The Superimmunity Gene sim of Bacteriophage P1 Causes Superinfection Exclusion

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Previous work has shown that the sim gene of bacteriophage P1, if cloned into a multicopy vector, confers immunity against P1 infection to cells. We show that a 1.85-kb DNA fragment from the sim region of P1 (in the multicopy plasmid pMK4) expresses immunity and encodes three proteins with molecular weights of about 25, 24, and 15 kDa. Deletion of 650 bp from the sim region abolished synthesis of all three proteins and of the sim phenotype. Expression of sim did not prevent adsorption of P1 to cells. Successful transfection with linear P1 DNA suggests that the recombinational circularization of P1 DNA is not inhibited in the presence of sim. Plasmid pMK4 and a P1 prophage can be stably maintained in the cell indicating that replication of the prophage is not disturbed by sim. The prophage can be induced in the presence of sim. This shows that the sim phenotype is not caused by preventing lytic replication or phage maturation. In cells with pMK4 there is no expression of genes from infecting phages and transduction frequency is drastically reduced. We suggest that sim functions as a superinfection exclusion system by preventing transfer of DNA from the adsorbed phages into the cytoplasm.

INTRODUCTION

The immunity system of bacteriophage P1 consists of at least two independent regulatory circuits. The related genes are located in the regions immC and immI (for review see Scott, 1980; Stemberg and Hoess, 1983). Within the immC region there is the c1 repressor gene and some of its regulating functions. The c1 repressor maintains the lysogenic state by repressing lytic genes. Since P1 lysogens are not immune to the closely related phage P7 although the c1 genes of both phages are interchangeable, it became clear that there must be a further immunity function. This function is provided by the c4 repressor (Scott et al., 1978). The c4 gene is located within the immI region on the left of the gene(s) ant coding for an antirepressor. The c4 repressor prevents the expression of the antirepressor and thereby sustains the maintenance of lysogeny. The c4 repressor also determines the specificity of immunity by preventing expression of the antirepressor gene(s) of a superinfecting homologous phage.

The sim gene of bacteriophage P1 was detected during attempts to clone the c1 gene (Devlin et al., 1982). After shotgun cloning, transformed cells were screened for the active repressor by testing for immunity to P1 infection. Surprisingly the immune clones contained two different kinds of plasmids. One kind of plasmid carried the c1 gene as expected (EcoRI-7 fragment); the other kind carried the EcoRI-9 fragment of P1 (Bachi and Arber, 1977). When the EcoRI-9 fragment was inserted into a multicopy vector it did not only confer immunity to wild-type P1 phage but also to the mutants c1 and vir and to the heteroimmune phage P7. On the basis of its phenotype the gene was termed sim (superimmunity). In a low copy number vector the sim gene did not express the superimmunity phenotype. Although closely linked, c4 and sim are two separate genes transcribed from different promoters. Transposon mutagenesis led to the suggestion that the minimal size of the sim gene is about 1 kbp.

Several possible mechanisms for sim-mediated superimmunity have been discussed (Devlin et al., 1982). The phenotype could result from the overproduction of a structural phage protein which would prevent correct phage assembly. Another possibility is that sim could be a nontranscribed region on the genome which would titrate proteins engaged in replication. Finally the sim gene might code for a repressor regulating the switch from early to late functions during lytic development.

We have cloned a P1 DNA fragment expressing the sim phenotype on a multicopy plasmid. With this plasmid we have examined several possible ways in which the superimmunity could be exerted by the sim gene. The data led us to conclude that sim acts by a superinfection exclusion mechanism.

MATERIALS AND METHODS

Bacterial strains

If not mentioned otherwise all experiments were performed with Escherichia coli C600 F−, thi-1, leuB6,

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lacY1, supE44, tonA21. Minicells were isolated from E. coli DS410 minA, minB, Str, lacY, xyl, mtl, thi. Ca^{2+} cells for transfection of P1 DNA were prepared from WA236 recBC21, sscB15, recC22, arg, his, pro, thr, leu, thi. The recipient strain for bacterial transduction was E. coli AB1157 pro, leu, thr, arg, his, thi, ara, xyl, mtl, lac, gal.

**Phage strains**

Phages P1Cmc1.100 (encodes a thermosensitive repressor), P1c4.32, and P1vir were from J. Scott via A. Pühler. Phage P7 was from W. Arber; Mu from R. Simon; T4, T5, and T7 from W. Rüger; and λ and ϕX174 from R. Eichenlaub.

**Media**

Because phage P1 needs Ca^{2+} for adsorption most cultures were grown in RGMC medium: 10 g tryptone, 1 g yeast extract, 8 g NaCl, 1 g glucose, 1 g MgCl_2, 0.5 g CaCl_2 per liter. Phage dilutions were performed in SM-Ca: 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM MgCl_2, 5 mM CaCl_2.

Transformation of Ca^{2+} cells was performed according to the method of Maniatis et al. (1982). Transfection of Ca^{2+} cells was performed as described previously (Mandel and Higa, 1970).

**Transduction of bacterial markers**

Phage P1Cmc1.100 was grown in E. coli C600 and the lysate was diluted to a titer of 5 x 10^{9}ml. An overnight culture of E. coli AB1157 was washed twice in SM-Ca and diluted to a titer of 5 x 10^{9}ml. To 1.8-ml aliquots of cells, 0.2 ml of phage-lysate was added. Preadsorption was performed at 37°C for 20 min without shaking. Samples were plated on appropriately supplemented M9 plates and incubated for 48 hr at 37°C.

Isolation of minicells from E. coli DS410 was performed as described previously (Mandell and Higa, 1970).

**Radioactive labeling of plasmid-encoded proteins in minicells**

Five hundred microliters of minicells (1 x 10^{10} cells) was mixed with 150 μl methionine assay medium (Difco) and 10 μCi [35S]methionine (Amersham, op oct 1350 μCi/mm mol) and incubated at room temperature for 1 hr. After centrifugation cells were lyzed and prepared for SDS-PAGE.

**SDS-PAGE**

Polyacrylamide slab gels (14–20%, 1-mm thick, 20-cm long) were prepared according to the method of Laemmli (1970). Electrophoresis was performed for 16 hr at 130 V, 20 mA. Gels were dried, treated with Amplify (Amersham), and autoradiographed on Cronex 2 X-ray films (DuPont). A molecular-weight-marker mix of 14C-methylated proteins was from Amersham: myosin (mol wt 200,000), phosphorylase B (mol wt 97,400), bovine serum albumin (mol wt 68,000), chymotrypsinogen (mol wt 25,700), β-lactoglobulin (mol wt 18,400), lysozyme (mol wt 14,300).

**RESULTS**

**Identification of proteins required for the sim phenotype**

The multicopy plasmid pMK4 consists of the vector pUC13 (Vieira and Messing, 1982) and a 1.85-kb NruI–PvuII DNA fragment of the EcoRI-9 of P1 DNA (Fig. 1). This plasmid expresses the superimmunity phenotype but does not carry the c4 gene excluding any interference of c4 activity with immunity (Baumstark and Scott, 1987).

Before looking for a sim gene product, it was necessary to limit the insertion of plasmid pMK4 to the region essential for expression of superimmunity. Therefore deletion derivatives of plasmid pMK4 were produced by the method of Yanisch-Perron et al. (1985). Plasmid pMK4 was digested with the restriction endonucleases BamHI and SacI. The linearized molecules, with one protruding 5' end at the insert site and a 3' protruding end at the vector site, were partially degraded by exonuclease III followed by exonuclease VII. After religation a population of plasmids was obtained with various deletions extending from the NruI site of the P1 DNA insert. The size of deletion was determined and the expression of immunity was tested (Fig. 1). Plasmids with deletions up to 550 bp from the NruI site (pMK4d6) still conferred immunity to the cell. Deletions of 650 bp and larger resulted in a loss of the immunity phenotype (pMK4d7). These results are in agreement with data of Devlin et al. (1982) who used transposon mutagenesis to show that one end of the sim gene was located at approximately 560 bp from the NruI site.

The synthesis of plasmid-encoded proteins in minicells was used to identify the gene product of sim. With plasmid pMK4 in minicells of E. coli DS410 we detected three protein bands on the autoradiogram in addition to the two major proteins (β-lactamase) encoded by the vector. The three proteins have molecular weights of about 25, 24, and 15 kDa. These proteins are not expressed by the deletion derivatives pMK4d7 and pMK4d8. There are also two minor signals in the position of a 24-kDa protein and a 14.5-kDa protein with only vector pUC13. To verify that the 24-kDa protein is also sim specific and not a result of an overpro-
duction of a vector-encoded protein, the insert of pMK4 was also introduced into both the Cm and the Tc resistance genes of vector pACYC184. Figure 3 shows that cells carrying the vector pACYC184 did not express the 24-kDa protein and the 14.5-kDa protein, but after insertion of the 1.85-kb fragment the three proteins were observed. They were not expressed from deletion derivatives of pMK4 which had lost the sim phenotype indicating that superimmunity must be related to at least one of the proteins. The disappearance of all three proteins upon removal of about 100 nucleotides

![Fig. 3. Expression of the sim gene(s) after insertion into vector pACYC184. Autoradiogram of [35S]methionine-labeled proteins from cells with plasmid: (1) pMK5 = pACYC184 with a 1.85-kb Nru–PvuII P1 DNA fragment in the HindIII site of the Tc gene; (2) pMK8 = pACYC184 with a 3.5-kb EcoR1 fragment of P1 DNA in the EcoR1 site of the Cm gene; (3) pACYC184; (4) Marker proteins. Proteins were separated by electrophoresis through a 14–20% SDS–polyacrylamide gel.](image)

![Fig. 2. Expression of genes from plasmid pMK4 and its deletion derivatives in minicells. The autoradiogram of a 14–20% SDS–polyacrylamide gel shows the proteins synthesized in cells with the following plasmids (1–10): 1, pMK4d8; 2, pMK4d7; 3, pMK4d6; 4, pMK4d5; 5, pMK4d4; 6, pMK4d3; 7, pMK4d2; 8, pMK4d1; 9, pMK4; 10, pUC13; and 11, marker proteins. The two largest proteins expressed from all plasmids are the β-lactamase proteins.](image)

![Fig. 1. Deletion derivatives of plasmid pMK4 and their phenotype. (A) The insert size was determined from the mobility of DNA fragments during electrophoresis on a 1% agarose gel. λ DNA digested with EcoRI and HindIII was used as the molecular weight marker. (B) Single colonies from plates incubated overnight were cross-streaked against P1 Cm1.100 (5 × 10⁷/ml) on a RGMC plate and incubated overnight at 40°. +, growth of bacteria across the phage streak (immunity); −, no growth across the phage streak (no immunity). (C) Dilutions (10⁻¹) of a P1 Cm1.100 lysate were spotted on RGMC plates with 0.2 ml overnight culture and 3 ml soft agar and incubated overnight at 40°. The e.o.p. of 1 was determined with E. coli C600 containing the vector pUC13 and corresponds to a titer of 5 × 10⁸/ml.](image)
TABLE 1

EFFECT OF \( \text{sim} \) ON THE LYSOGENIZATION OF CELLS AFTER INFECTION WITH PHAGE P1CMC1.100

<table>
<thead>
<tr>
<th>Strain</th>
<th>Infection</th>
<th>Survivors/ml</th>
<th>Lysogens/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600</td>
<td>-</td>
<td>( 6 \times 10^6 )</td>
<td>(&lt; 10^6 )</td>
</tr>
<tr>
<td>C600</td>
<td>+</td>
<td>( 1 \times 10^4 )</td>
<td>(&lt; 10^2 )</td>
</tr>
<tr>
<td>C600 pMK4</td>
<td>-</td>
<td>( 8 \times 10^3 )</td>
<td>(&lt; 10^2 )</td>
</tr>
<tr>
<td>C600 pMK4</td>
<td>+</td>
<td>( 7 \times 10^4 )</td>
<td>( 1 \times 10^2 )</td>
</tr>
<tr>
<td>C600 pBD1</td>
<td>-</td>
<td>( 8 \times 10^6 )</td>
<td>(&lt; 10^2 )</td>
</tr>
<tr>
<td>C600 pBD1</td>
<td>+</td>
<td>( 6 \times 10^6 )</td>
<td>( 6 \times 10^6 )</td>
</tr>
<tr>
<td>C600 pBD1 pMK6</td>
<td>-</td>
<td>( 6 \times 10^9 )</td>
<td>(&lt; 10^2 )</td>
</tr>
<tr>
<td>C600 pBD1 pMK6</td>
<td>+</td>
<td>( 5 \times 10^9 )</td>
<td>(&lt; 10^2 )</td>
</tr>
</tbody>
</table>

Note. Log cells (2 \( \times 10^9 \) ml) of Escherichia coli C600 with or without plasmid were infected with P1CMC1.100 (m.o.i. about 5) and incubated for 1 hr at 42°C. Appropriate dilutions were plated parallel on RGMC plates and RGMC plates with 50 \( \mu \)g/ml Cm and incubated overnight at 40°C. Plasmid pED1 which confers immunity to the cells by expression of the c1 repressor gene served for comparison. Plasmid pMK6 is described in the legend to Fig. 2.

Fig. 4. Synthesis of phage-specific proteins after infection of mini-cells. Mini-cells with plasmid pMK4 and cells without plasmid were infected with phage P1CMC1.100 with a m.o.i. of 10. After preadsorption for 20 min at 37°C proteins were labeled with \(^{35S}\)methionine. Samples were taken at various times after labeling, and proteins of the lysed cells were separated by electrophoresis through a 14–20% SDS–polyacrylamide gel. The autoradiogram shows proteins synthesized in cells without plasmid 0, 10, 20, 30, and 40 min after preadsorption (lanes 1–5); in cells with plasmid pMK4 without infection (lane 6); and in cells with plasmid pMK4 0, 10, 20, 30, and 40 min after preadsorption (lanes 7–11). Marker proteins (lane 12).
TABLE 2

<table>
<thead>
<tr>
<th>Marker</th>
<th>Transductants/ml</th>
<th>% Residual transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AB1157 pUC13 A</td>
<td>AB1157 pMK4 B</td>
</tr>
<tr>
<td>pro</td>
<td>$1.7 \times 10^3$</td>
<td>$1.1 \times 10^2$</td>
</tr>
<tr>
<td>his</td>
<td>$3.0 \times 10^3$</td>
<td>$2.1 \times 10^2$</td>
</tr>
<tr>
<td>arg</td>
<td>$7.1 \times 10^3$</td>
<td>$3.8 \times 10^2$</td>
</tr>
</tbody>
</table>

Note. The donor phage was grown in E. coli C600. The m.o.i. was about 1 and the titer of the recipient cells (AB1157) was $5 \times 10^9$/ml.

for example, by inhibiting the cre protein (Sternberg and Hamilton, 1981). To test this, E. coli recBC with and without pMK4 was transfected with linear P1Cmcl.100 DNA using the Ca2+ method (Mandel and Higa, 1970). After plating with indicator cells, incubation was at 42°C to force all successfully transfected cells into the lytic cycle of phage development. The efficiency of transfection of cells carrying pMK4 (2.0 $\times 10^{-9}$/cell) was not lower than that of cells without the plasmid (0.9 $\times 10^{-6}$/cell). This shows that linear P1 DNA can enter the cell (differently from normal infection) and can perfectly initiate replication in the presence of pMK4 by the required recombinational circularization.

The above observations exclude several hypotheses on sim action and leave the possibility that sim prevents the successful injection of phage DNA during infection, such that the DNA does not enter the cytoplasm. We examined this possibility in two different ways. If P1 DNA can enter the cytoplasm after infection of E. coli pMK4 cells and be maintained at least temporarily, we would expect expression of at least some phage genes. This was not observed. Within 40 min after infection of cells without plasmid, numerous phage-encoded proteins appeared in the cell, while in cells with pMK4 no expression of P1-specific genes was detected (Fig. 4). In other experiments we observed that bacterial transduction was greatly inhibited by sim. Table 2 shows that the transduction frequencies of the three markers tested (pro+, his+, arg+) were reduced by more than one order of magnitude when pMK4 was present in the recipient cell. Since the presence of the sim gene product(s) in the cell inhibited P1 infection and bacterial transduction, it is concluded that the sim system blocks the transfer of DNA from the adsorbed P1 phage into the cytoplasm. The phage infection was reduced by a factor of about $10^{-6}$ and the bacterial transduction by a factor of about $5 \times 10^{-2}$ (Tables 1 and 2). This could indicate a discrimination of sim between P1 DNA and bacterial DNA, but other explanations are also possible.

### Specificity of sim function

Overexpression of the sim gene product inhibited the growth of the P1 wild type and all P1 mutants tested (P1α, P1g, P1c, P1l, P1 vir+) as well as the heteroimmune phage P7. Other phages tested (λ, T4, T5, T7, φX174, and Mu) had normal efficiencies of plating on cells with plasmid pMK4 (data not shown).

### DISCUSSION

We have tried to find out which step of phage development is blocked by the sim function. We show that sim interferes with neither the maintenance or replication of prophage nor the vegetative replication, maturation, and release of progeny phages. Adsorption of phages to cells with plasmid pMK4 was normal. Transfection experiments demonstrated that P1 DNA can enter the cell and subsequently efficiently initiate and complete vegetative phage growth. This argues against the possibility that recombinational circularization of P1 DNA is blocked by the sim function. Phage-encoded proteins were not detected in P1-infected pMK4 cells, and transduction of host cells by the Cm gene of P1 or by chromosomal markers was greatly reduced. We conclude from these data that sim must interfere with some early step between adsorption of phage and circularization of the injected DNA. We favor the hypothesis that sim codes for a function which specifically blocks DNA injection into the cytoplasm after adsorption of phage P1 to the receptor site. Such a mechanism of superinfection exclusion would resemble those observed with phages T4 and P22 (Anderson and Eigner, 1971; Susskind et al., 1974). There are several similarities between the sieA system of phage P22 of Salmonella thyphimurium and the sim system of phage P1. The sieA gene is located in the imml region upstream of the genes mnt and ant (Susskind et al., 1971). The ant gene of P22 codes for an antirepressor and the mnt gene for a repressor of the ant gene (Susskind and Botstein, 1975). The imml region of phage P1 is organized in a manner similar to that of phage P22. The gene antp is the analog of the antirepressor gene of P22 (antp22) and the P1 gene c4 is analogous to the P22 gene mnt. The three P1 genes are located on the genetic map in the sequence sim, c4, ant (Yarmolinsky, 1987).

Apart from their corresponding locations on the genome there are also some similarities in effects of sim and sieA:

1. Adsorption of infecting phage is not prevented in either system.
(2) Neither sieA+ cells nor sim+ cells are killed after infection with phage.

(3) The failure to kill the cells cannot be explained by quantitative lysogenization of the cells by superinfecting phages.

(4) Thermal induction of prophages is not blocked.

(5) SieA as sim strongly reduce transduction frequencies (Ebel-Tsipis and Botstein, 1971).

(6) There is no expression of gene products by superinfecting phages.

Our results show that the immunity phenotype caused by the sim gene of bacteriophage P1 is correlated with the presence of three proteins with molecular weights of 25, 24, and 15 kDa. So far we do not know whether all three proteins are required for immunity. It appears that these proteins are transcribed from one promoter. Cells carrying the plasmid pMK4d6 still synthesize the three proteins although the coding capacity of 1300 bp is not sufficient for all of them. Therefore we assume that one of the proteins (probably the 25-kDa protein) is a precursor protein perhaps with a hydrophobic leader sequence at the N-terminal end. The processed protein could be the 24-kDa protein.

This interpretation is consistent with the idea that the sim protein prevents a successful injection of DNA after P1 adsorption and is located in the periplasmic space or is associated with the phage receptor. The DNA sequence of the sim region and isolation and sequencing of the N-terminal end of the 25- and 24-kDa proteins will help to determine whether this hypothesis is correct.

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REFERENCES


