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Degrees of relatedness of T-even type *E. coli* phages using different or the same receptors and topology of serologically cross-reacting sites

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The relatedness of a series of T-even like phages which use the *Escherichia coli* outer membrane protein OmpA as a receptor, and the classical phages T2, T4 and T6 has been investigated. Immunoelectron microscopy and the pattern of phage resistance in bacterial mutants revealed that: (i) phages of this morphology do not necessarily cross-react serologically; (ii) phages using different receptors may bind heterologous IgG everywhere except to the tip (comprising ~ 10% of one fiber polypeptide) of the long tail fibers; (iii) cross-reacting OmpA-specific phages may bind heterologous IgG only to the tip of these fibers: (iv) OmpA-specific phages not cross-reacting at the tip of the tail fibers use different receptor sites on the protein. Absence of cross-reactivity appears to reflect high degrees of dissimilarity. A DNA probe consisting of genes encoding the two most distal tail fiber proteins of T4 detected homologies only in DNA from phages serologically cross-reacting at this fiber. Even under conditions of low stringency, allowing the formation of stable hybrids with almost 30% base mismatch, no such homologies could be found in serologically unrelated phages. Thus, in the collection of phages examined, there are sets of very similar and very dissimilar tail fiber genes and even of such gene segments.

Key words: DNA hybridization/immunoelectron microscopy/OmpA protein/phage receptors/T-even phages

Introduction

The classical phages T2, T4 and T6 are very close relatives: most combinations of mixed genes derived by recombination between different phages are compatible with viability (Russel, 1974). This close genetic similarity is corroborated by electron microscopic heteroduplex studies using DNA from the three phages (Kim and Davidson, 1974; all heteroduplexes show >85% homology) and the long known strong serological cross-reactivity of these phages (Adams, 1952).

We have isolated, from a local sewage plant, several *Escherichia coli* phages which use one or other of the so-called major outer membrane proteins as receptors (Henning and Haller, 1975; Datta *et al.*, 1977). Morphologically, all of them are indistinguishable from the T-even phages. It was initially observed that one of these new phages, using the outer membrane protein OmpA as a receptor, could not be inactivated with anti-T4 serum and that antiserum raised against the phage did not neutralize T2, T4 or T6. Since absence of neutralization need not demonstrate absence of immunological cross-reaction (Lanni and Lanni, 1953; Jerne and Avegno, 1956), precipitation was tested. The antisera mentioned, however, were also unable to precipitate the heterologous phages. This somewhat unexpected situation prompted a more detailed investigation.

Results

OmpA-specific phages

Phages TuII*-6 (identical to TuII*; Henning and Haller, 1975), K3 (Hancock and Reeves, 1975) and Ox2 (Kay and Fildes, 1962) have been shown to use the *E. coli* outer membrane protein OmpA as their receptor (Manning *et al.*, 1976; Schweizer *et al.*, 1978). TuII*-6 was isolated from local sewage, whereas K3 and Ox2 were isolated in Stockholm (then called phage V; Hammarström, 1949) and in Oxford, respectively. Using the same technique as described for TuII*-6, we have isolated four additional OmpA-specific phages (TuII*-24, -26, -46, -60; see Table I) from the sewage plant at the rate of one phage per year. Thus, all OmpA-specific phages are entirely independent isolates. Mutants resistant to any one of these phages are *ompA*; they usually lack the OmpA protein and have become resistant to all other OmpA phages including the host-range mutants of K3 (Manning *et al.*, 1976). Additional evidence for the OmpA specificity is given below (Table III).

Rates of phage inactivation by homologous and heterologous antisera

The results of these experiments are shown in Table I. Serologically, four groups could be distinguished. The first, comprising T2, T4, T6, Ox2 and TuII*-26, reacts with anti-T4 and anti-K3 but not with anti-TuII*-6 sera. The second, consisting of K3 and TuII*-24, reacts with all three sera although the heterologous reactions with anti-TuII*-6 are rather weak. The third group, represented by TuII*-6 and -60, does not react with anti-T4 serum and only weakly so with anti-K3 serum. The fourth consists of TuII*-46 alone which is inactivated only by anti-TuII*-6 serum.

Topology of cross-reacting sites

All of the phages studied are indistinguishable morphologically and, particularly in view of their unique shape, complete absence of immunological cross-reactivity would appear somewhat surprising. Failure to neutralize a phage need not, however, indicate such absence, since e.g., cross-reacting capsid proteins would not, under the experimental conditions employed (very low phage titer, see Materials and methods), inactivate the phage. We have therefore examined selected phages, treated with antisera, by electron microscopy. Results of these experiments are summarized in Table II and examples are shown in Figure 1. Firstly, these data confirm those of Table I in that the absence of neutralization is always accompanied by the lack of recognizable binding of antibodies to any part of the phage. Thus, phages which exhibit identical morphology and use the same receptor can be completely unrelated serologically (K3 and TuII*-46). Secondly, phage neutralization always involves binding of antibodies to the long tail fibers. The most conspicuous such cross-reactivities are those where the sera contain antibodies which recognize only the ends of the long tail fibers as was observed, e.g., in the reaction of anti-TuII*-6 serum with phage K3, or

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anti-K3 serum with phages TuII*-6 and TuII*-60 (Table II and Figure 1). It is very likely that the cellular receptor is recognized by the distal end of the tail fibers as certainly seems to be the case for T4 (see Discussion). These limited cross-reactions would then suggest that the phages in question, although largely unrelated serologically, possess very similar recognition areas. If this interpretation is correct we may expect that antibodies against the OmpA-specific phage K3 which bind practically all over T4 (which uses lipopolysaccharide as a receptor, see Table I) did not contain a species directed against the ends of the tail fibers of T4 and *vice versa*. Because of the cross-linking caused by complete IgG molecules it was difficult to find phages which have bound antibody to the tail fibers but not to their ends (although some clear examples were obtained; see Figure 1, reaction of anti-TuII*-6 serum with TuII*-46). Therefore, we have used the corresponding Fab fragments and in each case these unambiguously allowed us to decide whether or not antibody binds to these ends, and, as can be seen from Figure 1, anti-K3-Fabs do not bind to the ends of the tail fibers of T4.

Table I. Rate constants of phage inactivation

Phage	Antiserum		
	α-T4	α-K3	α-TuII*-6
T2	1000	220	<0.2[a]
T4	2400	340	<0.2
T6	1200	108	<0.2
Ox2	25	660	<0.2
TuII*-26	30	70	<0.2
K3	360	900	9
TuII-24*	260	30	8
TuII*-6	<0.2	7	1500
TuII*-60	<0.2	4	1200
TuII*-46	<0.2	<0.2	1300

Receptors for T2, T4 and T6 are protein OmpF (Hantke, 1978), lipopolysaccharide (Wilson *et al.*, 1970), and protein Tsx (Manning and Reeves, 1978), respectively; all other phages use the OmpA protein.
[a]No phage inactivation was observed after incubation with undiluted serum for 30 min.

It is evident, however, that a simple relationship, such that the same receptor is always recognized by serologically similar tail fiber recognition areas, does not hold. This is indicated by the lack of reaction between anti-K3 serum and TuII*-46 or anti-TuII*-6 serum and Ox2 as all of these phages use the OmpA protein as their receptor. Moreover, although anti-TuII*-6 antibodies do bind to the tail fibers of TuII*-46, they do not do so at the ends of these fibers (Figure 1, Table II).

Finally, a rather unexpected result of these studies was the apparent unrelatedness of the head of T4 with those of T2 and T6; this will be taken up in the Discussion.

Phage resistance patterns

The absence of cross-reaction between phages which use the same receptor or the non-binding of antibodies to the ends of tail fibers may indicate that these phages recognize different sites on the OmpA protein. It should then be possible to isolate *ompA* mutants which are resistant to, e.g., TuII*-46 but have remained sensitive to K3.

Selection for resistance to phages TuII*-6, TuII*-46 and Ox2 has so far yielded the classes of mutants shown in Table III (all these mutants possess the wild-type complement of the OmpA protein, see Materials and methods). Mutants selected for resistance to phage TuII*-46 which remained sensitive to K3 and TuII*-24 were recovered, whereas mutants selected for resistance to TuII*-6 were found which, while also resistant to other OmpA-specific phages, were still sensitive to Ox2. On the other hand, a mutant resistant to Ox2 had remained sensitive to all of the other phages tested. These data strongly support the view that phages K3, TuII*-46 and Ox2 use different sites on the OmpA protein.

TuII*-46 shows no reaction at the ends of its tail fibers when anti-TuII*-6 serum is used while TuII*-60 or K3 do (Table II). We might therefore have expected that mutants which were resistant to TuII*-46 and sensitive to K3 should also be sensitive to TuII*-6 and TuII*-60. However, this is not the case. We reported earlier a class of mutants resistant to TuII*-6 and sensitive to K3 (Henning *et al.*, 1978). This indicates that these two phages also do not use exactly the same site on the OmpA protein as their receptor; no such information is available for TuII*-60.

DNA hybridizations

Most of the distal half of the T4 fiber consists of two pro-

Table II. Topology of cross-reacting sites

Phage	Antiserum								
	α-T4			α-K3			α-TuII*-6		
	head	tail fiber	tip	head	tail fiber	tip	head	tail fiber	tip
T2	−	+	n.d.	−	+	−	−	−	−
T4	+	+	n.d.	+	+	−	−	−	−
T6	−	+	−	−	+	−	−	−	−
Ox2	+	+	−	+	+	−	−	−	−
K3	+	+	−	+	+	+	−	−	+
TuII*-24	−	+	n.d.	+	+	n.d.	−	−	+
TuII*-6	−	−	−	−	−	+	+	+	+
TuII*-60	−	−	−	−	−	+	−	+	+
TuII*-46	−	−	−	−	−	−	−	+	−

+, −: The antiserum does or does not react, as determined by electron microscopy (cf., Figure 1), with the parts of the phages indicated. Tip: distal ends of the long tail fibers. n.d.: not determined.

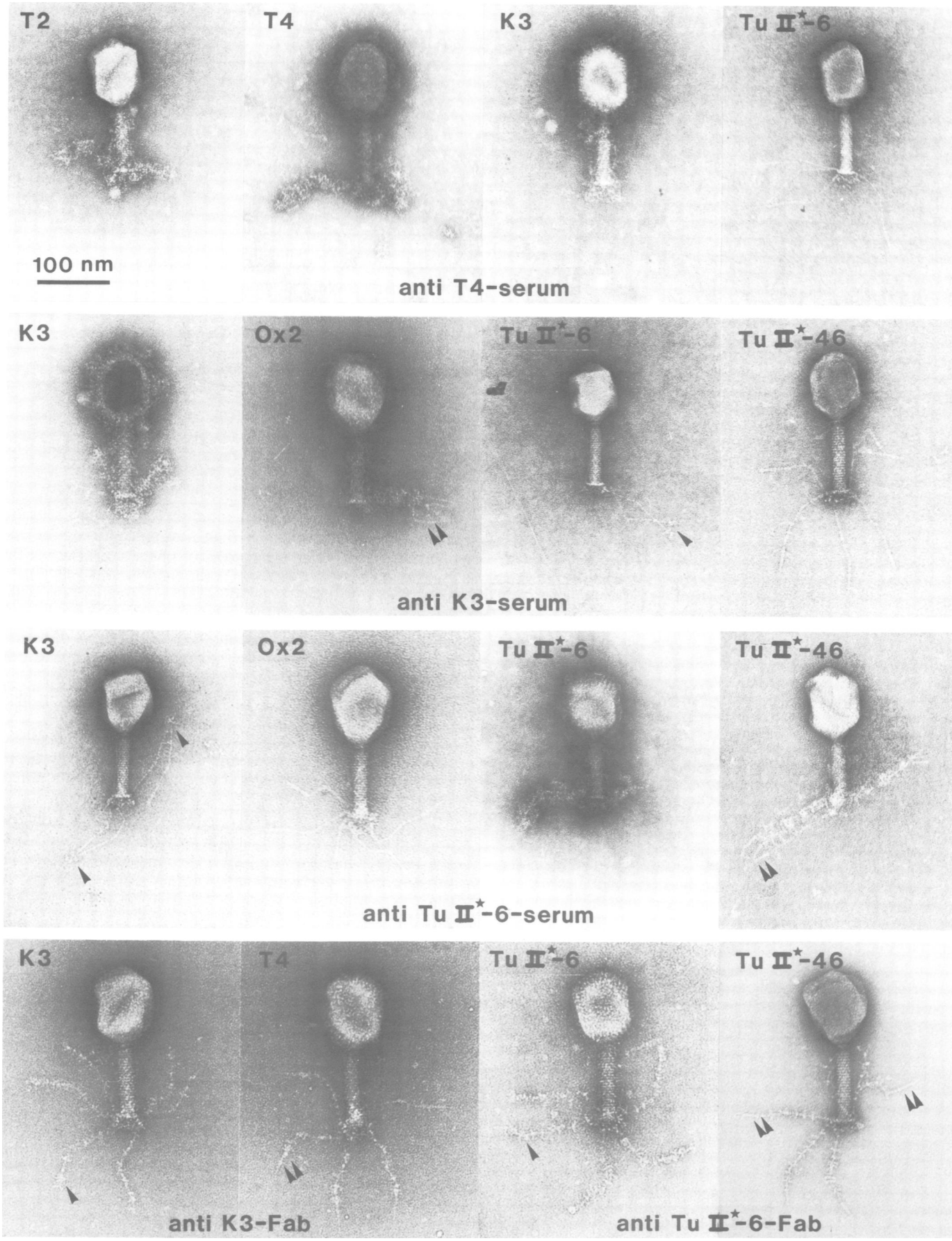

Fig. 1. Examples for the topology of cross-reacting sites. The phages and the antisera (IgG or Fab) used are indicated. Note (cf., Table II) the presence (one arrow) or the absence (two arrows) of IgG or Fab fragments at the ends of the long tail fibers.

Table III. Patterns of phage resistance to OmpA-specific phages

Selection for resistance to	Resistance (R) or sensitivity (S) to						
	TuII*-6	TuII*-24	TuII*-26	TuII*-46	TuII*-60	K3	Ox2
TuII*-6 (1)	R	R	R	R	R	R	S
TuII*-46 (5)	R	S	R	R	R	S	R
TuII*-46 (2)	R	R	R	R	R	R	R
Ox2 (2)	R	R	R	R	R	R	R
Ox2 (1)	S	S	S	S	S	S	R

Numbers in brackets: number of independent isolates for each given class.

teins, distally (and determining the host-range) of the 1026 residue gene 37 product and proximally of the 221 residue gene 36 product (Bishop *et al.*, 1974; Oliver and Crowther, 1981). These genes, together with some other T4 DNA, have been cloned into phage λ (Revel, 1981). From this hybrid phage we have subcloned a *Hind*III fragment (Revel, 1981) carrying gene 36 and almost all of gene 37 into plasmid pUC8. The hybrid plasmid was used as a source of hybridization probe to detect homologies, in Southern (1975) transfers, of phage DNA digested with *Taq*I (Huang *et al.*, 1982). The results (Figure 2) confirm and extend those of Table II. With minor variations, the patterns obtained under high and low stringency conditions (under the latter allowing stable hybrids containing almost 30% base mismatch to be formed, see Materials and methods) were the same. The T4 probe hybridized to restriction fragments from all phages reacting with anti-T4 serum. No hybridization at all was seen with the DNA from the non-cross-reacting phages TuII*-6, TuII*-46 and TuII*-60, proving that their relevant tail fiber genes are only rather distantly, if at all, related to those of T4.

Discussion

We interpret our results by assuming that the host-range of all of the phages examined is determined by the distal end of the long tail fibers and we do so in analogy to other phages where good evidence for this assumption is available. In phage T4, gene 37 codes for the 1026 residue (mol. wt. 109 000) protein, a dimer of which constitutes ~80% of the length of the distal half fiber; the two polypeptides are arranged in parallel, the tail fiber ends with their carboxy termini, and the host-range of the phage is determined by a segment (segment d) at this terminus (Beckendorf *et al.*, 1973; Beckendorf, 1973). The corresponding protein of phage T2 had a mol. wt. of ~120 000, and the region of size discrepancy to the T4 protein resides in the carboxy-terminal, host-range determining segment (Beckendorf *et al.*, 1973). It is also known that this host-range region is heterologous in DNA sequence as examined by electron microscopic heteroduplex analysis (Beckendorf *et al.*, 1973; Kim and Davidson, 1974).

A comparable situation exists for gene J encoding the protein of the tail fiber of lambdoid phages (Hendrix, 1971; Murialdo and Siminovitch, 1971; Simon *et al.*, 1971). Host-range mutants of λ map to the carboxy-terminal end of the polypeptide (Shaw *et al.*, 1977). Heteroduplex analysis of phage λ DNA and that from phages 21, 82 and 434 suggested that all four phages have identical sequences in the amino-terminal region of the J proteins, that 434 and 82 (having the same host-range but different from λ) have identical J proteins, and that λ differs from 21, 82 and 434 in a region close to the carboxyl terminus of the J protein (Simon *et al.*, 1971). (Interestingly, λ and 21 have the same host-range, and this may be a similar case to TuII*-6 *versus* TuII*-46.)

Our results fit into this general picture and reveal an additional remarkable diversity. We find that phages of T-even morphology may not cross-react immunologically at all. Non-cross-reacting phages using the same receptor may use different sites on the OmpA receptor protein. If such phages do cross-react two essential variations are found. When the cellular receptors differ, otherwise extensively cross-reacting sera may not contain antibodies against the heterologous ends of the tail fibers. If the same receptor is used, such sera may contain no antibodies other than those recognising the ends of the tail fibers of the heterologous phage. One finding that is inconsistent with these data is the fact that K3 and TuII*-6 possess cross-reacting tail fiber ends yet mutants exist which are resistant to TuII*-6 and sensitive to K3, indicating that the two phages do not use the same site on the OmpA protein. It was found recently, however, that a strain resistant to both phages has suffered a single amino acid substitution in the OmpA protein (Cole *et al.*, 1983). Thus, it seems most likely that the receptor areas on OmpA for K3 and TuII*-6, while not identical, are overlapping. We cannot comment on such possible overlaps for most OmpA-specific phages because the alterations in the corresponding OmpA proteins are not yet known.

What degree of non-homology is indicated by the absence of cross-reactivity, particularly in view of the fact that we did not observe binding of anti-T4 IgG to the heads of T2 or T6? In those cases where antibodies bound to phage heads, the heads were always completely covered with the immunoglobulin suggesting that the major head antigen was most likely the major capsid polypeptide. There is no indication that the genes for the major capsid proteins (gene 23 of T4) of T2, T4 and T6 possess extended non-homologous regions as far as could be seen by electron microscopic DNA heteroduplex analysis (Kim and Davidson, 1974) and the corresponding gene products of phage T2 and T4 do not differ significantly in size (Beckendorf *et al.*, 1973). It could well be that a relatively small immunodominant area on the phage head exists and that only a few amino acid substitutions cause absence of cross-reactivity.

It is most unlikely that the same consideration applies to the tail fibers. Those of T4 contain four proteins. It has been shown that along the dimer of the T4 polypeptide 37, 4–5 different antigenic determinants are distinguishable (Beckendorf, 1973). Considering the arrangement (Beckendorf, 1973; Earnshaw *et al.*, 1979) and amino acid sequence (Oliver and Crowther, 1981) of this long protein fiber it is likely that more determinants are present, and one would of course also

predict a fair number of these for the other fiber proteins. No such determinants were detected in several cases (Table II), and the results of the DNA hybridization experiments indicate that the absence of cross-reactivity along the distal half fiber represents a rather distant relatedness, if any: a probe containing the T4 gene 36 and most of gene 37 did not detect any homologies in the DNA of phages TuII*-6, TuII*-60 or TuII*-46 even under conditions of low stringency hybridization.

In the collection of phages examined there are then sets of very similar and dissimilar genes. More remarkably there are even sets of such gene segments which code for the ends of the tail fibers. If we accept these ends as being the receptor recognition areas we may consider the molecular organisation of the T-even phage tail genes to be analogous to the immunoglobulin genes. (From the known extent of the heterology of this segment of gene 37 from phages T2 and T4 it was first suggested in 1974 that these regions 'may have arisen by insertion of heterologous DNA, rather than by successive small mutations'; Russel, 1974.) One may then ask if a given phage possesses more than one gene region coding for the distal end of its tail fiber. There is no evidence for this. Host-range mutants exist which can use more than one receptor (Wandersman and Schwartz, 1978; Moreno and Wandersman, 1980; Beher and Pugsley, 1981; for a discussion of such cases, see Schwartz, 1980) but these have usually, yet not always, retained the ability to use the receptor of the parent phages. We have repeatedly attempted (involving $\sim 10^{12}$ phages from eight different lysates) to find host-range mutants of phage K3 able to grow on a host lacking the OmpA protein, but these attempts have not been successful.

Experiments are now in progress to clone and analyze, by DNA sequencing, the corresponding genes of the phages described here. Such sequence analyses should help answer some of the intriguing questions: what constitutes the receptor recognition area of a fiber, what is the basis and the origin of the very different recognition areas and whole tail fiber genes, and how related are these areas from phages which use different parts of the same receptor?

Materials and methods

Phages and selection of mutants

Phage T2 was obtained from K. Hantke, T4 and T6 were from the collection of the late W. Weidel, λ761-29 was from H. Revel. TuII*-6 is identical to TuII* (Henning and Haller, 1975), the other TuII phages are described in Results. Phages were purified by precipitation with polyethyleneglycol (Yamamoto *et al.*, 1970) and subsequent CsCl density gradient centrifugation to equilibrium. Phage-resistant mutants were selected by plating $10^7 - 10^8$ cells of the K12 strain P400 (Skurray *et al.*, 1974) together with $10^9 - 5 \times 10^9$ p.f.u. of phages on to nutrient broth agar (Difco). On this medium, *ompA* mutants missing protein OmpA grow slower than those still producing it; thus, picking large colonies enriches for the desired class. Such isolates were screened for the presence of wild-type amounts of the protein by SDS-polyacrylamide gel electrophoresis of their cell envelopes (Datta *et al.*, 1976).

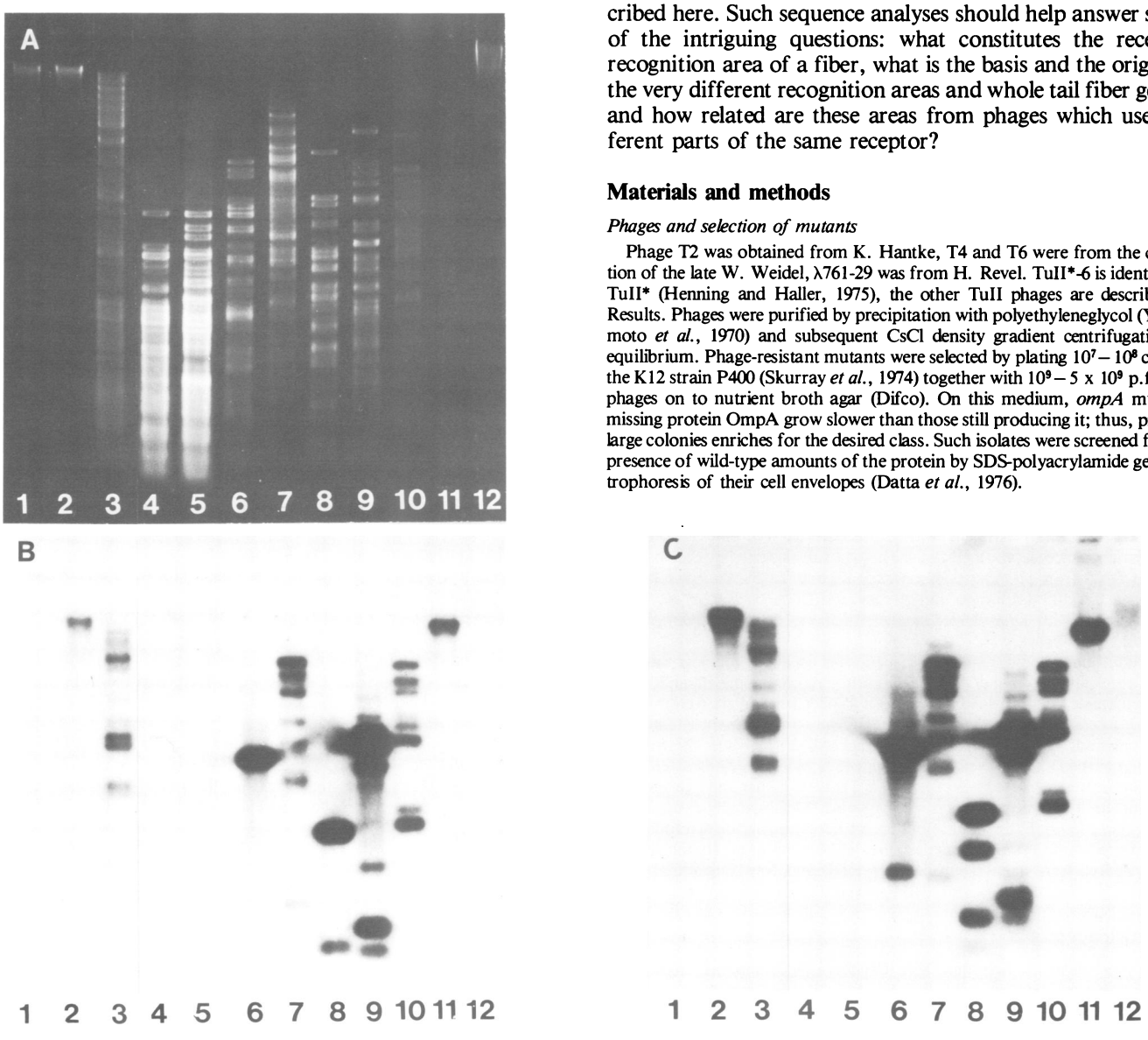

Fig. 2. DNA hybridizations. **(A)** Ethidium bromide-stained agarose electrophoretogram of *Taq*I digests of phage DNA. **Lanes: 1** TuII*-6; **2**, TuII*-24; **3**, TuII*-26, **4**; TuII*-46; **5**, TuII*-60; **6**, K3; **7**, Ox2; **8**, T2; **9**, T4, **10**, T6. Controls: **11**, undigested λ761-29 (harboring the DNA fragment used as a probe); **12**, undigested *E. coli* DNA (strain P400). The DNA from TuII*-6 and TuII*-24 proved to be rather (not completely) resistant to*Taq*I. **(B)** Autoradiogram of a Southern blot of gel **A** and probed with the T4 tail fiber genes 36 plus 37 under highly stringent conditions. **(C)** Same blot hybridized under conditions of low stringency; note that this condition already allows for some non-specific reaction with the chromosomal DNA but still none with those of phages TuII*-6, -46 and -60. The size of the fragments ranges from ~20 (beginning of the resolving power of the 1% gel) to ~0.2 kb.

Antisera and neutralization of phages

In initial experiments, two anti-T4 sera which were a gift from E. Kellenberger, one from a rabbit and one from a horse, were used. Later, anti-T4, K3, and TuII*-6 sera were raised in rabbits: for one challenge $1-4 \times 10^{11}$ p.f.u. were used, emulsified with complete Freund's adjuvant, and an immunization scheme was followed as described (Henning *et al.*, 1979). The Fab fraction was prepared essentially according to Porter (1959), it was concentrated to 10 mg/ml by ultrafiltration.

Phage inactivation by these sera was measured at 37°C by standard methods (Adams, 1959) using phage concentrations of 10^3-10^4/ml. The rate constants K given in Table I were calculated according to:

$$\frac{P_t}{P_o} = e^{-\frac{K.t}{D}}$$

where D = final dilution of serum in the phage-serum mixture, t = time of incubation, P_o = initial titer, P_t = phage titer after time t.

Electron microscopy

Phages ($\sim 10^{11}$ p.f.u./ml) were incubated with the same volume of antiserum or Fab solution for 30 min to 6 h at room temperature and washed six times by centrifugation (Eppendorf 3200 centrifuge). The immunocomplexes were adsorbed to glow-discharged, carbon-coated grids, negatively stained with 1% uranyl acetate and examined in a Philips 201 electron microscope at 40 or 60 KV.

In our experience, immunolabelling and washing the complexes in suspension prior to mounting on grids as first described by Höglund (1967) avoids any background of non-specifically adsorbed immunoglobulins. It therefore appears to be preferable to applying highly diluted antibodies on phages mounted already as used by Yanagida and Ahmad-Zadeh (1970) and Beckendorf (1973).

DNA hybridization

The 3.6-kb *Hind*III fragment from λ761-29 carrying T4 gene 36 and most of gene 37 was ligated into plasmid pUC8 (Vieira and Messing, 1982) which was then transformed into strain $F^- Z^-$ $\triangle M15$ (Rüther *et al.*, 1981). Bacteria harboring hybrid plasmids were detected as white colonies (Gronenborn and Messing, 1978; Rüther *et al.*, 1981) on L broth agar containing ampicillin (20 μg/ml), isopropylthiogalactoside (1 mM), and 5-bromo-4-chloro-indolyl-galactoside (40 μg/ml). The presence of the genes mentioned was verified by transforming plasmids into the *rec*$^+$, *sup*^{0} strain JC6256 (Achtman *et al.*, 1971) followed by a simple marker rescue test (drops of phage onto a lawn of plasmid-harboring cells) using T4 amber mutants in genes 37 and 36 (N52 and E1, respectively; cf., Revel, 1981). The *Hind*III fragment, isolated from such a plasmid, was labelled with [α-^{32}P]dATP (Amersham) by nick-translation and used to probe (Southern, 1975) the various phage DNAs digested with *Taq*I (Haung *et al.*, 1982). As judged from the size of the fragments produced (Figure 2), digestion with this endonuclease recognizing four bases (Sato *et al.*, 1977) remained incomplete in all cases. This was substantiated by the observation that DNA from phages (tested only for K3 and TuII*-60) grown on the *galU* strain CA7105 (leading to non-glycosylated DNA; Wilson *et al.*, 1977) upon treatment with *Taq*I yielded smaller fragments than that from phages grown on *galU*$^+$ hosts.

Hybridization of these fragments after transfer onto nitrocellulose was performed according to Wahl *et al.* (1979) in solutions containing either 20% or 50% formamide. After hybridization (16 h at 42°C) the filters were washed with both solutions (Wahl *et al.*, 1979) at 20°C only. In our mixtures and with 50% formamide at 42°C, hybridization proceeds 20°C below the melting point (T_m) of the DNA allowing hybrids with 13% or less base mismatch to be stable; in the presence of 20% formamide T_m is 22°C higher and hybrids containing up to 28% base mismatch will be stable (McConaughy *et al.*, 1969; Howley *et al.*, 1979). Experiments not detailed here were performed following standard procedures essentially as described in Maniatis *et al.* (1982); the enzymes required were from Boehringer (*Hind*III) and New England Biolabs (*Taq*I).

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