Replication of the broad host range plasmid RSF1010 in cell-free extracts of *Escherichia coli* and *Pseudomonas aeruginosa*

Ramon Diaz* and Walter L. Staudenbauer[†]

Max-Planck-Institut für Molekulare Genetik, Abt. Schuster, Ihnestr. 63-73, D-1000 Berlin 33, FRG

Received 8 June 1982; Revised and Accepted 6 July 1982

ABSTRACT

Replication of exogenous RSF1010 DNA can be carried out by soluble enzyme systems from *Escherichia coli* and *Pseudomonas aeruginosa*. It requires the function of RSF1010-encoded replication protein(s), which is not expressed in extracts from plasmid-free bacteria. In contrast to previously described *in vitro* systems for plasmid replication, initiation of RSF1010 DNA synthesis is independent of transcription catalyzed by host RNA polymerase. This is indicated by the insensitivity of RSF1010 replication to rifampicin as well as to RNA polymerase antibodies. It is proposed that a host RNA polymerase transcription-independent initiation mechanism might be a general property of broad host range plasmids.

INTRODUCTION

RSF1010 is a 8.9 kb multicopy plasmid conferring resistance to streptomycin and sulfonamides (1), which belongs to the incompatibility group Q (2). Like other members of this group RSF1010 is capable of stable maintenance in various Gram-negative bacteria including the enterobacteria and pseudomonads (3). It is conceivable that this broad host range character is determined by some plasmid-encoded replication function enabling the RSF1010 replicon to interact with the replication machineries of a variety of hosts. The replicative properties of RSF1010 are therefore of special interest.

Replication of RSF1010 in *Escherichia coli* is initiated at a fixed origin located at a distance of 2.6 kb from the single *Eco* RI site and proceeds via θ -shaped replication intermediates either unidirectionally or bidirectionally with about equal frequency (4). RSF1010 replication differs in several aspects from that of ColE1-type plasmids, the host range of which is limited to enterobacteria. Thus RSF1010 DNA synthesis does not continue

in the absence of de novo protein synthesis and requires only low levels of DNA polymerase I (5). Unlike ColE1-type plasmids, which depend completely on the replication machinery of the host, RSF1010 encodes a trans-acting replication function (6).

Elucidation of the molecular basis of the broad host range character of RSF1010 requires the development of cell-free replication systems for RSF1010. This communication describes the preparation and properties of soluble enzyme systems from E.coli and Pseudomonas aeruginosa that can carry out the replication of exogenous RSF1010 DNA.

EXPERIMENTAL PROCEDURES

Nucleic acids. Plasmid DNA was isolated by established procedures (7,8) from the E.coli strains listed in Table 1. DNA of the phages M13 and ϕ X were the kind gift of T.Boeldicke and E.Lanka respectively.

Preparation of cell extracts. 2 l cultures were grown in 5 l flasks with shaking at 37°C until O.D.₆₀₀ = 1.0. Cells were then poured onto 600 g crushed ice, sedimented, washed once with 30 ml buffer A (25 mM HEPES-KOH pH 8.0, 100 mM KCl, 1 mM dithiothreitol) and resuspended in buffer A (1 ml per gram of wet cells). All the operations were performed at 0-2°C as quickly as possible. Lysis was carried out by the freeze-thaw method (11) in the presence of

Table 1. Bacterial strains and plasmids

Strain designation	Plasmid	Source (Reference)
<u>E.coli</u> K12 C600 <u>thr-1, leu-6, thi-1,</u> <u>SupE44, lacY-1,</u> <u>tonA21, hsdR⁺,</u> <u>hsdM⁺</u>		(9)
C600	RSF1010	M.Bagdasarian
C600	pKN177	S.Molin (10)
YS10 <u>thr, leu, minA, str,</u> <u>thi, end, hsdR⁺,</u> <u>hsdM⁺</u>	ColE1	J.Tomizawa
<u>P.aeru-</u> <u>ginosa</u> PAO1162 <u>leu-38, hsdR, hsdM⁺</u>		M.Bagdasarian (3)
PAO1162	RSF1010	M.Bagdasarian

1 mM EDTA. Extracts were divided into small aliquots and stored in liquid nitrogen. This crude extract is referred to in the test as Fraction I. From it, a Fraction II preparation was obtained by adjusting the KCl and EDTA concentrations to 0.2 M and 5 mM respectively followed by fractionation with streptomycin sulfate and ammonium sulfate as described (12). Fraction II was also divided into small aliquots and stored in liquid nitrogen.

Assay of DNA synthesis. Standard reaction mixtures (25 μ l) contained final concentrations of 50 mM HEPES-KOH pH 8.0, 75 mM KCl, 10 mM Mg-acetate, 15 mM creatine phosphate, 0.1 mg/ml creatine kinase, 2 mM ATP, 0.4 mM each of CTP, GTP, and UTP, 0.05 mM each of NAD and cyclic AMP, 0.025 mM each of dATP, dCTP, dGTP, and 3 H-dTTP (500 cpm per pmol) and 2.5% polyethylene glycol 6000. The optimal amounts of extract and exogenous plasmid DNA had to be determined for each extract preparation. For replication of RSF1010 in fractions of P.aeruginosa, KCl concentrations were adjusted to final value of 20 mM. Incubations were performed at 30°C for the times indicated and the incorporation of 3 H-dTMP into acid-insoluble material determined as described previously (13).

Alkaline CsCl gradient centrifugation. Standard reaction mixtures were layered directly on preformed 4.2 ml alkaline gradients (density 1.2 - 1.4 g CsCl/ml in 0.2 N NaOH) and centrifuged for 35 minutes at 40,000 rpm in a Beckman SW50.1 rotor at 5°C. Fractions were collected from the bottom of the tubes and assayed for acid-insoluble radioactivity.

Enzymes and material. Creatine phosphate, creatine kinase and nucleoside triphosphates were obtained from Boehringer. Antisera against E.coli RNA polymerase holoenzyme and the corresponding preimmune control sera were the kind gifts of H.Lother. Streptolydigin was kindly supplied by B. Van Dorp. 3 H-dTTP was obtained from Amersham. The sources of all other reagents have been described (11).

RESULTS

Replication capacity of extracts from E.coli and P.aeruginosa

When DNA synthesis was assayed in crude extracts (Fraction I) from E.coli C600 (RSF1010), it was found that this soluble enzyme

Table 2. Replication capacity of extracts

Source of extract	Fraction	³ H-dTMP incorporated (pmol)	
		Plasmid DNA added	
		None	RSF1010
<u>E.coli</u> C600 (RSF1010)	I	14.9	38.8
<u>P.aeruginosa</u> PA01162 (RSF1010)	I	2.5	4.2
<u>E.coli</u> C600	I	0.9	1.0
<u>P.aeruginosa</u> PA01162	I	0.8	0.9
<u>E.coli</u> C600 (RSF1010)	II	0.9	43.0
<u>P.aeruginosa</u> PA01162 (RSF1010)	II	0.9	11.8

DNA synthesis was carried out for 60 min at 30°C in standard reaction mixtures (25 µl) without or with RSF1010 DNA at final concentrations of 1-2 µg/ml for Fraction I and 15-25 µg/ml for Fraction II. Protein concentration in the reaction mixtures was (4-6 mg/ml).

fraction supported the replication of endogenous RSF1010 DNA and that the incorporation could be increased more than two-fold by addition of exogenous RSF1010 DNA (Table 2). A small but significant amount of DNA synthesis also occurred in extracts from P.aeruginosa PA01162 (RSF1010). On the other hand, extracts from plasmid-free E.coli or P.aeruginosa cells could not support the replication of exogenous RSF1010 DNA. In order to ascertain that the observed incorporation did not merely result from the elongation of replicative intermediates already preformed in vivo, plasmid DNA synthesis was assayed after removal of endogenous nucleic acids by precipitation with streptomycin sulfate (see Experimental). The protein fraction obtained (designated Fraction II) was devoid of endogenous template but nevertheless fully retained the replication capacity of the crude extract for exogenous RSF1010 DNA (see Table 2). Optimal DNA synthesis occurred at a protein concentration of 4-6 mg per ml, higher concentrations of Fraction II being inhibitory. These results clearly indicate that the soluble enzyme systems both from E.coli and P.aeruginosa are capable of initiating RSF1010 replication in vitro.

Product analysis by alkaline gradient centrifugation (Fig.1)

Fig. 1. Alkaline CsCl gradient centrifugation of reaction products. A 150 μ l standard incubation mixture containing 5 mg/ml protein of Fraction II of C600(RSF1010) was supplemented with 20 μ g/ml of RSF1010 DNA and incubated for 60 min at 30°C. Incubation was stopped by the addition of EDTA to final concentration of 50 mM and the total reaction mixture was subjected to analysis by alkaline gradient centrifugation as described in the experimental section. The ordinate values indicate incorporation of ³H-dTMP into acid-insoluble material. Sedimentation is from right to left. The arrows indicate the positions of fully replicated closed-circular (CC) and open-circular (OC) monomeric plasmid DNA.

showed that most of the RSF1010 DNA synthesized in the *E.coli* system consisted of fully replicated closed-circular (CC) and open-circular (OC) monomeric plasmid DNA. Only a minor portion of the radioactive label was found in DNA fragments of less than unit length presumably derived from replicative intermediates. Analogous results were obtained for the *P.aeruginosa* system (data not shown).

The replication specificity of Fraction II prepared from RSF 1010-carrying as well as plasmid-free *E.coli* and *P.aeruginosa* cells was examined with various double-stranded and single-stranded DNA templates. The results are presented in Table 3. As in the case of crude extracts, Fraction II from plasmid-free bacteria was totally inactive for RSF1010 replication. This deficiency indicates that *in vitro* replication of RSF1010 requires at least one plasmid-encoded replication function, which is not expressed

Table 3. Template specificity of DNA synthesis

DNA added	³ H-dTMP incorporated (pmol)			
	Source of Fraction II			
	C600	C600(RSF1010)	PAO1162	PAO1162(RSF1010)
None	0.8	20.3	0.8	4.8
RSF1010	0.7	56.0	0.9	12.3
pKN177	0.9	0.9	0.9	0.9
ColE1	147.3	158.1	0.8	0.9
M13	189.2	200.3	39.0	26.3
φX174	35.6	32.5	49.5	58.7

DNA synthesis was carried out for 60 min at 30°C in standard reaction mixtures (25 µl). DNA was added to give final concentrations of 15 - 25 µg/ml (RSF1010, pKN177 and ColE1 DNAs), or 25 - 30 µg/ml (single-stranded DNA of phages M13 and φX174). Protein concentration in the reaction mixtures was 4 - 6 mg/ml.

in extracts from plasmid-free cells. The replication function appears to be specific for RSF1010 since it could not replace the repA gene product of the mini-R1 plasmid pKN177 (10) and had no stimulatory effect on ColE1 replication. It should be noted that the inability of ColE1 to replicate in the P.aeruginosa system is not simply due to a restriction barrier since the extract was prepared from a mutant defective in restriction and no degradation of the template DNA was observed. Fraction II from E.coli as well as P.aeruginosa was capable of converting the single-stranded viral DNA of the E.coli phages M13 and φX174 to the respective double-stranded replicative form (RF) DNA. M13 RF DNA synthesis (but not that of φX174) was sensitive to rifampicin both with extracts prepared from plasmid-free and RSF1010-carrying cells (data not shown).

Comparison of RSF1010 replication in E.coli and P.aeruginosa extracts

The time course of RSF1010 DNA synthesis carried out by Fraction II from E.coli and P.aeruginosa respectively is shown in Fig.2. After an initial lag period both systems supported plasmid replication at nearly linear rate for about 30 min at 30°C.

During further incubation the incorporation leveled off and reached a plateau after 60 min. Compared to the *E.coli* system both the rate and total amount of DNA synthesis carried out by *P.aeruginosa* extracts were markedly lower corresponding to about 20% of that obtained with *E.coli* extracts. It was noticed that the *P.aeruginosa* extracts but not the *E.coli* ones, were inactivated after storage for a few hours at 0°C.

The requirements of both systems were in general quite similar (Table 4). DNA synthesis was strictly dependent on Mg^{2+} ions with a rather sharp concentration optimum around 7.5 mM. ATP was required at an initial concentration of 2 mM, that had to be maintained by an ATP-regenerating system. Addition of all four ribonucleoside triphosphates further increased the activity of both systems. A stimulatory effect was also noted upon adding cAMP. The *E.coli* system was only partially dependent on all four deoxy-

Fig. 2. Kinetics of RSF1010 DNA synthesis. 25 μ l standard reaction mixtures containing 5 mg/ml proteins of Fraction II plus 20 μ g/ml RSF1010 DNA were incubated at 30°C; at the times indicated, the reactions were stopped and the acid-insoluble radioactivity assayed. Extracts were prepared from C600(RSF1010) (o—o) or PA01162(RSF1010) (●—●).

Table 4. Requirements of RSF1010 DNA synthesis

Omissions	³ H-dTMP incorporation (%)	
	Source of Fraction II	
	C600 (RSF1010)	PA01162 (RSF1010)
None	100	100
MgCl ₂	2.1	7.1
ATP	9.9	11.5
Creatine phosphate	11.8	10.6
ATP, creatine phosphate	2.3	7.1
CTP, GTP, UTP	74.8	69.9
cAMP	56.7	60.2
dATP, dCTP, dGTP	53.7	8.0
Polyethylene glycol	38.7	41.2

DNA synthesis was carried out for 60 min at 30°C in standard reaction mixtures (25 µl) with the addition of RSF1010 DNA to final concentration of 15-25 µg/ml. 100% synthesis corresponds to a dTMP incorporation of 53 pmoles (C600(RSF1010) fraction) or 12.1 pmoles (PA01162(RSF1010) fraction). Protein concentration in the reaction mixtures was 4-6 mg/ml.

ribonucleoside triphosphates presumably due to efficient enzymatic ribonucleotide reduction in the presence of dithiothreitol. Including polyethylene glycol (2.5%) in the reaction mixture stimulated the incorporation approximately two-fold. Stimulation of DNA synthesis by polyethylene glycol has been observed previously for mini-R1 (14) and oriC plasmid replication (15).

The E.coli and the P.aeruginosa system differed substantially in their salt sensitivity (Fig.3). RSF1010 replication in E.coli extracts was stimulated by increasing the KCl concentration up to 75 mM. In contrast, plasmid DNA synthesis in P.aeruginosa extracts was optimal around 20 mM and inhibited by 50% at 75 mM KCl. Standard reaction mixtures therefore employed different salt concentrations for E.coli and P.aeruginosa extracts.

The effect of template DNA concentration on RSF1010 replication is shown in Fig.4. Optimal incorporation occurred in both systems at a DNA concentration of 20 µg/ml. Higher concentrations of RSF1010 DNA were inhibitory presumably due to the dissipation

Fig. 3. Effect of salt concentration on RSF1010 synthesis. Standard incubation mixtures (25 μ l) containing 5 mg/ml protein of Fraction II plus 20 μ g/ml of RSF1010 DNA, were incubated for 60 min at 30°C with various concentrations of KCl. Extracts were prepared from C600(RSF1010) (o—o) or PA01162(RSF1010) (●—●).

of replication proteins bound to the DNA. This effect appears to be replicon-specific, since Cole1 replication in the *E.coli* system was hardly affected by increasing the DNA concentration above the optimal value.

Effect of inhibitors

The effect of various inhibitors on RSF1010 replication is summarized in Table 5. Arabinosylcytosine triphosphate completely blocked DNA synthesis at concentrations consistent with those that preferentially inhibit DNA polymerase III (16). Novobiocin and oxolinic acid, which interfere with plasmid replication by blocking DNA gyrase, also reduced the incorporation to background levels. On the other hand, antibiotics that inhibit protein biosynthesis had little or no effect on RSF1010 replication. This indicates that the replication of RSF1010, unlike that of R1 (10), is not coupled to the concomitant synthesis of an essential replication protein. Furthermore, rifampicin, a specific inhibitor

Fig. 4. Effect of DNA concentration on RSF1010 and ColE1 DNA synthesis. Standard incubation mixtures (25 μ l) containing 5 mg/ml protein of Fraction II of C600(RSF1010) were incubated for 60 min at 30°C with various concentrations of ColE1 (Δ — Δ) or RSF1010 DNA (o—o). Fraction II of PAO1162(RSF1010) was tested in a similar way with various concentrations of RSF1010 DNA (●—●).

of bacterial RNA polymerases, had no inhibitory effect on RSF1010 replication *in vitro*. On the contrary, plasmid DNA synthesis in the *P.aeruginosa* system could be stimulated occasionally up to two-fold upon addition of the drug.

The failure of rifampicin to inhibit RSF1010 replication is surprising, because an obligatory function of RNA polymerase has been demonstrated for ColE1 replication (17) and implicated in several other plasmid replication systems (10,15,18,19). We therefore compared the sensitivity of ColE1 and RSF1010 replication to rifampicin and streptolydigin, both of which inhibit RNA polymerase by binding to the β subunit. It can be seen in Fig.5A that low concentrations of rifampicin (0.1 μ g/ml) caused a drastic inhibition of ColE1 replication in extracts from RSF1010-carrying *E.coli* cells. On the other hand, even 100-fold higher concentra-

Table 5. Effect of antibiotics on RSF1010 DNA synthesis

Addition	³ H-dTMP incorporation (%)	
	Source of Fraction II	
	C600 (RSF1010)	PAO1162 (RSF1010)
None	100	100
ara CTP (100 µM)	7.3	5.0
Novobiocin (25 µg/ml)	2.5	6.6
Oxolinic acid (50 µg/ml)	18.3	15.6
Chloramphenicol (100 µg/ml)	87.2	84.4
Streptomycin (100 µg/ml)	89.3	91.0
Rifampicin (25 µg/ml)	92.3	149.6

DNA synthesis was carried out for 60 min at 30°C in standard reaction mixtures (25 µl) with the addition of RSF1010 DNA to final concentration of 15-25 µg/ml. 100% synthesis corresponds to a dTMP incorporation of 50 pmoles (C600(RSF1010) fraction) or 12.5 pmoles (PAO1162(RSF1010) fraction). Protein concentration in the reaction mixtures was 4-6 mg/ml.

tions of rifampicin had no inhibitory effect on RSF1010 replication. Streptolydigin interfered with the replication of both plasmids, although to a different extent (Fig.5B). ColE1 replication was inhibited at drug concentrations consistent with those that block transcription (20), whereas RSF1010 replication was clearly less sensitive.

The partial inhibition of RSF1010 replication by streptolydigin could point to the involvement of an altered form of RNA polymerase functional in RSF1010 replication (but not in ColE1 replication), which is less susceptible to inhibitors interacting with the β subunit. In order to evaluate this possibility, we tested the effect of antisera against RNA polymerase holoenzyme on plasmid DNA replication using the corresponding preimmune serum as control. As shown in Fig.6A, ColE1 replication was specifically blocked by small quantities of RNA polymerase antiserum. On the other hand, the effect of these antibodies on RSF1010 replication was indistinguishable from that of a preimmune serum (Fig.6B). Furthermore, we were unable to detect any RSF1010-induced alteration of RNA polymerase subunits by an immunological

Fig. 5. Effects of rifampicin and streptolydigin on ColE1 and RSF1010 synthesis. Standard incubation mixtures containing 5 mg/ml protein of Fraction II of C600 (RSF1010) were incubated for 60 min at 30°C in the presence of 20 μg/ml of RSF1010 (o—o) or ColE1 DNA (▲—▲). The effects of various concentrations of rifampicin or streptolydigin on RSF1010 or ColE1 DNA synthesis are shown in Fig.5A and 5B respectively. 100% synthesis correspond to 56 pmoles and 46 pmoles of dTMP incorporated in the presence of RSF1010 or ColE1 DNA respectively.

assay done basically as described by Towbin et al. (21) (data not shown). The interference of streptolydigin with RSF1010 replication must therefore be attributed to some secondary effect of this drug.

DISCUSSION

The results presented in this paper show that soluble enzyme systems prepared from *E.coli* and *P.aeruginosa* cells carrying the broad host range plasmid RSF1010 can support complete rounds of replication of exogenous RSF1010 DNA. The lower replicative capacity of the *P.aeruginosa* system as compared to that from *E.coli* apparently results from low stability and increased salt sensitivity of the *P.aeruginosa* replication enzymes. Otherwise the requirements of the two systems as well as their sensitivities to antibiotics are nearly identical suggesting that RSF1010 DNA synthesis in *E.coli* and *P.aeruginosa* occurred by basically the same mechanism. Consistent with this assumption is the finding that

Fig. 6. Effect of antibodies against *E. coli* RNA polymerase holoenzyme on RSF1010 and ColE1 DNA synthesis. Standard incubation mixtures containing 5 mg/ml protein of Fraction II of C600 (RSF 1010) were incubated for 60 min at 30°C in the presence of 20 µg/ml of ColE1 DNA (Fig. 6A) or RSF1010 (Fig. 6B). The effects of antibodies against *E. coli* RNA polymerase holoenzyme on ColE1 (▲—▲) or RSF1010 DNA synthesis (●—●) are compared. Open symbols (Δ—Δ, o—o) represent synthesis of ColE1 and RSF1010 DNA respectively in the presence of different amounts of preimmune control serum. 100% synthesis correspond to 60 pmoles and 52 pmoles of dTMP incorporated in the presence of RSF1010 or ColE1 DNA respectively.

RSF1010 replication in *E. coli* as well as *P. aeruginosa* involves θ -shaped replicative intermediates and starts from the same primary origin both *in vivo* and *in vitro* (unpublished results).

In contrast to RSF1010, ColE1 DNA is replicated only by the *E. coli* system. The deficiency of *P. aeruginosa* extracts to support ColE1 replication is in line with the inability of ColE1 to establish itself as a replicon in this organism either by mobilization or by transformation. The molecular basis of this replication barrier is not understood. Since the *P. aeruginosa* system supports the conversion of single-stranded ϕ X174 DNA to duplex DNA, one should expect that it is also capable of supporting ColE1 lagging strand synthesis (22). Thus the deficiency in ColE1 replication results most likely from a defect in leading strand synthesis. Preliminary results indicate that ColE1 replication in *P. aeruginosa* extracts can be partially restored by combined addi-

tion of *E.coli* DNA polymerase I and *E.coli* DNA gyrase, both of which have been implicated in the initiation of ColE1 leading strand synthesis (23). Conceivably, differences in the specificities of these enzymes among Gram-negative organisms could limit the host range of ColE1-type plasmids to enterobacteria.

The failure of extracts from plasmid-free bacteria to replicate RSF1010 DNA correlates well with in vivo data showing that RSF1010 replication requires at least one trans-acting plasmid-encoded factor (6). In this respect RSF1010 differs from ColE1-type plasmids and resembles R1 and R6K, which also require a plasmid-encoded protein for replication (10,24). Partial purification of the RSF1010-encoded replication factor by Heparin-Sepharose chromatography indicates that more than one protein component is involved (unpublished results). These proteins function presumably in the initiation stage of RSF1010 replication after binding to some recognition sequence(s) specific for RSF 1010 DNA.

The independence of RSF1010 replication from transcription catalyzed by host RNA polymerase provides a clue to the possible role of the RSF1010-encoded replication proteins in the initiation of plasmid DNA synthesis. Transcription of the origin region of various replicons is thought to be required for the formation of a primer RNA and/or for opening up the duplex DNA structure (transcriptional activation). Both functions could conceivably be performed by the RSF1010-encoded replication proteins making RSF 1010 replication less dependent on specific interactions with host enzymes. It is pertinent to note here that RSF1010 and the closely related plasmid R300B do not code for a primase supporting initiation of complementary strand synthesis of phage M13 single-stranded DNA in the presence of rifampicin (25). However, the replication proteins of RSF1010 might prime replication of the double-stranded RSF1010 DNA. It is tempting to speculate that a host RNA polymerase transcription-independent initiation mechanism might be a general property of broad host range plasmids.

ACKNOWLEDGEMENTS

We thank Dr. H.Schuster for support and encouragement, Dr. M. Bagdasarian for strains and DNA, Dr. E.Lanka for advice with

immunological tests, and Dr. G.Hillenbrand for critical reading of the manuscript. R.D. is also grateful to the Comision Asesoradora de Investigacion Cientifica y Tecnica (Spain) and to EMBO for additional support.

*Present address: Centro de Investigaciones Biologicas (C.S.I.C.), Instituto de Biologia Celular, C/Velazquez 144, Madrid-6, Spain.

*Present address: Lehrstuhl für Mikrobiologie, Technische Universität München, Arcisstrasse 21, D-8000 München 2, FRG.

REFERENCES

1. Guerry, P., Van Embden, J., and Falkow, S. (1974) *J. Bacteriol.* 117, 619-630
2. Grinter, N.J., and Barth, P.I. (1976) *J. Bacteriol.* 128, 394-400
3. Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F.C.H., Bagdasarian, M.M., Frey, J., and Timmis, K.N. (1981) *Gene* 16, 237-247
4. De Graaff, J., Crossa, J.H., Heffron, F., and Falkow, S. (1978) *J. Bacteriol.* 134, 1117-1122
5. Grindley, N.D.F., and Kelley, W. (1976) *Mol. Gen. Genet.* 143, 311-318
6. Bagdasarian, M., Bagdasarian, M.M., Lurz, R., Nordheim, A., Frey, J., and Timmis, K.N. (1982) in *Bacterial Drug Resistance* S.Mitsubishi (Ed.) Japan Sci. Soc. Press, Tokyo
7. Guerry, P., LeBlanc, D.J., and Falkow, S. (1973) *J. Bacteriol.* 116, 1064-1066
8. Humphreys, G.O., Willshaw, G.A., and Anderson, E.S. (1975) *Biochim. Biophys. Acta* 383, 457-463
9. Bachmann, B.J. (1972) *Bacteriol. Reviews* 36, 525-557
10. Diaz, R., Nordström, K., and Staudenbauer, W.L. (1981) *Nature* 289, 326-328
11. Staudenbauer, W.L. (1976) *Mol. Gen. Genet.* 145, 273-280
12. Conrad, S.E., and Campbell, J. (1979) *Nucleic Acids Res.* 6, 3289-3303
13. Schuster, H., Mikolajczyk, M., Rohrschneider, J., and Geschke, B. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3907-3911
14. Diaz, R., and Staudenbauer, W.L. (1982) *J. Bacteriol.*, in press
15. Fuller, R.S., Kaguni, J.M., and Kornberg, A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 7370-7374
16. Staudenbauer, W.L. (1976) *Mol. Gen. Genet.* 149, 151-158
17. Itoh, T., and Tomizawa, J.I. (1980) *Proc. Natl. Acad. Sci. USA* 77, 2450-2454
18. Inuzuka, M., and Helinski, D.R. (1978) *Biochemistry* 17, 2567-2573
19. Anderl, A., and Klein, A. (1982) *Nucleic Acids Res.* 10, 1733-1740
20. Cassani, G., Burgess, R.R., Goodman, H.M., and Gold, L. (1971) *Nature New Biol.* 230, 197-200
21. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4350-4354
22. Böldicke, T.W., Hillenbrand, G., Lanka, E. and Staudenbauer, W.L.

- (1981) Nucleic Acids Res. 9, 5215-5231
23. Hillenbrand, G., and Staudenbauer, W.L. (1982) Nucleic Acids Res. 10, 833-835
24. Inuzuka, M., and Helinski, D.R. (1978) Proc. Natl. Acad. Sci. USA 75, 5381-5385
25. Lanka, E., and Barth, P.T. (1981) J. Bacteriol. 148, 769-781