST1; lane 2: Aaϕ23; lane 3: Y4; lane 4: ST1; lane 5: ZIB1001; lane 6: ZIB1015; M: 1 kb DNA ladder as size marker.

including the well-characterized *Salmonella typhimurium*. More than 70% of natural isolates of *S. typhimurium* have been shown to carry lysogenic phages that are capable of generalized transduction [24]. The evolution of virulence in *Salmonella* appears to be driven by horizontal gene transfer [25]. However, the best-studied generalized transduction models are the coliphages P1 and Mu, and the *Salmonella* phage P22 [26, 27]. These temperate phages were thoroughly investigated in the laboratory, while little is known on transduction in natural environments [28].

The Aaϕ23-like temperate bacteriophages studied here were able to transduce antibiotic resistance markers located on the chromosome or on a plasmid. The transduction frequencies ranging from 2×10^{-5} to 5×10^{-8} transductants per pfu were in the same range as those measured in *Escherichia coli* using the temperate phages P1 (10^{-4} to 10^{-6} transductants per pfu) or Mu (10^{-7} to 10^{-8} transductants per pfu) [26]. These were perhaps not maximum frequencies obtainable, because growth of *Aa* strains and phages showed considerable variations even under standard conditions, and donor and recipient *Aa* were probably less well adapted to laboratory conditions compared with the respective *E. coli* strains. Evidence presented suggests that Aaϕ23 are generalized transducing phages. This is compatible with the observation that phage particles contain linear DNA molecules which are terminally redundant and circularly permuted (K. Willi, M. Maeder and J. Meyer, unpublished observations). Thus DNA is packaged by the headfull mechanism into Aaϕ23 phage particles, analogous to coliphage P1 [27].

The conjugative tetracycline resistance transposon Tn*916* was transduced to *Aa* recipients as a unit. Transfer by transformation or conjugation was experimentally excluded. Tn*916* integrated at different sites within the *Aa* genome, suggesting an integration by transposition rather than by homologous recombination of flanking sequences. Sato et al. [18] observed also various different integration sites of Tn*916* in SPA-defective *Aa* mutants. It cannot be decided based on the few derivatives studied whether the *Aa* genome carries preferred target sequences or even hot spots for Tn*916* integration as does *Bacillus subtilis* [29].

Tetracyclines are the antimicrobial agents most frequently used in combination with debridement to eliminate putative periodontal pathogens. In addition, metronidazole and other antibiotics have also been used successfully [30]. However, these agents are not effective in some patients, and an increasing proportion of the periodontal microflora was reported to be resistant [31–36]. *Aa* has been found to be rather susceptible to tetracyclines [31, 37, 38], although standard procedures for the determination of the minimum inhibitory concentration have not been established. Some clinical isolates of *Aa* carried the *tet*B determinant which was transferable between *Aa* strains [39]. Since tetracycline resistance determinants are present among *Aa* strains, the fairly common temperate Aaϕ23 phages may contribute to their spread by generalized transduction.

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