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# Partial Characterization of R-Plasmids from *Pasteurella multocida* Isolated from Turkeys

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*Pasteurella multocida,* isolated from turkeys during an outbreak of septicemic disease ("fowl cholera"), was found to be resistant to tetracycline, streptomycin, and sulfonamides. Agarose gel electrophoretic analysis of DNA from these isolates indicated the presence of extrachromosomal elements. Plasmid DNA was isolated by cesium chloride-ethidium bromide density centrifugation. *Escherichia coli* was transformed to antimicrobic resistance with this DNA. Two plasmids were isolated. One of these plasmids had a buoyant density of 1.7158 g/cm$^3$ (56.9 mol % guanine plus cytosine) and a molecular weight of $4.4 \times 10^6$ and conferred resistance to tetracycline, streptomycin, and sulfonamides. The other, having a buoyant density of 1.7198 g/cm$^3$ (61 mol% guanine plus cytosine) and a molecular weight of $3.44 \times 10^6$, conferred resistance to streptomycin and sulfonamides. Streptomycin resistance was mediated by streptomycin phosphotransferase. Compatibility group testing indicated that neither plasmid belonged to any of 13 compatibility groups (of conjugal plasmids). Both plasmids were also found to be compatible with three small, nonconjugative resistance plasmids.

R-plasmids are stable, extrachromosomal, genetic elements which confer upon bacteria resistance to many different antimicrobial agents. Among members of the family *Enterobacteriaceae,* the incidence of R-plasmids is extremely high (1, 20). R-plasmids have been found in nonenteric genera of bacteria, most notably in *Pseudomonas* and *Staphylococcus* (18, 24). Recent clinical isolates of *Pasteurella multocida* and *Pasteurella haemolytica* have been found that are resistant to one or more antimicrobics, including tetracycline (5). Recently, *Pasteurella* have been reported to harbor plasmids which code for multiple drug resistance (R. P. Silver, B. Leming, and C. A. Hjerpe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, H36, p. 141).

In this paper we report the isolation and partial characterization of covalently closed circular plasmid DNA from naturally occurring isolates of *P. multocida* obtained from turkeys that had died from septicemic disease produced by the isolate. Two distinct species of plasmid DNA were isolated, both of which contain genes coding for resistance to antimicrobial agents.

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains utilized in this study were *Escherichia coli* C600 (*lac thr leu thi*) and *E. coli* C600-1 (a nalidixic acid-resistant mutant of C600, derived in this laboratory). *P. multocida* A3 (serotype 3) was isolated from the spleen of a turkey that had died of septicemic disease. The flock involved had not been given food or water containing antimicrobial agents for at least 1 year prior to this outbreak, except for the specific treatment of disease. For compatibility testing, a number of *E. coli* strains containing the following plasmids of known compatibility groups were utilized: F-Kan (FI), R7K (W), R144 (I$\alpha$), R66a-1 (I$\omega$), and R906 (P) (all obtained from S. Falkow), and R711b (X), R447b (N), R1-16 (FII), R40a (C), Rts1 (T), R723-1 (O), R714b (A-C), and R391 (J) (all obtained from N. Datta).

In addition, the following small, nontransmissible plasmids, which have not yet been grouped, were also tested: Ap101, NTP3, and NTP5. Strains containing these plasmids were obtained from the Plasmid Reference Center, Department of Medical Microbiology, Stanford University, Stanford, Calif.

*E. coli* CH12 and JR35, positive for streptomycin adenylate synthetase and streptomycin phosphotransferase, respectively, were generously donated by J. Davies.

**Media and growth conditions.** *Pasteurella* cultures were routinely maintained on blood agar slants. For all experiments, cultures were grown in brain heart infusion broth with shaking at 37°C. For transformations, M9 minimal medium was used to grow cells (22). For the streptomycin inactivation assays, cells were grown at 37°C in a medium containing the following (per liter): 6.7 g of glucose; 8.0 g of tryptone; 0.3 g of $MgSO_4$; 1 g of $NH_4Cl$; 3 g of $KH_2PO_4$; 5.8 g of $Na_2HPO_4$; and 3 mg of thiamine (pH 7.0).

**Preparation of DNA.** Bacteria were grown to late logarithmic phase in brain heart infusion broth. The cells were harvested by centrifugation and lysed with

Triton X-100 as described by Elwell et al. (9). Covalently closed circular plasmid DNA was purified by isopycnic centrifugation in a cesium chloride-ethidium bromide gradient (12). The final density was adjusted to 1.6199 g/cm$^3$ (refractive index, 1.3920), and the solution was centrifuged for 40 to 48 h at 15°C and 34,000 rpm in a Beckman type 40 rotor.

DNA bands in the gradients were located with a black-light lamp (UVS-11, Ultraviolet Products, Inc., San Gabriel, Calif.) Plasmid and chromosomal bands were removed by puncturing the side of the gradient tube with an 18-gauge needle attached to a syringe.

**DNA contour length.** Plasmid DNA was spread onto Formvar-coated electron microscope grids (300 mesh) according to Kleinschmidt (16). The covalently closed circular DNA was converted to the open circular form by heating for 15 min at 95°C (19). Well-isolated molecules were photographed under an electron microscope (AEI model 6B), and the developed negatives were projected through a photographic enlarger onto paper and traced. The contour length of the tracing was determined by using a map measurer. The true magnification of the electron microscope was determined by calibration with a diffraction grating (54,800 lines per inch, Pellco, Tustin, Calif.). The molecular masses of the plasmids were calculated by assuming 1 μm of DNA to be equivalent to $2.07 \times 10^6$ daltons (17).

**Transformation.** The transformation method used was that described by Cohen et al. (7). Following transformation, the mixture of cells and DNA was diluted 1:10 in L broth and incubated at 37°C for 3 to 4 h when selecting for tetracycline resistance (Tc$^r$), or overnight when selecting for streptomycin resistance (Sm$^r$). After incubation, cells were plated on MacConkey agar supplemented with streptomycin (20 μg/ml), tetracycline (10 μg/ml), or tetracycline and streptomycin, and incubated at 37°C for 24 to 36 h.

**Compatibility group testing.** Compatibility group testing was done as previously described by Coetzee et al. (6). In all but two cases, self-transmissible plasmids of known compatibility groups were transferred into *E. coli* C600-1 containing the *Pasteurella* plasmid. In the case of the plasmids R714b and R391, no transfer could be effected. Thus, testing was done by transformation of *E. coli* strains carrying these plasmids with *Pasteurella* plasmid DNA. Strains containing Ap101, NTP3, or NTP5 were also transformed with *Pasteurella* plasmid DNA for compatibility testing.

**Measurement of minimal inhibitory concentration.** An agar dilution method was used for all isolates (27). The medium used was Mueller-Hinton agar supplemented with laked horse blood (5%).

**Streptomycin inactivation assays.** The assay for streptomycin phosphotransferase was a modification of the procedure of Ozanne et al. (25). Cells were osmotically shocked by a variation of the procedure of Nossal and Heppel (23) as described by Benveniste et al. (4). The reaction mixture contained the following: 20 μl of osmotic shock fluid, 0.23 nmol of [γ-$^{32}$P]ATP (specific activity, 25 Ci/mmol), 17 nmol of streptomycin sulfate, 7.5 μmol of tris(hydroxymethyl)amino methane-hydrochloride (pH 8.0 at 30°C), 2.5 μmol of $MgCl_2$, and 1.5 μmol of β-mercaptoethanol in a total volume of 0.1 ml. The reaction was started by the addition of osmotic shock fluid. Samples of 10 μl were taken every 15 min and assayed as by Ozanne et al. (25).

The assay for streptomycin adenylate synthetase was that described by Benveniste et al. (4).

**Agarose gel electrophoresis.** Cells were screened for the presence of plasmids by the method of Meyers et al. (21).

**Moles percent G+C.** Plasmid DNA samples were obtained from cesium chloride-ethidium bromide gradients. The DNA was then centrifuged for 20 h at 44,000 rpm, at 25°C, in a Beckman model E analytical ultracentrifuge equipped with a UV optical system and scanned to determine the positions of the DNA bands. The buoyant density of the DNA was determined by a formula derived by Schildkraut et al. (26) using *Micrococcus lysodeikticus* DNA as an internal standard (buoyant density = 1.7311 g/cm$^3$). Moles percent guanine plus cytosine (G+C) was calculated from the buoyant density by a formula derived by Schildkraut et al. (26).

**Matings and mobilization studies.** The procedures used are those described by Datta and Hedges (8).

## RESULTS

Agarose gel electrophoresis of a lysate obtained from *P. multocida* A3 is shown in Fig. 1. Bands representing plasmid DNA and chromosomal DNA are evident.

**Transformation.** *E. coli* C600 was transformed with plasmid DNA from *P. multocida* A3. After incubation in antibiotic-free medium, 0.1-ml samples were plated out. Approximately $4 \times 10^3$ Tc$^r$ transformants per ml of transforming mixture appeared on MacConkey agar supplemented with tetracycline. Of 150 clones (Tc$^r$) selected and stabbed onto streptomycin-containing medium, all 150 were also found to be streptomycin resistant. Approximately 100 Sm$^r$ transformants per ml of transforming mixture arose on MacConkey agar supplemented with streptomycin. All of the 10 clones selected were found to be tetracycline susceptible. Agarose gel electrophoresis confirmed that clones with different phenotypes contained different plasmids (Fig. 1). The plasmid conferring resistance to Tc, Sm, and sulfadiazine (Su) was designated pVM100, and the plasmid conferring resistance to Sm and Su (Tc susceptible) was designated pVM101.

**Minimal inhibitory concentration.** Minimal inhibitory concentrations of tetracycline, streptomycin, and sulfadiazine were determined for the clinical isolate of *P. multocida* A3 and for the *E. coli* transformants (Table 1).

**Compatibility testing.** The results of compatibility testing of the *Pasteurella* plasmids indicated that pVM100 and pVM101 did not appear to belong to any of the 13 known compatibility groups tested. In addition, pVM100

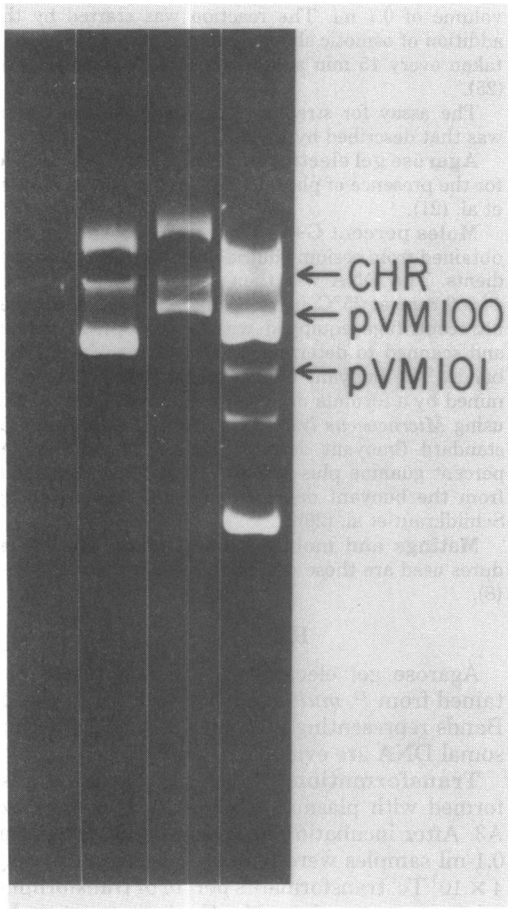


FIG. 1. *Agarose gel electrophoresis of ethanol-precipitated DNA from cleared lysates of P. multocida A3 and transformed E. coli C600. From left to right: C600, C600 (pVM101), C600 (pVM100), P. multocida A3.*

TABLE 1. *Minimal inhibitory concentrations (μg/ml)*

| Strain | Minimal inhibitory concn (μg/ml) | | |
|---|---|---|---|
| | Tc | Sm | Su |
| *P. multocida* A3 | 8 | 32 | >128 |
| *E. coli* C600 (pVM101) | 4 | >128 | >128 |
| *E. coli* C600 (pVM100) | 64 | 16 | >128 |
| *E. coli* C600 | 2 | 1 | 16 |

and pVM101 were found to be compatible with Ap101, NTP3, and NTP5.

**Contour length determinations.** The contour length of open circular forms of pVM100 and pVM101 is shown in Table 2.

**Streptomycin inactivation assays.** Figure 2A shows the results of an assay for streptomycin phosphotransferase. *E. coli* C600 (containing no plasmid) was also run through this assay and gave data similar to the negative control (data not shown). The assay for streptomycin adenylate synthetase was run and proved to be negative (Fig. 2B).

**Moles percent G+C.** pVM100 and pVM101 were shown to be 56.9% and 61% G+C, respectively, calculated from buoyant densities of 1.7158 and 1.7198 g/cm$^3$, respectively. The mole percent G+C for chromosomal DNA from *P. multocida* A3 was 40.3%.

**Transmission studies.** Matings of *Pasteurella* to *Pasteurella, Pasteurella* to *E. coli,* and *E. coli* to *E. coli* indicated that both pVM100 and pVM101 were nontransmissible. A triparental cross, utilizing an F-Kan plasmid as the transfer factor, showed that both pVM100 and pVM101 could be mobilized into another recipient.

## DISCUSSION

We have partially characterized two R-plasmids obtained from *P. multocida* isolated from turkeys dying during an outbreak of fowl cholera. These plasmids appear to be small, nontransmissible plasmids coding for resistance to tetracycline, streptomycin, and sulfonamides. Until now, the presence of plasmids in the genus *Pasteurella* isolated from avian sources had not been demonstrated. The presence of streptomycin phosphotransferase confirms that the streptomycin resistance is plasmid mediated (3).

There are two observations that require comment. One is the apparent contradiction concerning the phenotype expressed depending upon the antibiotic to which transformed strain C600 cells were exposed; i.e., all tetracycline-resistant clones chosen (selected on tetracycline) were streptomycin resistant, whereas all streptomycin-resistant clones chosen (selected on streptomycin) were susceptible to tetracycline. Second is the large difference in transforming frequency when selection for transformants was carried out on either streptomycin- or tetracycline-containing medium.

The apparent difference in the phenotype expressed when transforming mixtures were plated on a medium containing tetracycline or streptomycin is difficult to explain. It may reflect control of the expression of streptomycin resist-

TABLE 2. *Contour lengths and molecular weights of plasmid DNA from P. multocida*

| Plasmid | Contour length (μg) | Mol wt |
|---|---|---|
| pVM100 | 2.13 ± 0.34 | $4.40 \times 10^6$ |
| pVM101 | 1.66 ± 0.12 | $3.44 \times 10^6$ |

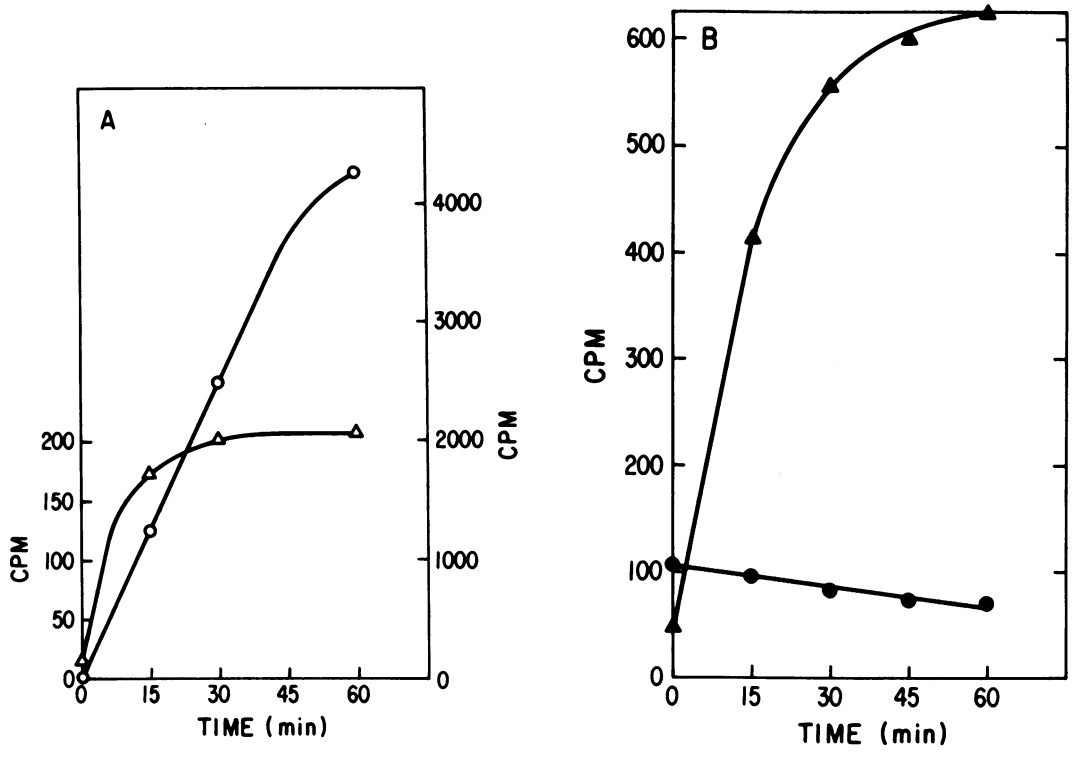


FIG. 2. *Streptomycin inactivation assays. (A)* Streptomycin phosphotransferase assay: (○) *E. coli JR35 (right-hand ordinate),* (△) *E. coli C600 (pVM101) (left-hand ordinate), (B) Streptomycin adenylate synthetase assay:* (▲) *E. coli CH12,* (●) *E. coli C600 (pVM101). The values for controls without osmotic shock fluid have been subtracted in both experiments.*

ance by the tetracycline resistance gene(s). If this is so, then the synthesis of the streptomycin phosphotransferase would only be initiated after the cell was first exposed to tetracycline. This phenomenon is currently under study in our laboratory, and further speculation, we believe, would serve no purpose.

The marked difference in transformation frequency for pVM100 (selected on medium containing tetracycline) and for pVM101 (selected on medium containing streptomycin) reflects the same phenomenon as described by Cohen et al. (7). That is, the expression of streptomycin resistance is a function of the length of time that the transformed cell has possessed the marker. In our experiments, no Sm-resistant clones were found when transforming mixtures were plated on a medium containing streptomycin after 4 h of growth in an antibiotic-free medium (results not presented here). Only after overnight incubation (15 to 18 h) were Sm-resistant clones detected by plating on a medium containing streptomycin.

pVM100 and pVM101 stably coexisted within all host strains containing conjugal plasmids of 13 known compatibility groups. Both plasmids also existed stably with three small, nontransmissible resistance plasmids. Indeed, these plasmids seem very much like the plasmid R300B of Grinter and Barth (11). R300B has been shown to be a small, nonconjugative plasmid coding for resistance to Sm and Su that was found to be compatible with all plasmids tested except for laboratory constructs of itself, such as Ap201 (14). Although Ap201 was not available to us, Ap101 (also an Am-transposon derivative of the Sm Su plasmid RSF1010) was available, and both pVM100 and pVM101 are compatible with it.

The moles percent G+C of pVM100 and pVM101 indicates to us that they are not of *Pasteurella* origin (40.3 mol %) (2). This brings up the question of what their origin might be. It is tempting to postulate an enteric origin for these plasmids, since their G+C content is within the general enteric range. Moreover, R-plasmids have now been detected in two other nonenteric, nonpseudomonad species, *Haemophilus influenzae* (9) and *Neisseria gonorrhoeae* (10). In both of these instances, ampicil-

lin resistance is mediated by a determinant (TnA) which codes for the TEM β-lactamase found in R-plasmids of enteric origin. It is clear now that many of the drug resistance genes in R-plasmids are carried on translocatable DNA sequences (15). In the same vein, one can speculate that these *Pasteurella* plasmids also may have originated from an enteric plasmid.

Whatever the origin of these R-plasmids, the occurrence in a heretofore susceptible animal pathogen is alarming. Animals such as turkeys are raised in lots of many thousands in this country. To stop outbreaks of disease having a high mortality, antimicrobial agents must be administered in a fashion that is quick and easy. Sulfaquinoxaline and tetracycline are two such agents and have been used in the past with great success in the treatment of fowl cholera. The finding that a strain of *P. multocida* now possesses a plasmid that codes for resistance to the drugs of choice for treatment is yet another indication of the growing problem of bacterial resistance to antimicrobial agents.

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## LITERATURE CITED

1. **Anderson, E. S.** 1968. The ecology of transferable drug resistance in the *Enterobacteriaceae*. Annu. Rev. Microbiol. **22**:132-180.
2. **Belozersky, A. N., and A. B. Spirin.** 1960. Chemistry of the nucleic acid of microorganisms. Nucleic Acids **3**:147-185.
3. **Benveniste, R., and J. Davies.** 1973. Mechanisms of antibiotic resistance in bacteria. Annu. Rev. Biochem. **42**:417-506.
4. **Benveniste, R., Y. Yamada, and J. Davies.** 1970. Enzymatic adenylylation of streptomycin and spectinomycin by R-factor resistant *Escherichia coli*. Infect. Immun. **1**:109-119.
5. **Chang, W. H., and G. R. Carter.** 1976. Multiple drug resistance in *Pasteurella multocida* and *Pasteurella haemolytica* from cattle and swine. J. Am. Vet. Med. Assoc. **169**:710-712.
6. **Coetzee, J. N., N. Datta, and R. W. Hedges.** 1972. R factors from *Proteus rettgeri*. J. Gen. Microbiol. **72**:543-552.
7. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110-2114.
8. **Datta, N., and R. W. Hedges.** 1972. Host ranges of R factors. J. Gen. Microbiol. **70**:453-460.
9. **Elwell, L. P., J. de Graaff, D. Seibert, and S. Falkow.** 1975. Plasmid-linked ampicillin resistance in *Haemophilus influenzae* type *b*. Infect. Immun. **12**:404-410.
10. **Elwell, L. P., M. Roberts, L. W. Mayer, and S. Falkow.** 1977. Plasmid-mediated beta-lactamase production in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **11**:528-533.
11. **Grinter, N. J., and P. T. Barth.** 1976. Characterization of Sm Su plasmids by restriction endonuclease cleavage and compatibility testing. J. Bacteriol. **128**:394-400.
12. **Guerry, P., J. van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619-630.
13. **Hedges, R. W.** 1974. R factors from *Providence*. J. Gen. Microbiol. **82**:171-181.
14. **Heffron, F., C. Rubens, and S. Falkow.** 1975. Translocation of a plasmid DNA sequence which mediates ampicillin resistance: molecular nature and specificity of insertion. Proc. Natl. Acad. Sci. U.S.A. **72**:3623-3627.
15. **Heffron, F., R. Sublett, R. W. Hedges, A. Jacobs, and S. Falkow.** 1975. Origin of the TEM beta-lactamase gene found on plasmids. J. Bacteriol. **122**:250-256.
16. **Kleinschmidt, A. K.** 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. Methods Enzymol., **12B**:361-377.
17. **Lang, D.** 1970. Molecular weights of coliphages and coliphage DNA. III. Contour length and molecular weight of DNA from bacteriophages T4, T5 and T7 and from bovine papilloma virus. J. Mol. Biol. **54**:557-565.
18. **Lowbury, E. J. L., J. R. Babb, and E. Roe.** 1972. Clearance from a hospital of gram-negative bacilli that transfer carbenicillin resistance to *Pseudomonas aeruginosa*. Lancet **ii**:941-945.
19. **Macrine, F. L., J. L. Ruder, S. S. Virgili, and D. J. Kopecko.** 1977. Survey of the extrachromosomal gene pool of *Streptococcus mutans*. Infect. Immun. **17**:215-226.
20. **Mercer, H. D., D. Pocurull, S. Gaines, S. Wilson, and J. V. Bennett.** 1971. Characteristics of antimicrobial resistance of *Escherichia coli* from animals: relationship to veterinary and management uses of antimicrobial agents. Appl. Microbiol. **22**:700-705.
21. **Meyers, J., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. A simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529-1537.
22. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Nossal, N. G., and L. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241**:3055-3062.
24. **Novick, R. P., and D. Bouanchaud.** 1971. Extrachromosomal nature of drug resistance in *Staphylococcus aureus*. Ann. N. Y. Acad. Sci. **182**:279-294.
25. **Ozanne, B., R. Benveniste, D. Tipper, and J. Davies.** 1969. Aminoglycoside antibiotics: inactivation by phosphorylation in *Escherichia coli* carrying R factors. J. Bacteriol. **100**:1144-1146.
26. **Schildkraut, C. L., J. Marmur, and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. **4**:430-433.
27. **Washington, J. A., II, and A. L. Barry.** 1974. Dilution test procedures, p. 410-417. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.