

Receptor Specificity of the *Escherichia coli* T-even Type Phage Ox2

Mutational Alterations in Host Range Mutants

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The T-even type *Escherichia coli* phage Ox2 uses the outer membrane protein OmpA as a receptor. The protein is recognized with the ends of the virion's long tail fibers. The 266 residue protein 38 is located at this site and acts as an adhesin. Host-range mutants had previously been isolated from Ox2. Mutant Ox2h5 is able to infect cells possessing an altered OmpA protein, which renders the cell resistant to Ox2. Ox2h10 was selected from Ox2h5. This phage recognizes the OmpC protein in addition to the OmpA protein. Ox2h12, which stems from Ox2h10, binds to OmpC with high affinity, but has lost efficient binding to OmpA. The mutational alterations caused in genes 38 are: Asp231 → Asn(h5) and His170 → Arg(h10). The triple mutant Ox2h12 possesses an insertion of a Gly residue next to Gly121. The three mutants have additionally acquired mutations affecting their base plate, making them "trigger-happy". When protein 38 was compared with the same protein derived from other *E. coli* phages, it was found to contain two constant and one variable domains, the latter harboring four hypervariable regions flanked by a largely conserved glycine-rich sequence. The h5 and h10 mutations occurred within two hypervariable areas, while the additional Gly residue was present in one of the flanking conserved sequences. On the basis of these results, as well as those obtained from host-range mutants analyzed previously, a model for such adhesins is proposed. Receptor recognition is most likely performed *via* the hypervariable regions, which may form loops held together in close proximity by the oligoglycine sequences. The latter may achieve this by being part of highly compact omega loops.

1. Introduction

T-even type *Escherichia coli* phages recognize their cellular receptors with their six long tail fibers (Kellenberger *et al.*, 1965; Wilson *et al.*, 1970; Beckendorf, 1973). Two different proteins of this fiber are known to mediate receptor binding here. In phage T4, the most distal part of the fiber consists of a dimer of the 1026 residue protein 37 (Ward *et al.*, 1970; Bishop *et al.*, 1974; Oliver & Crowther, 1981) and this phage recognizes its receptor with the free end of the dimer (Wilson *et al.*, 1970; Beckendorf, 1973). In phages T2, Ox2, K3 and M1 the situation is different: protein 38 is attached at the ends of their dimer of protein 37 and acts as an adhesin, i.e. binds to the cellular

receptor (Riede *et al.*, 1985, 1987a). Ox2 uses the outer membrane protein OmpA as a receptor (Morona *et al.*, 1984, 1985). From this phage host-range mutants have been isolated (Morona & Henning, 1984). The first-step mutant, Ox2h5, could utilize a mutationally altered OmpA protein which conferred resistance to phage Ox2. Ox2h10 was isolated from Ox2h5, the former still used the OmpA protein but could also recognize the outer membrane protein OmpC, which is quite unrelated to the OmpA protein, though it did so inefficiently. Finally, Ox2h12 was derived from Ox2h10; the former recognized the OmpC protein efficiently and had lost some affinity for the OmpA protein. We have shown that two host-range mutants of the OmpA-specific phage K3, capable of using mutationally altered OmpA proteins, have undergone mutations leading to altered proteins 38 (Riede *et al.*, 1987b). We have raised the question of

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which mutational alterations enable phage Ox2 to switch receptors and whether these alterations are also and only present in gene 38.

2. Materials and Methods

(a) Phages, bacterial strains, cloning and DNA sequencing

Phages T4, T2 and K3 (Schwarz *et al.*, 1983) as well as phage Ox2 (Kay & Fildes, 1962) and its host-range mutants (Morona & Henning, 1984) have been described. They were propagated, using L-broth (Miller, 1972), on strain P400 (Skurray *et al.*, 1974), except for Ox2h12 which was grown on P460 (where the OmpA protein is missing; Skurray *et al.* (1974)). All other procedures were exactly as described (Riede *et al.*, 1985, 1986). Plasmid pUC8 (Vieira & Messing, 1982), linearized with *Sma*I and dephosphorylated, was used as recipient for sonicated and size-fractionated phage DNA. Clone pTU Ox2h5-3 (cf. Fig. 1) has been described (Riede *et al.*, 1985), and pTU Ox2h10-4, h10-6 and h12-5 were detected by colony hybridization using radioactively labeled g38[†] from plasmid pTU Ox2h5-3 as probes. For DNA sequencing (Sanger *et al.*, 1977) either phages M13mp8, mp9 (Messing & Vieira, 1982), mp18 or mp19 (Norrander *et al.*, 1983) were used. The clones recovered and the sequencing strategies are shown in Fig. 1. All operations were performed essentially as described by Maniatis *et al.* (1982).

(b) Determination of the copy number of protein 38

As standard practice [³⁵S]methionine-labeled phage T2 was dissolved in SDS, and proteins 37 and 38 were precipitated with their respective antibodies, the precipitates were subjected to SDS/polyacrylamide gel electrophoresis, and the radioactivity in the 2 isolated proteins was measured. It was necessary to precipitate the two proteins under different conditions because gp38 was only poorly precipitable under the conditions described for gp37 (Drexler *et al.*, 1986); on the other hand, precipitation of gp37 under conditions optimal for precipitation of gp38 lacked specificity to an intolerable degree (i.e. adsorption of a number of other phage proteins by Staphylococci). T2 (250 ml culture) was labeled ([³⁵S]methionine, Amersham; 11 mCi/ml) essentially as described by Vanderslice & Yegian (1974). The phage was purified by precipitation with polyethylene glycol (Yamamoto *et al.*, 1970) and subsequent centrifugation into a CsCl step gradient followed by dialysis (Maniatis *et al.*, 1982). Phages were dissociated by boiling for 10 min in the presence of 0.9% (w/v) SDS and 22 mM-Tris·HCl (pH 7.5), viscosity was broken by several pulses of sonication. Proteins were precipitated with trichloroacetic acid (10% (w/v) final concentration, 1 h at 0°C), washed with acetone, then with ether and subsequently dried. They were then redissolved in 1.8% SDS in 44 mM-Tris·HCl (pH 7.5) (2 h at 70°C). To 150 µl (equivalent to 6 × 10⁹ phage) of this solution 110 µl of antiserum (anti-gp37, Drexler *et al.*, 1986; anti-gp38, Riede *et al.*, 1987b) was now added, plus 30 µl of 2 M-NaCl (in the case of gp38) or 85 µl of 2 M-NaCl (in the case of gp37). After 1 h at 37°C, 0.5 ml of 150 mM-NaCl (in the case of gp38) or water (in the the case of gp37) and

130 µl of cells of *Staphylococcus aureus* (10% (w/v) Pansorbin; Calbiochem) were added and incubation was continued for 1 h at 37°C. The cells were then washed twice with 1 ml of 10 mM-Tris·HCl (pH 7.5), 150 mM-NaCl (gp38) or with the same buffer containing 150 mM-NaCl, 0.02% SDS and 0.1 mM-EDTA (gp37). The pellets were boiled for 10 min in 200 µl of sample buffer and electrophoresed in a SDS/10% polyacrylamide gel (buffer and gel: Laemmli, 1970). The dried gel was fluorographed using EN³HANCE (New England Nuclear) for 3 days at -70°C. Bands corresponding to gp37 or 38 were cut out and rehydrated in water (1 ml, 5 h at room temperature). Addition of 4 ml of PROTOSOL (New England Nuclear) and incubation for 24 h at room temperature solubilized the radioactivity, which was then determined by scintillation counting.

(c) Electron microscopy

Phages (2 × 10¹⁰/ml) in M9 buffer (Yamamoto & Uchida, 1973) were kept at 55°C for 15 min, adsorbed onto glow-discharged, carbon-coated grids and negatively stained with 1% (w/v) uranyl acetate. They were visualized in a Philips 201 electron microscope at 60 kV.

3. Results

(a) Mutational alterations in genes 38 of host range mutants of phage Ox2

The nucleotide sequences determined for the cloned DNA are indicated in Figure 1. The sequence of the wild-type Ox2 g38, encoding a 266 residue protein, has been described (Montag *et al.*, 1987). The sites of the mutational alterations detected are also shown in Figure 1. In Ox2h5, a GAC codon had changed to AAC, causing the substitution of aspartic acid 231 for asparagine. In Ox2h10, in addition to this change the CAC codon specifying histidine 170 was replaced by CGC encoding arginine. In Ox2h12, a small duplication had occurred, altering the wild-type sequence GGT GGA to GGT GGT GGA, thus changing the amino acid sequence from -Gly121-Gly to Gly121-Gly-Gly-. No other alterations were found, including the part of gp37 sequenced from the Ox2h10-6 clone.

(b) Copy number of protein 38 in phage T2

The h5 and h10 mutational alterations are located in the respective proteins within an area previously suggested as being involved in receptor binding (Riede *et al.*, 1987a,b); on the other hand, one would not expect that the additional glycine residue in gp38 of Ox2h12 would alter the binding specificity directly (see Discussion). It is not known whether one or more copies of protein 38 are present at the tip of a tail fiber. If oligomers of this protein were indeed bound to the dimer of protein 37, amino acid substitutions would, perhaps, be more likely to cause long range effects (e.g. by altering the arrangement of subunits) than if they were present in a monomer. We therefore determined the copy number of protein 38 in phage T2. Phage T2 was used because the amino acid sequences of its gp37 and gp38 are known (Riede *et*

† Abbreviations used: g, gene; gp, gene product.

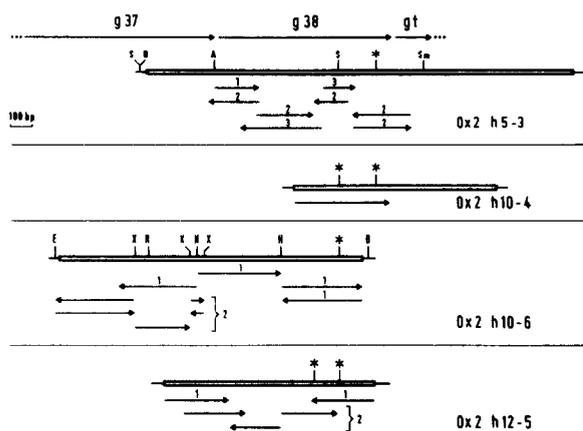


Figure 1. Clones and sequencing strategies. The cloned fragments, except for Ox2h10-6, are oriented in pUC8 such that the *lac* regulatory area is to the right. Restriction sites: A, *AhaIII*; B, *BamHI*; E, *EcoRI*; H, *HpaII*; S, *SalI*; Sm, *SmaI*; X, *XhoII*. The numbers indicate sequences (arrows) obtained in the following ways. Ox2h5-3: 1, primer and *AhaIII*-*SalI* fragment in M13mp9; 2, 3, shotgun cloning (M13mp8) of the *BamHI*-*SmaI* fragment digested with *HpaII* or *AbaI*, respectively. Ox2h10-4, reverse primer and plasmid. Ox2h10-6: the *EcoRI*-*BamHI* fragment was digested with 1, *HpaII* or 2, *XhoI* and the resulting fragments were cloned into M13mp18 or 19. Ox2h12-5, 1, primer, reverse primer and plasmid; 2, shotgun cloning (M13mp8) of an *HpaII* digest of the cloned fragment. Thin line, vector DNA; dots, the respective genes continue in this direction; gt, lysis gene; asterisks, sites of mutational alterations. bp, base-pairs.

al., 1986, 1987b) and also because antisera against both proteins were available. Since proteins 38 of T2, K3 and Ox2 are exchangeable (Riede *et al.*, 1985, 1987a) there is no doubt that the results obtained also apply to the latter phage.

In the precursor of protein 37 contained in T2, 17 methionine residues are present. During phage morphogenesis, this protein is processed so as to remove about 120 residues from the COOH terminus (Drexler *et al.*, 1986). The 1341 residue precursor of gp37 of T2 possesses a single methionine residue at the COOH terminus. The next such residues exist at positions 1163 and 1096 (Riede *et al.*, 1986), i.e. 178 and 245 residues, respectively, located away from the COOH terminus. Thus, 32 methionine residues are present in the dimer of gp37. Protein 38 of this phage contains six methionine residues (Riede *et al.*, 1987b). The ratio of methionine residues in the two proteins would be $32/6 = 5.33$ or $32/12 = 2.67$ or $32/18 = 1.78$, depending on whether one, two or three copies, respectively, of protein 38 are present in one tail fiber. (It may be noted that, even in the very unlikely event of removal of two methionine residues upon processing, these ratios would not be altered to a significant degree.) The ratio was determined for the two proteins from phage propagated in the presence of [^{35}S]methionine, as

described in Materials and Methods. The results of four independent experiments (Table 1) demonstrate that only one copy of protein 38 is present per tail fiber.

(c) *Phages Ox2h5, Ox2h10 and Ox2h12 carry an additional mutation(s) which alter(s) infection efficiency*

The h5, h10 and h12 alleles were separated from each other and their effect on the host ranges of the corresponding recombinant phages was analyzed (K. Drexler, unpublished results). The efficiency of these phages on various indicator strains turned out to depend on the combination used, i.e. some proteins 38 carrying these individual alterations were more efficient when present at protein 37 of phages Ox2h5, h10 or h12 than when bound to the fibers of phage Ox2. It therefore appeared that Ox2h5 had actually undergone more than one mutation. The data presented earlier (Riede *et al.*, 1987b) as well as in this paper strongly argue against the possibility that protein 37, in addition to protein 38, has a binding site(s) for the OmpA protein. Moreover, a second mutation was not found that would have altered protein 37 at or near its COOH terminus (see above: the two polypeptides of the dimer are arranged in parallel, with the COOH terminus pointing to the free end of the fiber (Beckendorf, 1973; Beckendorf *et al.*, 1973)). Any larger deletion or insertion/duplication involving about 20 or more residues within protein 37 of the host-range mutants could also be excluded. The sizes of the wild-type proteins and the mutants, as determined by SDS/polyacrylamide gel electrophoresis, were found to be identical (data not shown). We have therefore asked if this second mutation(s) may be causing "trigger-happiness" and may consist in an alteration of a base plate component (Dawes & Goldberg, 1973; Yamamoto & Uchida, 1973, 1975; Arscott & Goldberg, 1976; Goldberg, 1983). Base plate mutants of this type exhibit a property that can easily be determined: the mutant phages are thermolabile, due to contraction of the tail sheath being triggered at elevated temperatures.

The effect of infectivity of incubation of various phages at 55°C is shown in Figure 2. The data show that the thermostability of Ox2 does not represent

Table 1
Ratio of methionine residues in protein 37 to those in protein 38

Expt no.	gp37†	gp38†	gp37/gp38
1	476.3	86.4	5.5
2	579.9	96.9	5.98
3	473.8	86.4	5.48
4	469.6	92.9	5.1

† Cts/min in immunoprecipitated protein (see Materials and Methods).

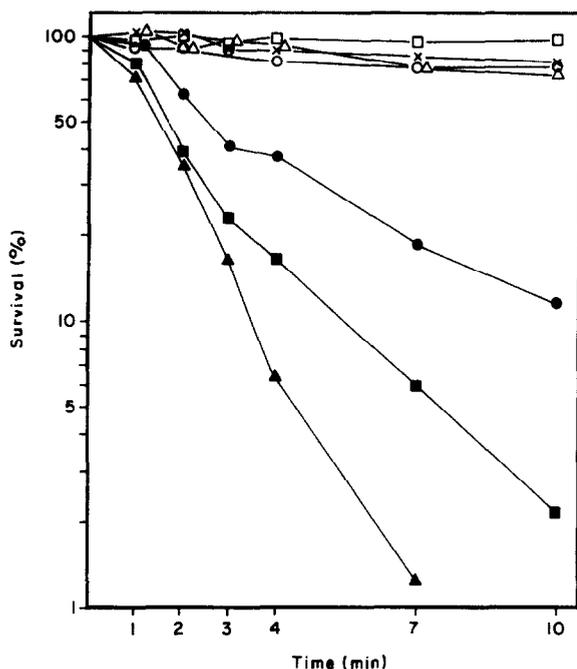


Figure 2. Heat treatment of phage. Phage in M9 buffer (10^6 /ml) were kept at 55°C for the times indicated and titrated immediately. (□) T4; (×) K3; (○) T2; (△) Ox2; (■) Ox2h5; (●) Ox2h10; (▲) Ox2h12.

anything unusual; indeed it is practically indistinguishable from that of phages T2, T4 or K3. Clearly, however, all three Ox2 host range mutants were thermolabile, although to different degrees. The electron microscopic appearance of phages treated in this way is shown in Figure 3. Nearly 100% of the three host-range mutants exhibit contracted tail sheaths after the heat treatment. In all cases, the base plate remains attached to the contracted sheath, indicating that the additional mutation(s) have affected the base plate proteins 6, 7, 10, 25 or 27 (Yamamoto & Uchida, 1973).

For phage Ox2h5, another property characteristic of such mutants could be demonstrated. "Trigger-happiness" of a T4 mutant was shown by Crawford & Goldberg (1977) to enable the phage to infect cells with a lower cellular concentration of receptor than was required for the wild-type parent to bind irreversibly. When grown in the presence of glucose, cells not producing the chromosomally encoded *ompA* gene and carrying a plasmid with this gene under the control of the *lac* regulatory elements (Freudl *et al.*, 1985) are resistant to OmpA-specific phages. The ability of this strain to inactivate either Ox2 or Ox2h5 was tested after various times of induction of OmpA synthesis, mediated by isopropylthiogalactoside. It was found that less growth time (1.5 h) in the presence of the inducer was required for these cells to be able to inactivate Ox2h5 than was necessary (2.5 h) for Ox2. With the strains available the same test could not be performed with Ox2h10 or h12 because of the presence of the OmpC protein.

4. Discussion

(a) Receptor specificity and structure of adhesins

In the primary structures of the receptor-recognizing proteins 38 deriving from phages Ox2, M1, K3 and T2, three domains are distinguishable (Montag *et al.*, 1987). Two more or less well-conserved regions exist, encompassing about 120 NH_2 -terminal and 25 COOH -terminal residues; they were therefore termed constant regions. Between these the variable part is located, flanked and interrupted by highly conserved glycine-rich stretches. Following each of the first four such stretches there are four hypervariable sequences (Fig. 4, I–IV), where a very large number of amino acid substitutions, deletions and/or insertions have occurred. On the basis of this basic situation, as well as the location within the hypervariable regions of the alterations in two host range mutants of phage K3 (Riede *et al.*, 1987b), we concluded that these regions represent the receptor-recognizing domain. This conclusion is further strengthened by the location of the mutational alterations leading to mutants Ox2h5 and Ox2h10. A domain structure of the protein is also quite evident from the predicted secondary structure, in addition to being evident from the degrees of hydrophilicity and hydrophobicity along the chain (Fig. 4). The predicted receptor-recognizing area of the polypeptide is, in contrast to the two constant domains, mostly hydrophilic and very rich in potential turns.

The location of the h12 alteration was unexpected. An additional glycine residue is present within the first conserved glycine-rich sequence. It appears rather unlikely that the insertion at this site changes receptor specificity directly, i.e. is located within the receptor binding region. Two explanations for the influence of this additional residue may be offered. We have suggested that the glycine-rich sequences contribute to the flexibility (wobble) of the protein, thus facilitating it in its orientation and binding during the process of reaching a final position at the receptor (Riede *et al.*, 1987b). The glycine residue in question may then further increase intra-chain mobility and allow the protein, together with the h5 and h10 mutations, to bind to the OmpC protein. However, the concept of omega loops has been introduced (Leszczynski & Rose, 1986). The residue most frequently found in such loops is Gly, followed by Pro, Tyr, Asp, Asn and Ser. Such loops, previously regarded as random coils, were shown to be highly compact substructures with a packing efficiency similar to that of a β -sheet. Should the glycine-rich stretches indeed be part of omega loops, the wobble hypothesis would not apply. The whole variable domain could then be viewed as a highly compact, stiff structure, with the addition of the glycine residue somewhat distorting the whole domain. The likelihood would be that the conserved oligoglycine sequences serve to bring the hypervariable regions into close proximity, possibly by forming four loops (Fig. 4(c)). In such a model, invoking the omega

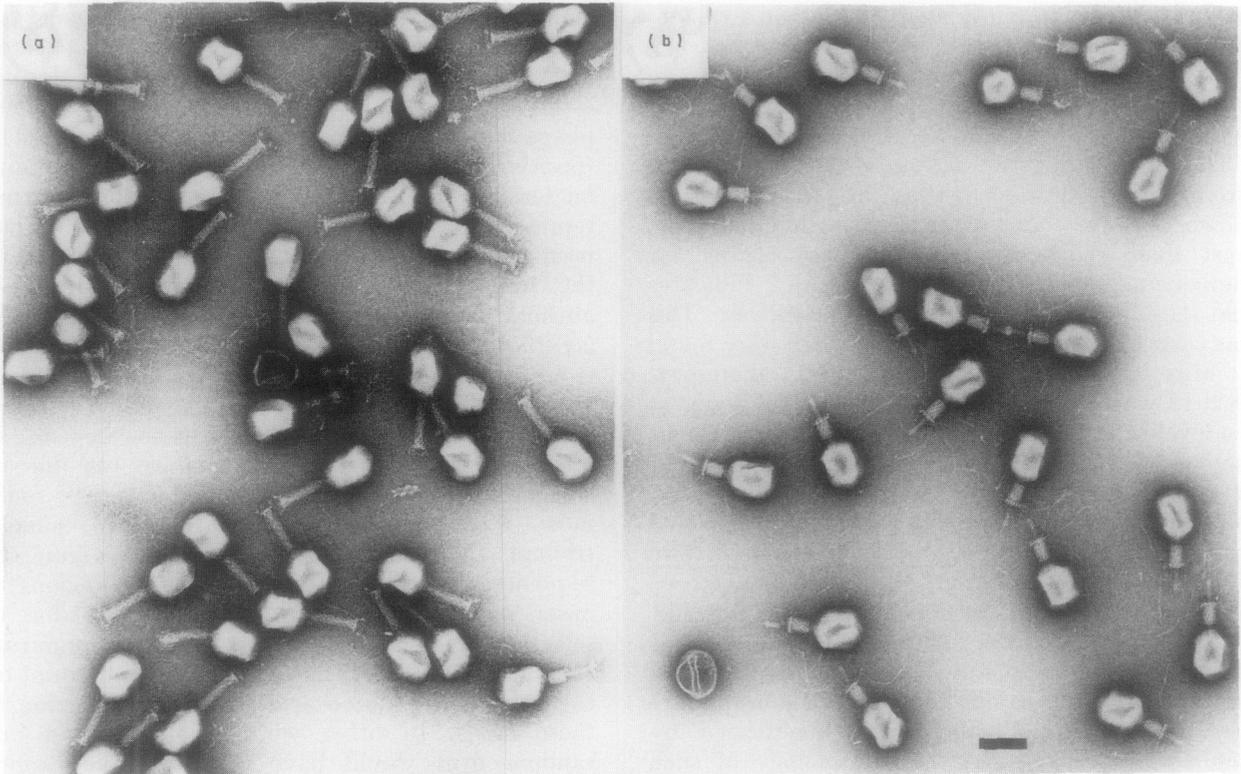


Figure 3. Electronmicrographs. Phage Ox2 (a) or (b) Ox2h5 upon heating for 15 min at 55°C. The appearance of Ox2h10 or Ox2h12 after this treatment was identical with that of Ox2h5. The bar represents 100 nm.

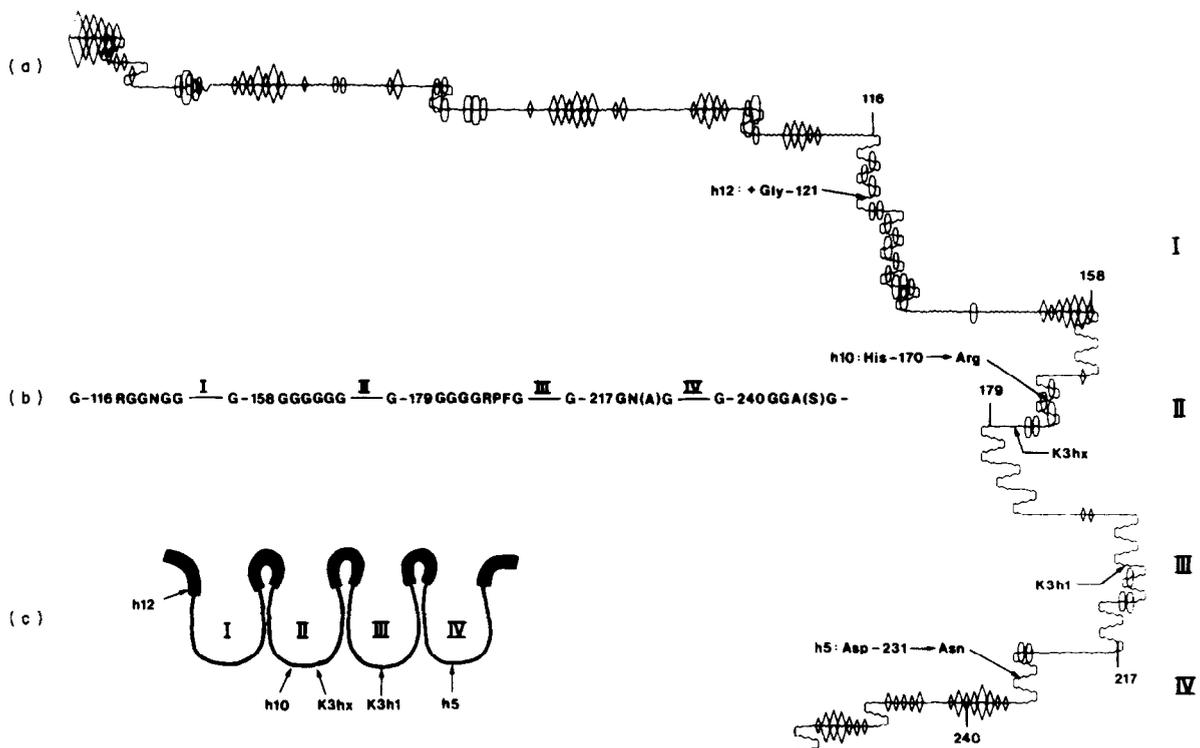


Figure 4. Features of protein 38 of phage Ox2. (a) Computer print out of the predicted secondary structure (Chou & Fasman, 1978) and the hydrophatic profile (Kyte & Doolittle, 1982) of the protein. Diamonds, hydrophobic residues; circles, hydrophilic residues; short wave-length lines, β -sheet; long wave-length lines, α -helix. Arabic numbers, amino acid positions. The sites of alterations in host-range mutants are indicated. (b) The highly conserved glycine-rich stretches (residues in brackets are not conserved) flanking and interrupting the variable and hypervariable regions I to IV. (c) The loop model. The thick lines represent the conserved sequences shown in (b).

loop would also be more appealing as an explanation than floppiness caused by these sequences.

(b) "Trigger-happiness"

The three host-range mutants carry mutational alterations in addition to those in g38. It could be shown that they are of the "trigger-happy" type, most likely harboring altered genes coding for components of the base plate. Figure 2 indicates that these mutations are different in each case. This was surprising since Ox2h5 and Ox2h10 were found to arise at frequencies around 10^{-6} ; moreover, Ox2h12 was present in lysates of Ox2h10 even at the level of 10^{-3} (Morona & Henning, 1984). Thus, the base plate mutants either were already present in considerable numbers in populations of the respective parental phages or were rapidly selected for during isolation of the host-range mutants. Two different host-range mutants of phage K3 have been described that do not use another receptor but, like Ox2h5, can recognize altered OmpA proteins conferring resistance to K3 (Riede *et al.*, 1987b; cf. Fig. 4). It now turns out that the K3 mutants are also "trigger-happy": they were inactivated by incubation at 55°C and the morphology of such inactivated phages was identical with that shown in Figure 3(b).

Why have all these host-range mutants acquired base plate mutations in addition to those in g38? It was found (K. Drexler, unpublished results) that some of the mutational alterations in g38 of the Ox2 derivatives cause a decrease in the efficiency of receptor recognition. Obviously, this disadvantage is counterbalanced by the mutations causing "trigger-happiness". This character, however, is not necessarily advantageous *per se* for a phage, since there are also harsh treatments other than heat that may inactivate it. For instance, only a small fraction of Ox2h10 survived CsCl density gradient centrifugation, and most phages purified this way had contracted tail sheaths. It is therefore not surprising that none of the naturally occurring OmpA-specific phages investigated was inactivated at 55°C. The evolution leading to such phages with their hypervariable regions in g38 may then have started with simple alterations, such as those described both here and earlier (Riede *et al.*, 1987b), accompanied by base plate mutations. Subsequently, additional alterations in g38 may have restored the somewhat defective affinity for the receptor, the result being that "trigger-happiness", now becoming a burden, may have disappeared again.

(c) *The role of the OmpA protein in the evolution of genes 38*

Comparison of amino acid sequences of OmpA proteins from several Enterobacteriaceae has shown that sequence hypervariability exists for the cell surface exposed regions of the protein (for a

summary, see Braun & Cole, 1984). Moreover, the same areas exhibit alterations in mutants selected for resistance to phages (for a summary, see Morona *et al.* 1985). We have therefore viewed a given phage receptor system as the result of a permanent (positively Darwinian) struggle between phage and bacterial mutants resistant to it, with one partner representing the selective force for the other. It has been argued that this may not apply with regard to the OmpA protein (van der Ley *et al.*, 1986). These authors have reported that smooth *E. coli* strains (synthesizing a lipopolysaccharide with long O-antigenic polysaccharide chains) were resistant to various phages, including OmpA-specific virions, which use outer membrane proteins as receptors. It was also shown that this resistance was due to shielding of the proteins by the O-antigens. Since most wild-type isolates of *E. coli* are smooth (though these are usually of clinical origin) the sequence variability of surface-exposed regions of outer membrane proteins would not be due to selective pressure by phages (or colicins requiring them for entry) but rather to a lack of constraint on the part of the amino acid sequences occurring in these areas. In other words, not selection but random drift would have operated. We cannot exclude the possibility that such drift may have contributed to this sequence variability, but we still think that the major force indeed was selection.

In the local sewage plant (at least during the summer months) phages using *E. coli* outer membrane proteins were extraordinarily abundant. Over a period of five years, in this plant we have isolated 10 phages (all of T-even type morphology) which were specific for one or the other *E. coli* outer membrane proteins (Henning & Haller, 1975; Datta *et al.*, 1977; Riede *et al.*, 1984; and unpublished results). The phage titer of the sewage, as determined on a rough *E. coli* strain, was usually found to be around 10^2 /ml. OmpA, OmpC or OmpF-specific phages represented about 2% of these sewage virions. The plant received sewage at a rate of 2.7×10^7 to 3×10^7 l/day. Assuming the samples taken were representative for the whole plant, 10^{10} to 10^{11} outer membrane-specific phages were thus circulating through the plant per day. All the isolates were different from each other as well as from those described by other investigators (see Schwarz *et al.*, 1983; Riede *et al.*, 1984). All were new; hence, they did not stem from local laboratories. Clearly, there must be enough bacteria with accessible outer membrane proteins existing in the wild, meaning that enough opportunities for selection of mutants of such proteins should exist; otherwise, it would be rather difficult to offer an explanation for the generation of the type of genes 38 described so far (Montag *et al.*, 1987; Riede *et al.*, 1987b).

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