APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1987, p. 105–109
0099-2240/87/010105-05$02.00/0

Vol. 53, No. 1

Measuring Genetic Stability in Bacteria of Potential Use in Genetic Engineering

MICHAEL V. WALTER, ARLENE PORTEOUS, AND RAMON J. SEIDLER*

Corvallis Environmental Research Laboratory, Biotechnology/Microbial Ecology Program, U.S. Environmental Protection Agency, Corvallis, Oregon 97333

Received 13 June 1986/Accepted 7 October 1986

Four commonly used conjugation techniques, colony cross streak (CCS), broth mating (BM), combined spread plate (CSP), and membrane filtration (MF), were compared with each other regarding reliability, sensitivity, and complexity in evaluating the transfer of conjugative plasmids. Five plasmids representing several incompatibility groups plus a variety of laboratory and environmental isolates were used as mating pairs. The suitability of each method was evaluated for use in a routine assessment of the genetic stability of genetically engineered microorganisms. By the CSP and MF techniques with laboratory strains such as *Escherichia coli* and *Pseudomonas* species as recipients, transconjugants were usually produced in 100% of the mating trials. However, when environmental strains isolated from plants and soil were used as recipients, transconjugants were detected in 100% of some crosses and in as little as 30% in other crosses depending on the plasmid and recipient used. In general, differences in the percentage of successful matings between the CSP and MF techniques compared with the BM and CCS techniques were not statistically significant at the $P \leq 0.05$ level. Occasionally, certain mating pairs consistently produced transconjugants by CCS or BM but not by CSP or MF. Since any single conjugation mating technique is not completely reliable in detecting transconjugants, we have developed a combined mating technique which integrates the CCS, CSP, BM, and MF methods as a single procedure to assess the mobility of plasmid DNA of genetically engineered microorganisms.

Genetic engineering has led to the modification of microorganisms capable of performing functions such as pollution abatement, pest control, crop protection from frost, extraction and concentration of metals from ore, and enhanced recovery of oil (13). The use of such organisms requires their release into the environment. The possible detrimental consequences of released genetically engineered microorganisms (GEMs) has caused concern regarding what impact their long-term survival would have on the environment (15). One concern about the release of GEMs is their genetic stability, that is, the potential for transfer of DNA from GEMs to indigenous microflora (14). The hazards of interspecies movement of DNA may be greater if engineered DNA in a GEM is transferred into organisms which colonize the natural environment. Following transfer into indigenous species, potential risks would arise from increases in gene expression or changes in substrate specificity of gene products. There is also concern regarding the possible establishment of engineered genes in new species causing unintended exposure of species and ecosystems to novel products of genetic engineering.

GEMs may be evaluated for plasmid mobility by conducting mating experiments with laboratory strains as recipients and indigenous microflora recently isolated from environments where the GEM would be released. It would be useful to have a series of techniques to quantitatively evaluate plasmid mobility under highly controlled laboratory conditions, to evaluate whether a GEM has the ability to undergo conjugation. Thus, the present study was designed to (i) compare four established laboratory techniques regarding sensitivity and consistency for producing transconjugants; (ii) evaluate these laboratory techniques as to expense and technical difficulty; and (iii) compare results obtained with laboratory strains with those obtained with environmental isolates as recipients.

* Corresponding author.

MATERIALS AND METHODS

Media and cultures. Bacteria, plasmids, and genotypes are listed in Table 1. Working cultures were maintained on Luria-Bertani (LB) agar (11) containing antibiotics to which they were resistant. Stock cultures were stored at −80°C in 15% glycerol. Recipient laboratory strains included *Escherichia coli* HB101 for crosses involving plasmids Sa, RK2, and pRK2013, *Pseudomonas cepacia* PC012 for crosses involving plasmid R388::Tn*1721*, and *P. aureofaciens* for crosses involving plasmid R40a. A total of 22 environmental isolates were obtained from soil and plants at the greenhouse of the Environmental Protection Agency Corvallis Environmental Research Laboratory. These strains were gram-negative aerobic heterotrophs.

Medium selection of transconjugants for crosses involving plasmid R388::Tn*1721* was M-9 glucose mineral salts medium (11) containing trimethoprim, 50 μg/ml, and tetracycline, 100 μg/ml. *P. cepacia* PC012(pR388::Tn*1721*) will not grow on M-9 glucose mineral salts medium. For crosses involving the other four plasmids, LB agar containing the following antibiotics (Sigma Chemical Co.) was used: pSa—kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), streptomycin (25 μg/ml), ampicillin (200 μg/ml); pRK2—streptomycin (25 μg/ml), tetracycline (15 μg/ml); pRK2013—kanamycin (50 μg/ml), streptomycin (25 μg/ml); pR40a—kanamycin (50 μg/ml), chloramphenicol (25 μg/ml). Inspection of phenotypes in Table 1 provides information on how counterselection of mating participants was achieved. Plates were incubated at 30 ± 2°C and examined after 24 and 48 h.

Mating procedures. In the colony cross streak technique (CCS), separate cultures of donor and recipient were incubated at 30 ± 2°C on agar containing antibiotics to which they were resistant. A selective agar plate was then

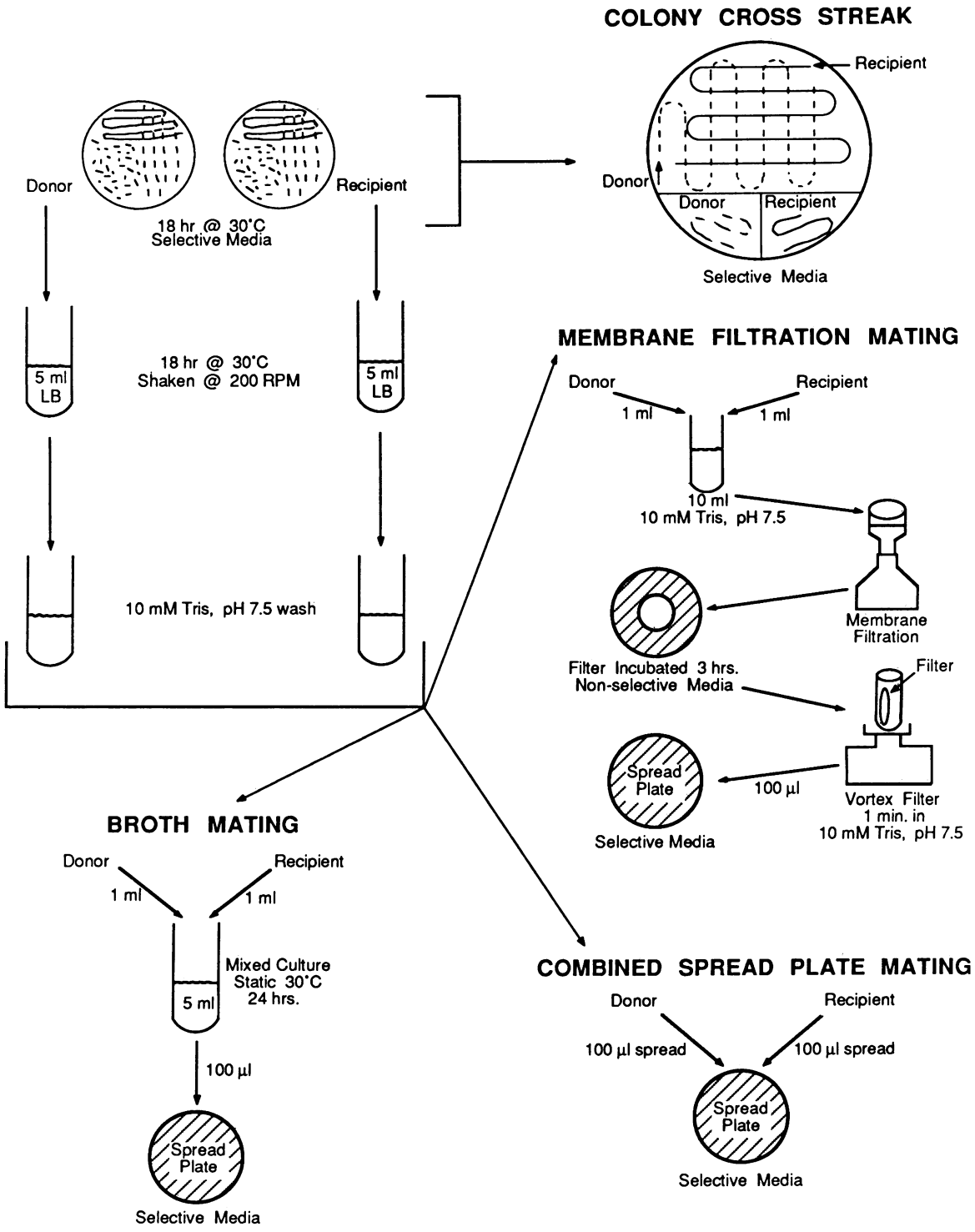

FIG. 1. Combined mating technique integrating the four procedures into a combined operation for assessing conjugal transfer of DNA among test donor and recipient strains. A single source of washed cell suspensions is utilized in executing the BM, CSP, and MF techniques.

streaked, first with the recipient and then with the donor, as illustrated in the upper right-hand corner of Fig. 1. The donor only was streaked on one sector and the recipient only was streaked on the other, to serve as negative controls.

Broth matings (BM) were performed by using a modification of the technique described by Hinshaw et al. (10). Donors and recipients were incubated for 24 h at 30 ± 2°C with shaking in 5 ml of LB broth containing antibiotics to which they were resistant. Both were washed by centrifugation through sterile 10 mM Tris buffer (pH 7.5) and sus-

TABLE 1. Microorganisms, plasmids, and relevant genotypes utilized in this study

Species	Source	Plasmid	Incompatability group	Genotype[a]
E. coli K-12	J. Kaper, University of Maryland	Sa	W	Km Cm Sm
P. cepacia PCO12	R. Olsen, University of Michigan	R388::Tn*1721*	W	Tp Tc Pro
E. coli C600	ATCC 37125	RK2	P	Ap Km Tc
E. coli K-12	M. Nishibuchi, University of Maryland	pRK2013	P	Km
E. coli K-12	R. Grant, Toronto, Ont., Canada (16)	R40a	C	Km Sm

[a] Km, Cm, Sm, Tp, Tc, and Ap designate resistance to kanamycin, chloramphenicol, streptomycin, trimethoprim, tetracycline, and ampicillin, respectively. Pro, Proline auxotroph.

pended in 5 ml of the same buffer. A 1-ml aliquot of both donor and recipient was inoculated into 5 ml of LB broth, incubated statically at 30 ± 2°C for 24 h, serially diluted in Tris, and spread plated on selective agar plates (Fig. 1).

The combined spread plate (CSP) technique was a modified version described by Tardif and Grant (16). Donors and recipients were incubated under conditions described for BM. A 1.5-ml aliquot of each culture was microfuged, washed in Tris buffer, and suspended in 1.5 ml of the same buffer. A 100-μl aliquot of the recipient was spread plated onto a selective agar plate and allowed to dry for 5 min, and a 100-μl aliquot of the donor was then spread plated over the recipient.

The membrane filtration (MF) technique was modified from the procedure described by Chatterjee and Starr (4). Donor and recipient cultures were incubated and washed as described for BM. Aliquots, 1 ml, of donor and recipient suspensions were added to 10 ml of Tris buffer (pH 7.5) in a screw-cap test tube (20 by 150 mm) and then filtered through a 0.45-μm nitrocellulose filter (Millipore Corp.). Filters were transferred onto LB agar plates and incubated for 3 h at 30 ± 2°C, removed from plates, and vortexed for 1 min in 1 ml of Tris buffer (pH 7.5). Serial dilutions were then spread plated onto selective agar plates and incubated. Mating rates were expressed as transconjugants produced per initial donor cell at the beginning of the incubation period. Percentages of successful matings among the four mating techniques were evaluated by chi-square statistical analyses.

Plasmid verification. Presumptive transconjugants were verified in 75% of all crosses by demonstrating the physical presence of the plasmid in a few colonies from each mating. Plasmids were extracted by a modified alkaline lysis technique described by Portnoy and White (6). The method was modified by adjusting the pH of the lysis buffer to 12.6 to optimize the lysis of various organisms being studied. Plasmids were visualized by using standard techniques (1) following horizontal electrophoresis in 0.7% agarose gels and staining with 0.5 μg of ethidium bromide per ml. Representative presumptive transconjugants were also confirmed by colony hybridization for the appropriate labeled plasmids, using conventional methodologies (11). Filters were hybridized at 42 ± 2°C for 15 h in prehybridization buffer containing p32 labeled with nick-translated plasmid DNA (11). Following hybridization, filters were washed with three successive rinses of 0.1× standard saline citrate buffer (1× = 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C.

RESULTS

The results of the four techniques used to demonstrate conjugation are compared in Table 2. Each technique was capable of demonstrating transconjugant formation. The MF method produced transconjugants most consistently with the exception of matings involving laboratory strains as recipients and a donor carrying plasmid R40a. The effectiveness of the other three methods varied depending upon the choice of plasmid and the recipient strain used. Laboratory strains were generally more effective as recipients than environmental isolates were. However, plasmid R40a transferred more consistently into environmental isolates than into laboratory strains when either BM or MF was used. Statistically significant differences in the percentage of successful matings were generally not observed at the $P \leq 0.05$ level. Exceptions to this, however, were noted in comparisons of MF and CSP for plasmid R388::Tn*1721* and MF and BM for crosses involving pRK2013. In both cases these differences were only noted when environmental isolates were used as recipients. There was also a significant difference between MF and CSP with plasmid R40a when laboratory strains served as recipients.

The mean percent success rate for each mating technique generally illustrates the superior nature of *E. coli* and *P. cepacia* laboratory recipients. The differences between recipient laboratory strains and environmental strains were not statistically significant at $P \leq 0.05$, although laboratory strains demonstrated a higher success rate than environmental isolates in three of the four methods tested.

The frequencies of matings ± the standard error of the mean expressed as transconjugants per donor observed for each mating technique are listed in Table 3. Frequencies of DNA transfer varied 1,000-fold and more among the various techniques, even within the same donor plasmid group. For the most part, the highest rates of transconjugation for all techniques were observed with laboratory strains as recipients except when plasmid R40a was transferred. The highest frequencies of transconjugation were observed with the MF

TABLE 2. Percentage of successful matings by the four conjugal mating techniques

Plasmid	Recipient culture[a]	% Successful matings by:			
		CCS	BM	CSP	MF
Sa	LS	85	90	100	100
	EI	62	80	100	100
R388::Tn*1721*	LS	88	90	100	100
	EI	63	91	38[b]	80[b]
RK2	LS	83	46	61	100
	EI	78	37	32	92
pRK2013	LS	100	100	100	100
	EI	58	20[b]	33	100[b]
R40a	LS	60	30	85[b]	11[b]
	EI	50	84	50	100
Mean	LS	83	70	89	83
	EI	62	62	50	94

[a] LS, Laboratory strain; EI, environmental isolate.

[b] Significant difference at $P \leq 0.05$ in percentage of successful matings with different mating techniques. Each value represents the average of at least 10 experimental replications.

TABLE 3. Comparision of transconjugation mating frequencies by mating technique and plasmid type

Plasmid	Recipient strain[a]	Mating frequency (transconjugants per donor ± SE)[b] by:		
		BM	CSP	MF
Sa	LS	$7.5 \times 10^{-6} \pm 7.2 \times 10^{-7}$	$4.6 \times 10^{-3} \pm 1.0 \times 10^{-3}$	$6.1 \times 10^{-3} \pm 2.5 \times 10^{-3}$
	EI	$6.5 \times 10^{-8} \pm 2.7 \times 10^{-8}$	$2.6 \times 10^{-7} \pm 3.5 \times 10^{-8}$	$1.8 \times 10^{-5} \pm 4.2 \times 10^{-6}$
R388::Tn*1721*	LS	$3.2 \times 10^{-5} \pm 3.7 \times 10^{-6}$	$3.5 \times 10^{-4} \pm 9.0 \times 10^{-5}$	$1.2 \times 10^{-4} \pm 3.4 \times 10^{-5}$
	EI	$4.2 \times 10^{-8} \pm 1.0 \times 10^{-8}$	$2.7 \times 10^{-7} \pm 1.1 \times 10^{-7}$	$3.2 \times 10^{-4} \pm 1.6 \times 10^{-4}$
RK2	LS	$8.2 \times 10^{-8} \pm 5.2 \times 10^{-8}$	$1.9 \times 10^{-8} \pm 5.4 \times 10^{-9}$	$1.5 \times 10^{-7} \pm 2.7 \times 10^{-8}$
	EI	$3.2 \times 10^{-9} \pm 1.0 \times 10^{-9}$	$1.4 \times 10^{-8} \pm 8.1 \times 10^{-9}$	$6.1 \times 10^{-8} \pm 4.5 \times 10^{-8}$
pRK2013	LS	$1.7 \times 10^{-5} \pm 1.8 \times 10^{-6}$	$1.7 \times 10^{-3} \pm 2.8 \times 10^{-4}$	$3.3 \times 10^{-1} \pm 3.6 \times 10^{-2}$
	EI	$1.3 \times 10^{-9} \pm 8.0 \times 10^{-10}$	$3.5 \times 10^{-8} \pm 1.8 \times 10^{-8}$	$2.8 \times 10^{-8} \pm 4.9 \times 10^{-9}$
R40a	LS	$2.5 \times 10^{-8} \pm 2.1 \times 10^{-8}$	$8.6 \times 10^{-5} \pm 4.4 \times 10^{-5}$	$1.4 \times 10^{-9} \pm 1.4 \times 10^{-9}$
	EI	$2.9 \times 10^{-5} \pm 3.6 \times 10^{-5}$	$3.2 \times 10^{-8} \pm 9.1 \times 10^{-9}$	$1.9 \times 10^{-4} \pm 3.1 \times 10^{-5}$

[a] LS, Laboratory strain; EI, environmental isolate.
[b] Each value represents the average of at least 10 experimental replications.

method. In about 50% of the 30 data sets, standard errors were 10% of the mean.

Colonies representing presumptive transconjugants were removed from selective agar plates for further analysis. Either virtually all presumptive transconjugants exhibited an intense reaction with the p32 probe in colony hybridization or the appropriate acquired plasmid DNA bands were visible through gel electrophoresis.

DISCUSSION

Bacterial transfer of DNA, especially involving antibiotic resistance genes, has been the subject of much research and several reviews (2, 3, 8, 9). As a result, various methods have been developed to detect and evaluate in vitro conjugation events. The conjugal mating techniques utilized in this study were selected because they are representative of the diverse methods found in the literature. This study attempts to compare these methods regarding their sensitivity and reliability in side-by-side tests and to evaluate their suitability as preliminary screening techniques in testing genetic stability of GEMs.

Routine screening of donor and recipient microorganisms requires a method which is sensitive and provides reproducible results, but is also cost and time effective. The CCS technique is quick, simple, and inexpensive to perform. As a result, a large number of samples can be tested and replicated. However, because this technique utilizes donor and recipient cultures cross streaked onto a solid medium, it is not quantitative.

The BM technique is as reliable as the CCS but has the advantage of being quantitative. The results obtained with this method were in general agreement with results reported by others for the various plasmids used. Watanabe and Fukasawa (17) reported conjugal rates ranging from 10^{-3} to 10^{-8} transconjugants per donor with plasmid Sa, while our results with different cultures provided 10^{-6} to 10^{-8} transconjugants per donor. Datta and Hedges (7) reported conjugal rates between *E. coli* strains of 10^{-3} transconjugants per donor with plasmid R388. Our results wth *P. cepacia* ranged from 5.1×10^{-3} to 5.5×10^{-5} transconjugants per donor. Meyer et al. (12) reported that plasmid RK2 transferred at a rate ranging from 2.6×10^{-6} to 8.0×10^{-6} with various strains of *E. coli*; these rates are similar to our observed rate of 5.0×10^{-7} transconjugants per donor. The conjugal rates with BM were 10- to 100-fold less than those observed with the CSP and MF techniques.

The CSP technique is also quantitative and simple to perform, and many donor and recipient cultures can be screened rapidly. Tardif and Grant (16) demonstrated transfer rates of 9.0×10^{-7} transconjugants per donor by the CSP technique with plasmid R40a and a *P. aeruginosa* recipient. We observed a transfer rate of 4.4×10^{-5} transconjugants per donor when a *P. aureofaciens* strain was used as a recipient. The conjugal rates observed with CSP were generally slightly higher than those with BM and comparable to rates obtained with MF. The CSP technique was very reliable when laboratory cultures were used as recipients but gave variable results when environmental isolates were used.

MF was the most reliable of the four methods compared. Transconjugants were obtained in all crosses with all plasmids except R40a when laboratory strain recipient cultures were used. Coplin (5) reported conjugal rates with the MF technique, using various IncF and IncP group plasmids, of between 9.0×10^{-2} and 8.0×10^{-6} transconjugants per donor with *Erwinia stewartii* strains as recipients. We observed conjugal rates of between 2.0×10^{-1} and 3.0×10^{-7} transconjugants per donor for various plasmids when we used laboratory strains as recipients. While it is very reliable, the MF technique is more time-consuming and requires additional glassware and laboratory equipment. It would not lend itself well to use in routine screening of large numbers of donors and recipients or in the replication of measurements.

Standard errors varied over a wide range with the various data sets. There may be several reasons for this. The data used in the calculations include matings which did not produce transconjugants. Furthermore, variations in transconjugation frequencies will occur with the timing of initial transconjugant formation during the mating period. Early matings will produce more transconjugants and thus larger transconjugation frequencies.

No single mating technique was capable of demonstrating superior results with all plasmids when laboratory and environmental strains were used as recipients. Therefore, an integrated procedure was designed to incorporate the CCS, BM, CSP, and MF techniques in a single process for evaluating conjugal transfer of DNA (Fig. 1). This procedure is designated the combined mating technique. One source of

washed cell suspensions can be used to execute the quantitative procedures (BM, MF, and CSP). The combined mating technique provides both liquid and solid substrates for matings. This increases the chances of detecting transconjugants since certain plasmids transfer preferentially when mating pairs are incubated on solid surfaces (4).

ACKNOWLEDGMENTS

We thank Katie Barbour and Karen Armstrong for technical assistance and Bruce Caldwell at Oregon State University for statistical evaluation assistance. We also thank John Armstrong for his suggestions during the study. The editorial suggestions of John Armstrong and Charles Hendricks are appreciated.

LITERATURE CITED

1. **Amy, P. S., J. W. Schulke, L. M. Frazier, and R. Seidler.** 1985. Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. **49**:1237–1245.
2. **Anderson, E. S.** 1968. The ecology of transferable drug resistance. Annu. Rev. Microbiol. **22**:131–180.
3. **Broda, P.** 1979. Plasmids, p. 21–68. W. Freeman & Co., San Francisco.
4. **Chatterjee, A. K., and M. P. Starr.** 1973. Gene transmission among strains of *Erwinia amylovora*. J. Bacteriol. **16**:1100–1106.
5. **Coplin, D.** 1978. Properties of F and P group plasmids in *Erwinia stewartii*. Phytopathology **68**:1637–1643.
6. **Crosa, J., and S. Falkow.** 1981. Plasmids, p. 266–282. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
7. **Datta, N., and R. W. Hedges.** 1972. Trimethoprim resistance conferred by Enterobacteriaceae. J. Gen. Microbiol. **72**:349–355.
8. **Davies, J., and D. I. Smith.** 1978. Plasmid determined resistance to microbial agents. Annu. Rev. Microbiol. **32**:469–518.
9. **Helinski, D. I.** 1973. Plasmid determined resistance to antibiotics. Molecular properties of R factors. Annu. Rev. Microbiol. **27**:437–470.
10. **Hinshaw, V., J. Punch, M. J. Allison, and H. P. Dalton.** 1969. Frequency of R factor-mediated multiple drug resistance in *Klebsiella* and *Aerobacter*. Appl. Microbiol. **17**:214–218.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Meyer, R., D. Figurski, and D. R. Helinski.** 1975. Molecular vehicle properties of the broad host range plasmid RK2. Science **190**:1226–1228.
13. **Milewski, E.** 1985. Field testing of microorganisms modified by recombinant DNA techniques. Applications, issues, and development of "points to consider" document. Recomb. DNA Tech. Bull. **8**:102–108.
14. **Rissler, J.** 1984. Research needs for biotic environmental effects of genetically-engineered microorganisms. Recomb. DNA Tech. Bull. **7**:20–30.
15. **Sinclair, J. C., and M. Alexander.** 1984. Role of resistance to starvation in bacterial survival in sewage and lake water. Appl. Environ. Microbiol. **48**:410–415.
16. **Tardif, G., and R. B. Grant.** 1983. Transfer of plasmids from *Escherichia coli* to *Pseudomonas aeruginosa*: characterization of a *Pseudomonas aeruginosa* mutant with enhanced recipient ability for enterobacterial plasmids. Antimicrob. Agents Chemother. **24**:201–208.
17. **Watanabe, T., and T. Fukasawa.** 1961. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. J. Bacteriol. **81**: 661–678.