

Functional expression of individual plasmid-coded RNA bacteriophage MS2 genes

Erik Remaut, Peter De Waele, Anne Marmenout, Patrick Stanssens, and Walter Fiers*

Laboratory of Molecular Biology, State University of Ghent, Ledeganckstraat, 35, B-9000 Ghent, Belgium

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The genes of the RNA-containing bacteriophage MS2 were individually inserted into thermoinducible expression plasmids under control of the phage λ P_L promoter. Three phage-coded proteins (A-protein, coat protein, and replicase) were expressed at high efficiency. Induced cultures specifically complemented superinfecting amber mutants of phage MS2. Regulatory mechanisms operative during the natural infection cycle of the phage were reproduced by the plasmid expression system.

Key words: complementation/MS2 proteins/P_L promoter

Introduction

Since their discovery by Loeb and Zinder (1961), RNA-containing bacteriophages such as f2, MS2, Q β have been used as model systems for the study of numerous fundamental problems in molecular biology. The small size of their genome rendered them amenable to detailed structural and functional analysis (for review, see Zinder, 1975; Fiers, 1979). The phage particle constituted an excellent source of a well characterized pure mRNA species that greatly facilitated the elucidation of the mechanism of initiation, elongation, and termination (Kozak and Nathans, 1972; Steitz, 1979). Bacteriophage MS2 was the first organism for which the primary structure of the genome was unravelled at the nucleotide level (Fiers *et al.*, 1976). Genetic studies of these phages, however, have been hampered by several drawbacks related to the fact that all but one (the lysis gene) of the four known genes are indispensable for growth. As a consequence, only point mutations conferring a conditionally-lethal phenotype have so far been isolated. These have allowed the elucidation, in molecular terms, of the phenomenon of suppression, and they have revealed a complex system of gene expression regulated both in amount and in time.

We describe here the construction of plasmids that allow controlled, efficient expression of individual phage MS2 genes and show that the expressed proteins can functionally complement MS2 amber mutants. The system opens the possibility of isolating new types of mutants and may be a further tool in the study of the molecular biology of these organisms.

Results

Expression of individual phage MS2 genes

The expression vectors used in this study exploit the powerful leftward promoter (P_L) of phage λ and have been described by Remaut *et al.* (1981). Control over promoter activity is exerted by maintaining the plasmids in bacterial hosts that

supply a temperature-sensitive repressor from a defective lysogenic λ phage. Simple alteration of the temperature of incubation (42°C vs 28°C) turns on or off the P_L promoter activity. Thus, cloned genes having their own ribosome binding site but devoid of a promoter, as is the case for the MS2 genes, can be expressed from the extraneous P_L promoter in a controllable fashion. P_L-dependent synthesis of individual MS2 proteins was monitored as described in Materials and methods. The construction of the plasmids is described in the Materials and methods section. Figure 1 shows the relevant MS2 coding sequence present in each plasmid.

The replicase gene

Plasmid pPLaR1 contains the MS2 replicase cistron inserted in the sense orientation downstream from the P_L promoter. Upon induction this plasmid directed the synthesis of large amounts (up to 35% of total *de novo* synthesis) of a protein with an apparent molecular weight (mol. wt.) on SDS-polyacrylamide gels of ~59 K (Figure 2). This protein was absent in induced cultures containing a reference plasmid pPLa2311. The observed mol. wt. is in excellent agreement with the value for MS2-coded replicase protein, calculated from RNA sequence data (Fiers *et al.*, 1976), and with the size of the replicase polypeptide revealed in rifampicin-blocked cells (Remaut and Fiers, 1972). Complementation analysis showed that induced cultures of K12 Δ H1 Δ trpF⁺ (pPLaR1) were able to complement specifically an amber mutant in the replicase gene (Table I). Plaque assays for the complementation tests were performed at the intermediate temperature of 37°C. Indeed at 42°C strain K12 Δ H1 Δ trpF⁺ (pPLaR1) did not form a confluent lawn on the plates, nor was the strain able to form colonies upon continued incubation at 42°C. Presumably continued high-level production of MS2 replicase is lethal to the cell. The replicase of phage Q β , which is related to MS2, is known to form a complex with the elongation factors Tu-Ts (Kondo *et al.*, 1970; Kamen, 1970) and the ribosomal protein S1 (Wahba *et al.*, 1974). If the MS2 replicase has similar properties, overproduction of the protein might sequester these indispensable factors and conceivably lead to interference with protein synthesis.

Percentage synthesis of the MS2 replicase reaches 35% as early as 30 min after induction but then declines to 16% at 70 min (Figure 3). For comparison, K12 Δ H1 Δ trp (pPLa2311) synthesizes β -lactamase at ~30% of total *de novo* synthesis from 60 min until at least 150 min after induction (Remaut *et al.*, 1981).

The A-protein (maturation protein)

Plasmid pPLaA2 contains the MS2 A-protein cistron inserted in the sense orientation downstream from the P_L promoter. Plasmid pPLaA2 directed the synthesis at 42°C of a protein of ~44 K (Figure 2). The value agrees well with the mol. wt. of phage MS2 A-protein calculated from sequence data (Fiers *et al.*, 1975) and with the size of the A-protein polypeptide synthesized in rifampicin-blocked cells (Remaut *et al.*, 1972). The 44 K protein was absent in induced cultures containing either pPLa2311 or pPLaA17. The latter plasmid contains the A-protein cistron in the anti-sense orientation

*To whom reprint requests should be sent.

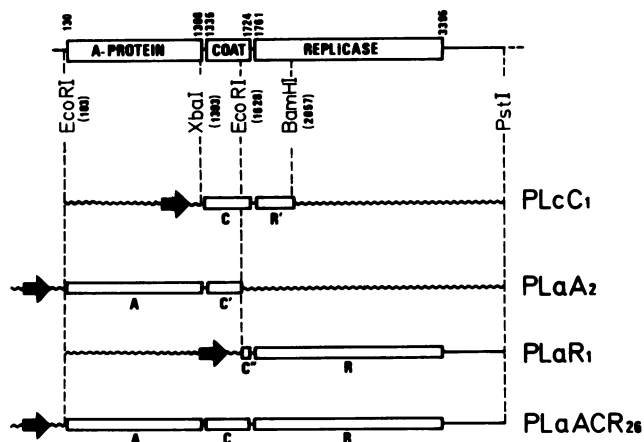


Fig. 1. Insertion of individual MS2 genes into P_L expression vectors. Details of the construction of the plasmids are given in Materials and methods. The heavy arrow indicates the position of the P_L promoter. The boxed areas represent phase MS2 genes. The numbers refer to the positions on the linear cDNA map of cloned MS2 RNA (Devos *et al.*, 1979a). A, A-protein; C, coat protein; R, replicase; R', part of the replicase; C' and C'', parts of the coat protein. The wavy line indicates the acceptor plasmid.

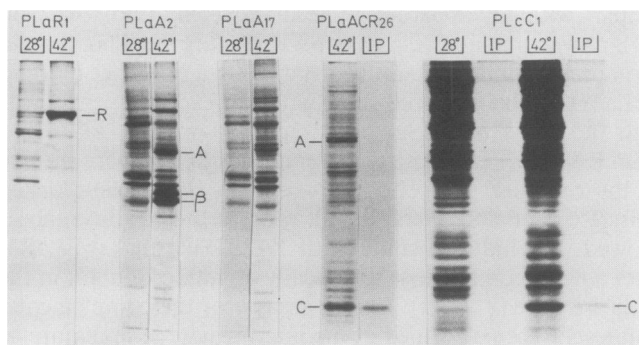


Fig. 2. Representative profiles of cloned MS2 phage proteins induced in whole cells by P_L expression vectors. The cells were labeled and their proteins electrophoresed in SDS-polyacrylamide gels as described in Materials and methods. The plasmid under study and the temperature of incubation are indicated above the lanes. The host was M5219 for pPLcC1 and was K12ΔH1Δtrp for all other plasmids. The lanes marked with the symbol IP show the patterns obtained after immunoprecipitation of extracts from induced cultures with anti-MS2 coat protein antiserum. Immunoprecipitation was done essentially according to Sekizawa *et al.* (1977) except that antigen-antibody complexes were collected by adsorption to formaldehyde-treated bacteria from the Cowan I strain of *Staphylococcus aureus*. A, A-protein; C, coat protein; R, replicase; β, β-lactamase and its precursor.

downstream from P_L . Percentage synthesis of the MS2 A-protein accounted for ~5% of *de novo* protein synthesis (Figure 3). Plasmid pPLaA2 also synthesized large amounts (30%) of the β-lactamase and its precursor. These products are visualized on the gels as the heavy bands running at 27.5 K and 30 K, respectively (Figure 2; see also Remaut *et al.*, 1981). The MS2 A-protein and the β-lactamase on pPLaA2 are present (in that order) on the same polycistronic messenger transcribed from P_L . Induced cultures of K12ΔH1Δtrp (pPLaA2) specifically complemented an amber mutant in the A-protein cistron (Table I). Complementation was not observed with either pPLaA17 or the parent pPLa2311.

In addition to the A-protein, plasmid pPLaA2 also codes for the ribosome-binding site and the first 97 amino-acid residues of the coat protein. From the known sequence around the *EcoRI* site in the coat cistron and the sequence of pBR322 (Sutcliffe, 1978) at the junction point, a theoretical reading

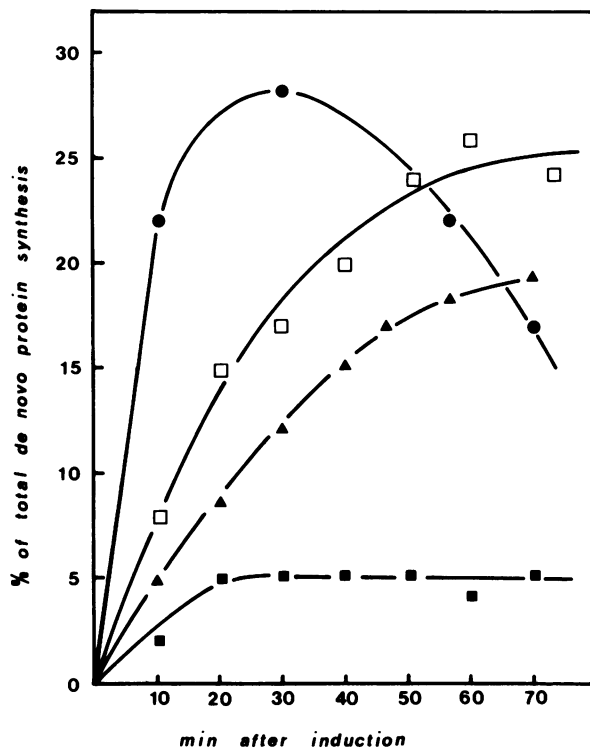


Fig. 3. Schematic presentation of the rate of expression of individual MS2 phage genes cloned on P_L expression vectors. Protein synthesis in induced cultures of strain K12ΔH1Δtrp harboring various P_L expression plasmids was followed as outlined in Materials and methods. Percentage synthesis of individual proteins was determined after cutting out the relevant protein bands from the dried gel and comparing the incorporated radioactivity to the total radioactivity recovered from the gel. Percentage synthesis is plotted against time after induction at 42°C. ●—●, replicase synthesis by pPLaR1; ■—■, A-protein synthesis by pPLaA2; □—□, synthesis of β-lactamase and its precursor by pPLaA2; ▲—▲, coat protein synthesis by pPLaACR26.

frame for a protein consisting of 98 amino acids can be predicted. When analyzed on 20% acrylamide gels, a protein of about the predicted size was indeed found in induced cultures of K12ΔH1Δtrp (pPLaA2) (data not shown). As expected this protein was absent in induced cultures of K12ΔH1Δtrp (pPLaA17). Immunodiffusion tests performed according to Skalka and Shapiro (1976) showed that induced cultures of K12ΔH1Δtrp (pPLaA2) were antigenically active against anti-MS2 coat protein antiserum but not against anti-MS2 particle antiserum. Full-size coat protein as synthesized by pPLaACR26 reacted with both antisera. This finding indicated that the amino-terminal coat fragment contains one or more antigenic sites not exposed in the virus particle.

The coat protein

Both pPLcC1 and pPLaACR26 could be thermoinduced to synthesize the MS2 coat protein, as illustrated by specific immunoprecipitation of a protein of ~13.5 K with anti-MS2 coat protein antiserum (a gift from J. Van Duin) or anti-MS2 particle antiserum (Figure 2). Their level of expression was, however, drastically different. pPLcC1 synthesized barely detectable amounts of coat protein. In fact the protein could be visualized only by continuously labeling the cells from 20 min to 120 min after induction. The percentage synthesis during that time interval accounted for ~2% of ongoing protein synthesis. Plasmid pPLaACR26 on the other hand induced coat protein synthesis to a level of 20% of total *de novo* synthesis as early as 60 min after induction. This differential

Table 1. Complementation plaque assays in strain K12ΔH1Δtrp⁺ containing P_L expression plasmids

Plasmid	wild-type	Infecting phage am901 (replicase)	am309 (A-protein)
pPLaR1	+	+	-
pPLaA2	+	-	+
pPLaA17	+	-	-
pPLa2311	+	-	-

Details of the assay are given in Materials and methods.

rate of expression presumably reflects the important role of secondary structure in determining the efficiency of translational initiation in different genes (Fiers, 1979). Plasmid pPLaACR26 contains the complete MS2 coding region. Hence the mRNA initiated from the P_L promoter presumably assumes a configuration similar to that of native MS2 RNA, thereby optimally exposing the coat protein initiation signal. Indeed, in an *in vitro* translation system supplemented with phage MS2 RNA, ribosomes are known to bind only to the initiation region of the coat protein (for review, see Kozak and Nathans, 1972). pPLcC1 on the other hand contains neither the A-protein region nor the larger part of the replicase gene. In this new context the coat protein initiation signal is apparently not very effective in directing initiation of protein synthesis. The replicase protein was not detectable in induced cultures of K12ΔH1Δtrp (pPLaACR26). The A-protein was synthesized in amounts comparable to the values obtained with pPLaA2.

Figure 3 summarizes the data on percentage synthesis of the plasmid-coded MS2 proteins based on incorporation of [³⁵S]methionine. The A-protein and the replicase each contain eight methionine residues. On the assumption that the amount of the P_L-directed mRNA transcribed from both plasmids is similar and that both proteins are equally stable in the cells, the actual ratio of initiation at their respective cistrons can be estimated on the basis of the percentage synthesis. The coat protein contains only two methionine residues: percentage synthesis is therefore an underestimation of the frequency of the initiation at this cistron relative to the A-protein and the replicase cistrons. Taking this into account it can be calculated that, at the point of maximum relative synthesis, initiation at the coat protein cistron in pPLaACR26 occurs 16 times more frequently than at the A-protein cistron and about three times more frequently than at the replicase cistron in pPLaR1.

Discussion

Here we report controllable, efficient expression of individual genes of the RNA bacteriophage MS2. Restriction fragments obtained from a plasmid carrying a nearly full-size cDNA copy of MS2 RNA (Devos *et al.*, 1979a) were inserted into plasmid expression vectors downstream from phage λ P_L promoter, the activity of which is controlled by a thermolabile repressor (Remaut *et al.*, 1981). The A-protein, coat protein, and RNA replicase of phage MS2 were expressed at high levels by the plasmid vectors. In the case of the A-protein, and even more so the replicase protein, the levels of expression were much higher than observed during the natural infection cycle of phage MS2 (Sugiyama and Stone, 1968). Especially in the case of the RNA replicase, these vector systems represent an attractive source of a protein that so

far has eluded several attempts at purification.

The A-protein and RNA replicase synthesized by the plasmid vectors specifically complemented MS2 amber mutants. Hitherto, the genetics of RNA phages has been limited to the isolation of conditionally lethal mutants of the suppressible-nonsense (Tooze and Weber, 1967) or temperature-sensitive type (Horiuchi *et al.*, 1966). The expression system presented here opens the possibility of isolating lethal mutants of an RNA phage, for example, deletions in essential regions, by supplying the mutant function in *trans* from a compatible plasmid. The principle of gene-specific suppressors was introduced by Humayun and Chambers (1978) who cloned gene G of φX174 onto pMB9 and showed that bacteria containing this recombinant plasmid complemented amber mutants in gene G. In this system the expression of the inserted gene cannot be experimentally controlled. The use of a thermoinducible P_L promoter allows the cloning of viral genes whose continuous expression is lethal to the host (Kastelein *et al.*, 1982). By varying the time period of induction or choosing a suboptimal temperature for induction the amount of plasmid-coded gene products accumulating in the cell can be experimentally varied.

Furthermore, we show that control mechanisms operative during the infection cycle of the phage are reproduced in the plasmid expression system. Earlier studies on phage MS2-infected cells have shown that functional coat protein represses initiation at the replicase cistron (see Kozak and Nathans, 1972). The same or a similar regulatory mechanism is observed in the plasmid expression system. Indeed, the replicase protein was not detectable in induced cultures of K12ΔH1Δtrp (pPLaACR26), which synthesize large amounts of coat protein, whereas in the absence of the latter protein, prominent synthesis of replicase was observed, as illustrated by plasmid pPLaR1. The synthesis of A-protein was not affected by concomitant synthesis of coat protein, again in agreement with what is known about the physiology of phage-infected cells.

During the infection cycle of phage MS2, the A-protein is synthesized at relatively low levels and is believed to be translated only from nascent RNA chains (Robertson and Lodish, 1970). It is thought that the ribosome binding site is quickly masked by the secondary structure of the growing RNA chain. Fiers *et al.* (1975) proposed a model whereby a segment located at about two-thirds distance in the gene can base pair with the region just before the initiating GUG of the gene. In agreement with these earlier findings, the plasmid coded A-protein is produced in low amounts (5% of the total *de novo* protein synthesis as compared to 30% for β-lactamase situated further downstream on the same mRNA transcribed from the P_L promoter); but more studies are needed to prove that the A-protein gene is autoregulatory at the level of the secondary structure of the messenger (or viral) RNA.

The study of translational regulation mechanisms in RNA phages is complicated by the fact that the viral RNA acts at the same time as a messenger and as a template for replication. This complication does not occur when non-replicating mRNAs are transcribed from the cloned genes. For example, Robertson and Lodish (1970) suggested that high levels of coat protein synthesis during infection could be attributed to the structure of replicative intermediates having exposed coat genes but buried replicase and A-protein genes. Our results argue that the efficiency of initiation at the coat protein cistron is regulated at the translational level, presumably by

secondary structure of the RNA. Plasmid pPLaACR26, carrying the nearly full-length MS2cDNA, expressed the coat protein with high efficiency (20% of total *de novo* protein synthesis), whereas plasmid pPLcC1, containing essentially only the coat protein gene, synthesized very low levels of coat protein. These “*in vivo*” results substantiate the results of “*in vitro*” experiments on the binding of ribosomes to viral RNA. Binding of ribosomes to the coat initiation region is very strong and selective for full-size viral RNA but decreases concomitant with increasing fragmentation, both for the MS2-related R17 (Adams *et al.*, 1972; Steitz, 1973) and for Q β (Porter and Hindley, 1973). Obviously, the plasmids described here can be further manipulated to vary the viral RNA content surrounding the coat protein cistron and enable studies to be made of its effect on the efficiency of initiation.

Recently, a peculiar phenomenon of frame-shifting in the coat protein cistron of phage MS2 was described (Atkins *et al.*, 1979). These studies involved an *in vitro* translation system. Two features of the vectors described in this paper allow the study of these problems *in vivo*: the coat protein cistron can be inserted on a variety of restriction fragments and the expression of this cistron can be maintained at a high level for several hours after induction. Neither of these requirements can be met during the natural infection cycle of the phage. The use of the vectors described here to investigate the intriguing mode of regulated synthesis of the fourth phage MS2 gene coding for the lysis protein has been reported elsewhere (Kastelein *et al.*, 1982).

Materials and methods

Materials

Restriction enzymes were obtained from New England Biolabs, MA, with the exception of *EcoRI* which was obtained from Boehringer, Mannheim. The linker sequences GGAATTC (*EcoRI*), CGGATCCG (*BamHI*) and CTCTAGAG (*XbaI*) were from Collaborative Research, MA. Prior to ligation the linker molecules were phosphorylated using T4 polynucleotide kinase (Boehringer, Mannheim).

Bacterial and phage stains

The *Escherichia coli* K12 strains, K12 Δ H1 Δ trp and M5219 were used as hosts for the expression vectors and have been described by Remaut *et al.*, (1981). Both strains harbor a defective λ prophage that supplies a temperature-sensitive repressor (cI857). The phage MS2 amber mutants am309 and am901 were obtained from M. van Montagu.

DNA methodology

Plasmid DNA was isolated from small scale cultures (20 ml) using the cleared-lysate technique, essentially as described by Kahn *et al.* (1979). The conditions for transformation have been described (Remaut *et al.*, 1981). All restriction enzymes were used according to the manufacturer's specifications. DNA fragments were ligated with T4 DNA ligase as described (Kahn *et al.*, 1979).

Construction of plasmids

Plasmid pMS2-7 (Devos *et al.*, 1979a) carries a nearly full-size cDNA copy of the MS2 phage RNA. The knowledge of the complete nucleotide sequence of the MS2 RNA allowed us to define restriction fragments carrying individual genes of the phage (see Figure 1). The presence of predicted restriction sites was verified by restriction analysis on pMS2-7 DNA.

Individual phage genes were inserted into controllable expression vectors carrying the leftward promoter of bacteriophage λ (Remaut *et al.*, 1981). Plasmid pPLaR1 was constructed by replacement of an *EcoRI-PstI* fragment of pPLa2311 (Remaut *et al.*, 1981) with an *EcoRI-PstI* fragment obtained from pMS2-7, carrying the distal part of the coat protein gene (32 amino acid residues), the complete coding region of the replicase gene and the untranslated 3' end of the phage. The *EcoRI* site is located at nucleotide 1628 (Devos *et al.*, 1979a) within the coat protein cistron. The *PstI* site originates from an IS1 insert fortuitously picked up in pMS2-7 (Devos *et al.*, 1979b). In pPLaR1 the replicase gene is inserted in the sense orientation with respect to the P_L promoter.

An *EcoRI* fragment carrying the complete coding region for the A-protein

(maturation protein) and an amino-terminal part of the coat gene (97 amino-acid residues) was inserted into the *EcoRI* site of pPLa2311. The orientation of the insert was determined by combined digestion with *AvaI* and *HindIII*. The A-protein cistron of phage MS2 contains a single *AvaI* site asymmetrically located at nucleotide 1098. The *HindIII* reference point is situated within the kanamycin resistance gene of pPLa2311. In pPLaA2 the A-protein is in the sense orientation with respect to the P_L promoter whereas pPLaA17 has the anti-sense configuration. MS2 DNA present on pPLaA2 runs from an *EcoRI* site at nucleotide 103 in the untranslated 5' region to an *EcoRI* site at nucleotide 1628 within the coat protein cistron. The plasmid contains 27 nucleotides from the untranslated 5' end, the complete coding region for the A-protein, the first intracistronic space, and 293 nucleotides from the coat protein cistron.

By combination of appropriate restriction fragments from pPLaR1 and pPLaA2 the plasmid pPLaACR26 was constructed, carrying the complete in-line coding sequence of phage MS2 downstream from the P_L promoter. Finally, the coat protein cistron was obtained from pMS2-7 as an *XbaI-BamHI* fragment and initially inserted into pBRX13, a derivative of pBR322 (Bolivar *et al.*, 1977) in which the *EcoRI-BamHI* fragment was replaced by the linker sequence *EcoRI-XbaI-BamHI*, resulting in loss of the tetracycline resistance (unpublished work). From this plasmid (pBRC31) an *EcoRI* fragment carrying the ribosome binding site and the amino-terminal part of the coat protein (97 amino-acid residues) was inserted into pPLc24 (Remaut *et al.*, 1981), a plasmid which already contains the carboxyl-terminal part of the coat protein (32 amino-acid residues), thus reconstituting the complete coding sequence. The plasmid was designated pPLcC1 and was shown to contain the *EcoRI* fragment in the sense orientation downstream from P_L by combined digestion with *SalI* and *BamHI*. The inserted fragment contains a single *SalI* site asymmetrically located at nucleotide 1365 within the coat protein cistron. MS2 DNA in pPLcC1 runs from an *XbaI* site at nucleotide 1303 (at the penultimate amino acid residue of the A-protein) to a *BamHI* site at nucleotide 2057 within the replicase cistron. In addition to the coat protein cistron the plasmid contains the coding sequence for the first 99 amino-acid residues of the replicase protein. Plasmids pPLaR1 and pPLaACR26 specify resistance to kanamycin (50 μ g/ml). Plasmid pPLcC1 specifies resistance to carbenicillin (100 μ g/ml). Plasmids pPLaA2 and pPLaA17 specify resistance to both kanamycin and carbenicillin.

Induction of plasmid-coded MS2 proteins

Inducible synthesis of individual MS2 phage proteins in *E. coli* strains K12 Δ H1 Δ trp or M5219, containing various expression vectors was measured essentially as described (Remaut *et al.*, 1981). Bacteria were grown at 28°C in LB-medium (1% Bacto tryptone; 0.5% yeast extract; 0.5% NaCl) without antibiotic to a density of 2×10^8 cells/ml. The cells were collected and resuspended in their original volume of magnesium salts supplemented with 1 mM MgSO₄, 0.2% glucose, 1% methionine assay medium (Difco) and 50 μ g/ml L-tryptophan. After continued incubation at 28°C for 60 min, one half of the culture was shifted to 42°C. At various times after induction, aliquots from the 28°C and 42°C cultures were labeled with 20 μ Ci/ml [³⁵S]methionine (600 Ci/mmol; The Radiochemical Centre, Amersham) for 5 min. Incorporation was terminated by phenol extraction. The proteins were precipitated from the phenol phase by addition of five volumes of ethanol and redissolved in 1% SDS, 1% β -mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8. The samples were boiled for 5 min, centrifuged at 12 000 g and electrophoresed in SDS-polyacrylamide gels (12.5%) according to Laemmli (1970). Following electrophoresis the gels were fixed in 10% TCA, treated with EN³HANCE (New England Nuclear, Boston, MA) and dried before autoradiography. To determine the percentage synthesis of individual MS2 proteins as compared to total *de novo* protein synthesis, the relevant protein bands were excised from the dried gel and their radioactivity compared to the total radioactivity recovered from the gel.

Complementation assays

Strain K12 Δ H1 Δ trp was made F⁺ by conjugation with NK3 (Durwald and Hoffmann-Berling, 1968) and then transformed with plasmids pPLa2311, pPLaA2, pPLaA17, and pPLaR1, respectively. For complementation assays, cultures of the various strains, grown overnight at 28°C in L-broth, were diluted 50-fold in fresh medium and incubated at 34°C for three generations to express the F-pilus. Aliquots of the cultures were infected with suitable dilutions of wild-type MS2 phage or its representative mutants and plated on LB plates at 37°C. Plaques were examined after 6 h incubation. Mutant am309 carries an amber mutation in the A-protein cistron (Vandamme *et al.*, 1972); mutant am901 has an amber lesion in the replicase cistron (M. Van Montagu, personal communication). The su-1⁺ strain CR63 (Appleyard, 1956) was used as reference permissive host for the amber mutants and the su⁻ strain C3000 (Loomis and Magasanik, 1967) as non-permissive host.

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