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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 *imm*C and *imm*I (for review see Scott, 1980; Sternberg and Hoess, 1983). Within the *imm*C region there is the *c*1 repressor gene and some of its regulating functions. The *c*1 repressor maintains the lysogenic state by repressing lytic genes. Since P1 lysogens are not immune to the closely related phage P7 although the *c*1 genes of both phages are interchangeable, it became clear that there must be a further immunity function. This function is provided by the *c*4 repressor (Scott *et al.*, 1978). The *c*4 gene is located within the *imm*I region on the left of the gene(s) *ant* coding for an antirepressor. The *c*4 repressor prevents the expression of the antirepressor and thereby sustains the maintenance of lysogeny. The *c*4 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 *c*1 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 *c*1 gene as expected (*Eco*RI-7 fragment); the other kind carried the *Eco*RI-9 fragment of P1 (Bächi and Arber, 1977). When the *Eco*RI-9 fragment was inserted into a multicopy vector it did not only confer immunity to wild-type P1 phage but also to the mutants *c*1 and *vir*^{s} and to the heteroimmune phage P7. On the basis of its phenotype the gene was termed *sim* (super*im*munity). In a low copy number vector the *sim* gene did not express the superimmunity phenotype. Although closely linked, *c*4 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 kb.

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, *leu*B6,

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*lac*Y1, *sup*E44, *ton*A21. Minicells were isolated from *E. coli* DS410 *min*A, *min*B, Str^r, *lac*Y, *xyl, mtl, thi.* Ca^{2+} cells for transfection of P1 DNA were prepared from WA236 *rec*BC21, *sbc*B15, *rec*C22, *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 P1Cm*c*1.100 (encodes a thermosensitive repressor), P1*c*4.32, and P1*vir*^{s} 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 m*M* Tris–HCl, pH 7.5, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 5 m*M* $CaCl_2$.

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 P1Cm*c*1.100 was grown in *E. coli* C600 and the lysate was diluted to a titer of 5×10^9/ml. An overnight culture of *E. coli* AB1157 was washed twice in SM-Ca and diluted to a titer of 5×10^8/ml. To 1.8-ml aliquots of cells, 0.2 ml of phage–lysate was added. Preadsorption was performed at 37° for 20 min without shaking. Samples were plated on appropriately supplemented M9 plates and incubated for 48 hr at 37°.

Isolation of minicells from *E. coli* DS410 was performed as previously described (Magazin *et al.*, 1978; Reeve, 1979).

Radioactive labeling of plasmid-encoded proteins in minicells

Five hundred microliters of minicells (1×10^{10} cells) was mixed with 150 μl methionine assay medium (Difco) and 10 μCi [^{35}S]methionine (Amersham, sp act 1350 μCi/mmol) and incubated at room temperature for 1 hr. After centrifugation cells were lysed 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 ^{14}C-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 *Nru*I–*Pvu*II DNA fragment of the *Eco*RI-9 of P1 DNA (Fig. 1). This plasmid expresses the superimmunity phenotype but does not carry the *c*4 gene excluding any interference of *c*4 activity with immunity (Baumstark and Scott, 1987).

Before looking for a *sim* gene product, it was necessary to limit the insert 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 *Bam*HI and *Sac*I. 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 *Nru*I 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 *Nru*I 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 *Nru*I 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-

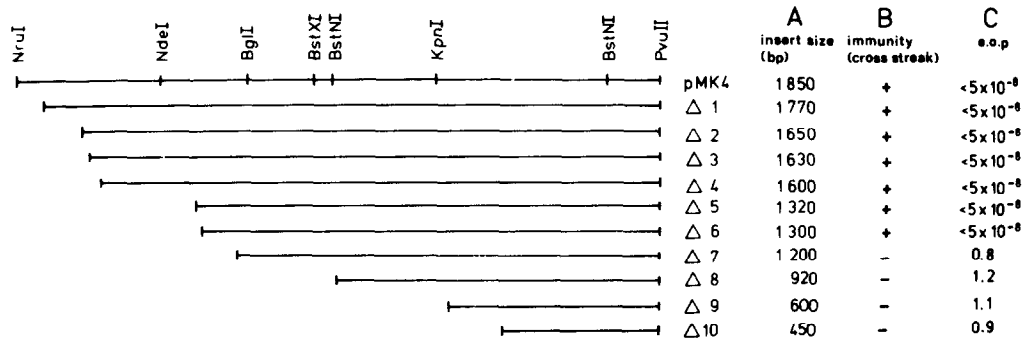

FIG. 1. Deletion derivatives of plasmid pMK4 and their phenotype. (A) The insert size was determined from the mobility of DNA fragments during electrophoresis on a 1% agarose gel. λ DNA digested with *Eco*RI and *Hin*dIII was used as the molecular weight marker. (B) Single colonies from plates incubated overnight were cross-streaked against P1Cm*c*1.100 (5×10^{10}/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 μl) of a P1Cm*c*1.100 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 5×10^{10}/ml.

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

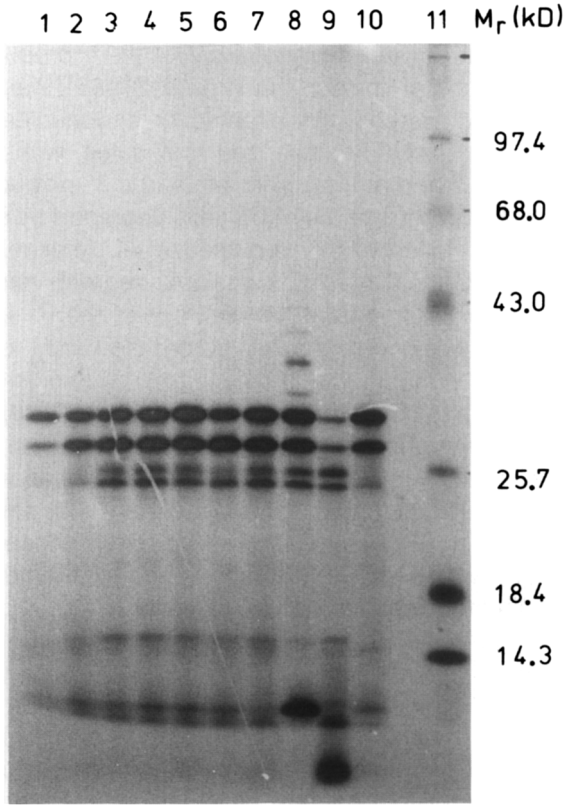

FIG. 2. Expression of genes from plasmid pMK4 and its deletion derivatives in minicells. The autoradiogram of a 14–20% SDS–polyacrylamide gel shows the proteins synthesized in cells with the following plasmids (1–10): 1, pMK4d8; 2, pMK4d7; 3, pMK4d6; 4, pMK4d5; 5, pMK4d4; 6, pMK4d3; 7, pMK4d2; 8, pMK4d1;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

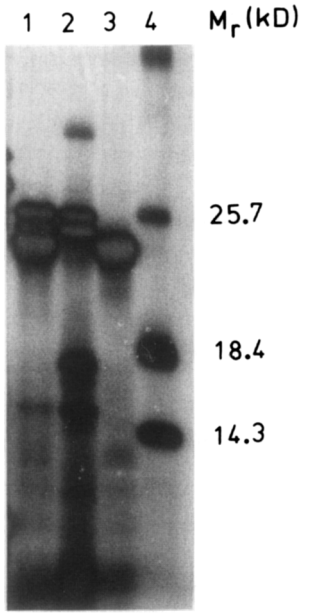

FIG. 3. Expression of the *sim* gene(s) after insertion into vector pACYC184. Autoradiogram of [^{35}S]methionine-labeled proteins from cells with plasmid: (1) pMK5 = pACYC184 with a 1.85-kb *Nru*I–*Pvu*II P1 DNA fragment in the *Hin*cII site of the Tc gene; (2) pMK6 = pACYC184 with a 3.5-kb *Eco*RI fragment of P1 DNA in the *Eco*RI site of the Cm gene; (3) pACYC184. (4) Marker proteins. Proteins were separated by electrophoresis through a 14–20% SDS–polyacrylamide gel.

TABLE 1

EFFECT OF *sim* ON THE LYSOGENIZATION OF CELLS AFTER INFECTION WITH PHAGE P1Cm*c*1.100

Strain	Infection	Survivors/ml	Lysogens/ml
C600	–	6×10^8	$<10^2$
C600	+	1×10^4	$<10^2$
C600 pMK4	–	8×10^8	$<10^2$
C600 pMK4	+	7×10^8	1×10^2
C600 pBD1	–	8×10^8	$<10^2$
C600 pBD1	+	6×10^8	6×10^8
C600 pBD1 pMK6	–	6×10^8	$<10^2$
C600 pBD1 pMK6	+	5×10^8	$<10^2$

Note. Log cells (2×10^8/ml) of *Escherichia coli* C600 with or without plasmid were infected with P1Cm*c*1.100 (m.o.i. about 5) and incubated for 1 hr at 42°. Appropriate dilutions were plated parallel on RGMC plates and RGMC plates with 50 μg/ml Cm and incubated overnight at 40°. Plasmid pBD1 which confers immunity to the cells by expression of the *c*1 repressor gene served for comparison. Plasmid pMK6 is described in the legend to Fig. 2.

from the insert of pMK4d6 and the absence of a truncated protein in cells with pMK4d7 and pMK4d8 suggest that the deletions cover the promoter of the *sim* gene(s) and that all three proteins originate from a common mRNA.

Infection of cells with pMK4 by phage P1

With the following experiments we tried to find out which step during the development of phage P1 is blocked by the action of *sim*.

The adsorption of phage P1 to cells with pMK4 was first tested. Growing cells were infected with phage P1 at a multiplicity of 1, and after preadsorption for 15 min at 37°, cells were removed by centrifugation and phages in the supernatant were quantitated. Adsorption of phage P1 to cells with and without pMK4 was about 97%. Thus *sim* seems not to prevent adsorption of phage P1. To test whether the *sim* protein acts as a "*c*1-like" repressor, cells with pMK4 were infected with phage P1Cm*c*1.100 and surviving and lysogenized fractions were determined on plates without and with chloramphenicol. For comparison cells overproducing the *c*1 repressor from plasmid pBD1 (Dreiseikelmann *et al.*, 1988) were also infected with P1Cm*c*1.100. The result is shown in Table 1. Cells expressing the *c*1 repressor are not killed because they are lysogenized. Cells expressing the *sim* gene product also escape killing, but in contrast do not carry a prophage. Cells harboring two plasmids, one expressing *c*1 (pBD1) and the other expressing *sim* (pMK5), again were not lysogenized after infection. From this observation we conclude that (i) the *c*1 repressor does not regulate *sim* expression and (ii) that *sim* acts at an earlier stage of P1 infection than the *c*1 repressor.

In another experiment P1Cm*c*1.100 lysogenic cells were transformed with plasmid pMK4. The transformation efficiency was normal (about 8×10^3 clones/μg DNA). Both prophage and plasmid pMK4 were stably maintained in the cells. This means that replication of the prophage is not hindered by the presence of a plasmid expressing *sim*.

After thermoinduction of P1*c*1.100 lysogenic cells carrying plasmid pMK4, normal lysis was observed and the titer of the lysate was nearly the same as of lysogens without pMK4. This shows that neither prophage maintenance or replication nor lytic replication or maturation of progeny phages is inhibited by *sim* function. The recombinational circularization of P1 DNA following the infection is an essential step for replication (Segev and Cohen, 1981; Sternberg *et al.*, 1981). It could be possible that *sim* prevents this circularization,

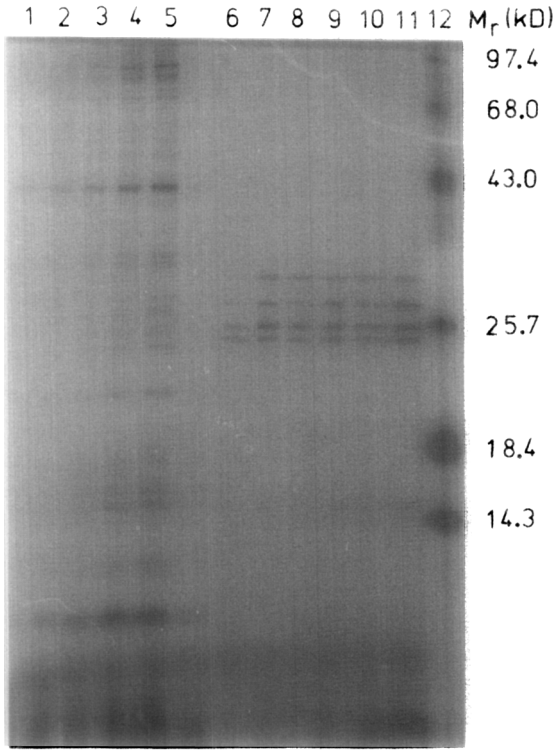

FIG. 4. Synthesis of phage-specific proteins after infection of minicells. Minicells with plasmid pMK4 and cells without plasmid were infected with phage P1Cm*c*1.100 with a m.o.i. of 10. After preadsorption for 20 min at 37° proteins were labeled with [^{35}S]methionine. Samples were taken at various 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

BACTERIAL TRANSDUCTION FOR *pro*$^+$, *his*$^+$, AND *arg*$^+$ IN pMK4 CELLS

Marker	Transductants/ml AB1157 pUC13 A	Transductants/ml AB1157 pMK4 B	% Residual transduction (A/B)
pro	1.7×10^3	1.1×10^2	6.5
his	3.0×10^3	2.1×10^2	7.0
arg	7.1×10^3	3.8×10^2	5.3

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 5×10^8/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 P1Cmc1.100 DNA using the Ca^{2+} method (Mandel and Higa, 1970). After plating with indicator cells, incubation was at 42° to force all successfully transfected cells into the lytic cycle of phage development. The efficiency of transfection of cells carrying pMK4 (2.0×10^{-6}/cell) was not lower than that of cells without the plasmid (0.9×10^{-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 5×10^{-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 (P1*c*4.32, P1*c*1.100, P1 *vir*^{s}) 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 *sie*A system of phage P22 of *Salmonella thyphimurium* and the *sim* system of phage P1. The *sie*A gene is located in the *imm*I 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 *imm*I region of phage P1 is organized in a manner similar to that of phage P22. The gene ant_{P1} is the analog of the antirepressor gene of P22 (ant_{P22}) and the P1 gene *c*4 is analogous to the P22 gene *mnt*. The three P1 genes are located on the genetic map in the sequence *sim*, *c*4, *ant* (Yarmolinsky, 1987).

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

(1) Adsorption of infecting phage is not prevented in either system.

(2) Neither *sie*A$^+$ 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) *Sie*A 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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