DETECTION OF AN INDUCIBLE MEMBRANE PROTEIN ASSOCIATED WITH R-FACTOR-MEDIATED TETRACYCLINE RESISTANCE

Stuart B. Levy and Laura McMurry

Departments of Molecular Biology and Microbiology
and of Medicine
Tufts University School of Medicine and
Tufts-New England Medical Center
Boston, Massachusetts 02111

Received December 28,1973

Summary: New synthesis of an R - factor-specific protein occurs in E. coli minicells containing tetracycline-resistant R factors when they are incubated in the presence of tetracycline. This protein is sometimes detected in small amounts in tetracycline-resistant minicells in the absence of the drug, but it is not detectable in minicells containing R factors which lack tetracycline resistance. The protein has a molecular weight of about 50,000 in sodium dodecyl sulfate (as determined by polyacrylamide gel electrophoresis) and is found preferentially in the minicell membrane.

INTRODUCTION

Resistance to multiple antibiotics in bacteria is linked to the presence of transmissible episomes, called R factors (1). One of the more common, if not most common, R-factor-determined resistance is that to tetracycline. Although degradative enzymes have been identified to explain resistance to some antibiotics (2), no degradative activity has been found for tetracycline. Here resistance may be explained by a decrease in the energy dependent (3) uptake of the antibiotic in resistant R^+ * bacteria (4, 5) as compared with isogenic R^- cells*. In tetracycline-resistant staphylococci and enteric rods, resistance is increased when the organisms are preincubated in small amounts of tetracycline (4, 5, 6).

This laboratory has been studying R factors by segregating them into DNA-less E. coli minicells (7) where they are the only piece of DNA present (8)

* R^+ = contain an R factor; R^- = lack an R factor

In the minicell, these R factors are capable of synthesizing various macromolecules (9) including functionally active proteins (10). Minicells without R factors carry out little or no such syntheses. Using this system, we can describe several R-factor-specific DNA-binding proteins and membrane-associated proteins (11). In the course of these studies, a new protein band was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis of labeled proteins synthesized in the presence of tetracycline by minicells carrying tetracycline-resistant R factors.

MATERIALS AND METHODS

E. coli Strains:

E. coli minicell producing strain χ984 (originally received from R. Curtiss) and its R^+ derivatives were used (Table). R factors R124 and R386 were received from N. Datta. R factor RM98 was received from R. Iyer. All R factors were introduced by conjugation into strain χ984. These strains were grown up overnight in L broth to late exponential phase before minicell purification (8).

Minicell purification and radioactive labeling of R-factor proteins.

Minicells were purified as previously described (9) except that only one 10 to 30% sucrose gradient was used. After washing, the minicells were resuspended at about 10^9 minicells/ml in 25% methionine assay media: (25 ml Methionine Assay (Difco): 75ml minimal media (9) and pyridoxine 25 μg/ml; adenine 25 μg/ml; glucose 0.1%) After incubation for 10 minutes, penicillin (1500 units/ml) was added for an additional 30 minutes to further eliminate contaminating cells (9). Following this prelabeling period, [^{35}S] - methionine (New England Nuclear) was added (10-50 μCi/.01-.05μg/ml) and incorporation of label proceeded at 37C for two hours. Where indicated, tetracycline (10-15 μg/ml) was added 30 minutes before the radioactive label. After labeling, the minicells were washed thoroughly, centrifuged into a pellet and frozen at -20C. The number of viable cells was determined by platings on nutrient agar before and after labeling. At no time were there more than 10^4 cells per 10^9 minicells.

Polyacrylamide gel electrophoresis.

Ten percent polyacrylamide gels with 0.1% SDS* in 0.05M phosphate buffer pH 7.0 were used (14). The labeled minicells were precipitated by 5% trichloracetic acid in the presence of sodium deoxycholate (50μg/ml) and were resuspended in 0.05M Tris-HCl pH 8.0. Any residual acid was neutralized with NaOH and the preparation was treated with 1% SDS, 1% β-mercaptoethanol

* SDS = sodium dodecyl sulfate

and immediately heated for 2 minutes at 100C. The material was then layered onto 1.5 mm slab gels (modified EC apparatus). These gels were run three to four hours at 100 milliampere per gel until bromphenol blue tracking dye had reached 10 cm from the origin. Bovine serum albumin, ovalbumin, myoglobin and lysozyme were used as standards for molecular weight determinations. Gels were subsequently dried onto Whatman 3MM filter paper under vacuum and steam heat and autoradiographed on Kodak No Screen or Blue Brand Medical X-ray film.

Lysis of minicells.

Minicells were resuspended at 10^9 minicells/ml in 2.5 ml of 0.05M Tris-HCl pH 8.0 and treated with 7 x 10^{-4}M EDTA and lysozyme (100 μg/ml) for 20 minutes at room temperature. The proteolytic inhibitors, tosyl-L-phenyl-alanylchlormethane and tosyl-L-lysyl-chlormethane (Calbiochem) were added at 10^{-4}M and the preparation sonicated 6 times for 30 seconds each time in an MSE sonifier set at maximum intensity. Samples were made 0.01M MgCl and treated with deoxyribonuclease (20μg/ml) for 10 minutes before being centrifuged at 15,000 rpm for 15 minutes in the RC2B ultracentrifuge at 4C. The supernatant was saved and the pellet was resuspended in Tris buffer and resonicated 6 more times; the optical density was less than 10% that of the original sample. The samples were recentrifuged in the RC2B centrifuge; the resulting pellet represents washed purified membranes. The two supernatants were combined and recentrifuged at 55,000 rpm in the SW65 (Beckman) rotor for two hours. From this lysis procedure three fractions were obtained: 1) 55,000 rpm supernatant; 2) 55,000 rpm pellet; and, 3) washed minicell membranes.

RESULTS

a) Detection of a tetracycline-inducible protein in minicells containing tetracycline-resistant R factor 222. Minicells from a D1-7 culture grown overnight in the absence of tetracycline were purified and labeled in the presence or absence of tetracycline (10μg/ml). A "new" protein of molecular weight 50,000 appeared clearly in the minicell preparation labeled in the presence of tetracycline (Fig. 1A). This protein achieves an intensity similar to the major R factor protein band seen among the proteins synthesized by R factor 222 and other R factors (11). Close scrutiny of the gel of proteins labeled in the absence of tetracycline demonstrated the presence of a very light band in the same position on the gel (Fig. 1A).

Cells from the minicell-producing D1-7 strain were also labeled in the presence and absence of tetracycline and the total proteins analyzed on poly-

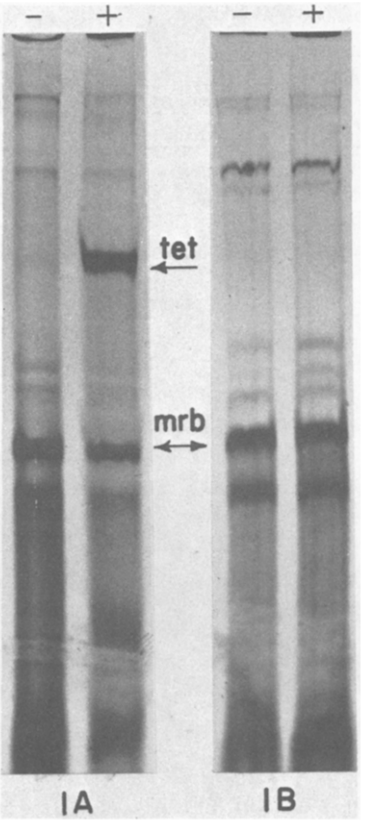

Fig. 1. R-factor proteins labeled in the presence or absence of tetracycline. Minicells were labeled in the presence (+) or absence (-) of tetracycline (10μg/ml). Samples were prepared and examined by SDS-gel electrophoresis. The same amount of radioactivity was placed on each gel. 1A - D1-7 minicells; 1B:D1-8 minicells: tetracycline inhibited 60% of protein synthesis. Tet = protein induced by tetracycline; mrb = major R factor protein band.

acrylamide gels. Under neither labeling condition was the tetracycline protein band detectable among all the other proteins synthesized by the cells.

b) Protein profile of minicells containing tetracycline-sensitive R factor 222-R3. Strain D1-8 contains R factor 222 which has spontaneously lost its tetracycline resistance marker and is 222-R3 (Table). Such tetracycline-sensitive segregants of 222 have been described by others (1, 15). The gel profile of the proteins of D1-8 minicells labeled with and without tetracycline

TABLE

E. coli Minicell Strains	R Factor	Compatibility Type[d]	Known R Factor Resistances [a]	Tet[b] Protein
χ^{984}	-	-	-	-
D1-7	222	F II	Cm Su Sm Tc	+
D1-8	222-R3	F II	Cm Su Sm	-
D1-26	R1	FII	Cm Su Sm Km/Neo	-
D1-41	R124	FIV	Tc	+
D1-42	R386	FI	Tc	+
D2-14	R64	I	Sm Tc	+
D3-2	RM98	N	Amp	-
D3-9	N-3	N	Tc	+
D6-2	CF-2[c]	?	Tc	+

[a] Cm = chloramphenicol; Su = sulfon amide; Sm = streptomycin; Tc = tetracycline; Km/Neo = kanamycin/neomycin; Amp = ampicillin

[b] presence (+) or absence (-) of inducible tetracycline protein

[c] R factor isolated in an *E. coli* from a patient (C.F.) with skin abscesses

[d] Refs. 12, 13

present is shown in Fig. 1B. No protein of molecular weight 50,000 is seen.

c) Membrane location of the tetracycline-inducible protein. Microdensitometer tracings of autoradiograms of the two membrane fractions and the supernatant from lysis of tetracycline-induced D1-7 minicells showed that about 70% of the tetracycline protein was in the membranes. The purity of the membrane fractions was indicated by the absence from them of many

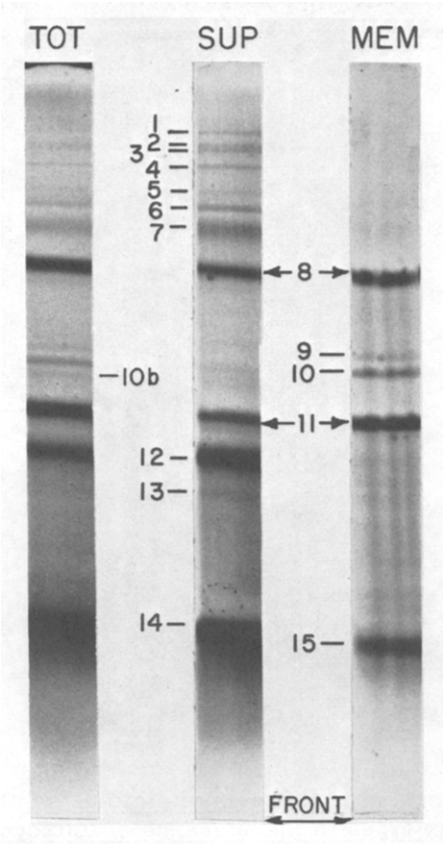

Fig. 2. Location of tetracycline protein in minicells. D1-7 minicells were lysed and the membranes and supernatants prepared as outlined in Methods. Aliquots of whole minicells (tot), the 55,000 rpm supernatant (sup) and purified membranes (mem) were analyzed on SDS polyacrylamide gels. Numbers refer to individual protein bands seen in the gels; 8 = tet; 11 = mrb (see Fig. 1).

supernatant bands seen in the gel (in particular, bands 1-7, 12 and 14, Fig. 2). Moreover, under similar lysis conditions less than 10% of the soluble protein β-galactosidase remained with the membrane fraction. These results suggest that the tetracycline protein is primarily in the membrane. Since these sonications may extract some of the protein normally associated with the membrane, we cannot yet define the distribution of this protein between soluble and membrane fractions in the minicell. About 10% of the total radioactivity incorporated by the minicell into protein is present in the tetracycline protein band.

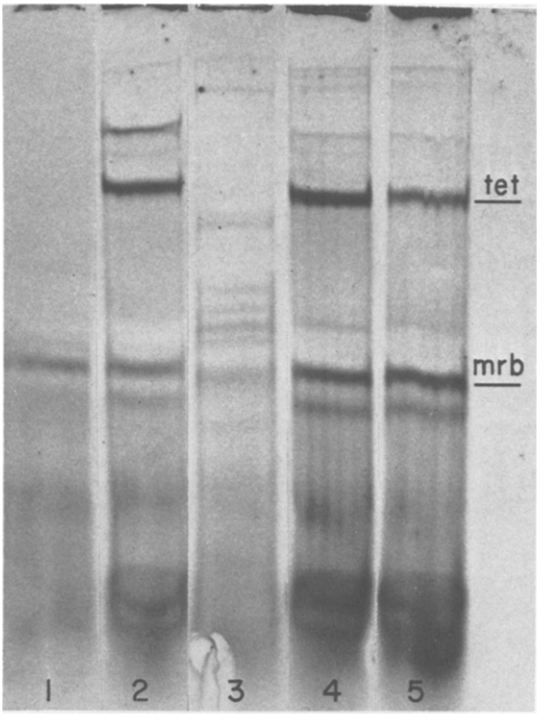

Fig. 3. SDS-gel electrophoretic patterns of proteins synthesized by R factors of different compatibility types in the presence of tetracycline. Samples were prepared as described in Fig. 1. R factors are described in Table. 1 = D1-26; 2 = D1-41; 3 = D3-2; 4 = D1-42; 5 = D3-9; tet, mrb as in Fig. 1.

d) Other tetracycline-resistant and sensitive R factors. R factors can be distinguished by their ability to co-exist in the same cell. Incompatible R factors represent the same compatibility type (12, 13). R factors from four other compatibility groups were labeled in the presence or absence of tetracycline as in previous experiments (Table, Fig. 3). With every tetracycline-resistant R factor tested, the tetracycline-induced protein band is prominent, but only in the presence of tetracycline. When minicells containing tetracycline-sensitive R factors were tested, this inducible protein band could not be demonstrated (Table, Fig. 3).

DISCUSSION

In these studies we have identified a protein band in SDS polyacrylamide gels which is 1) synthesized only by R factors resistant to tetracycline, 2) found closely associated with the minicell membrane where it could affect uptake, and 3) induced to higher amounts by tetracycline (as is phenotypic expression of tetracycline resistance in whole cells). These facts strongly imply a role for this protein in tetracycline resistance. The similar mobility on gel electrophoresis of a tetracycline-inducible protein from R factors of at least five different compatibility groups suggests a common origin of this gene product among R factors of seemingly different origin. We have recently isolated several conditional, temperature-sensitive mutants of 222 which become sensitive to tetracycline at 42C, but are resistant at 30C. The finding of such mutants, to be described in a separate report, further suggests that a protein is involved in this resistance.

Tetracycline induces resistance in tetracycline-resistant cells and this inducibility correlates with a decreased uptake of the drug by resistant cells (4, 5, 6). The antibiotic is not degraded. Although differences in gel electrophoretic patterns of polypeptides have been noted in comparing induced and uninduced tetracycline-resistant cells (16) or minicells (17), this is the first report of a single protein species which becomes newly synthesized in the presence of tetracycline and which is associated only with R factors mediating tetracycline resistance.

ACKNOWLEDGMENTS

The excellent technical assistance of Ms. Sylvia Rozbroj is greatly appreciated.

This work was supported in part by USPHS Grant # A1-10648, a grant from the University Cancer Committee (American Cancer Society, grant # IN 23-0) and Research Career Development Award to S.B.L. We thank Dr. J. King for making available to us the Joyce microdensitometer.

REFERENCES

1. Watanabe, T. (1963) Bacteriol. Rev. 27: 87-115.
2. Davies, J. E. and Rownd, R. (1972) Science 176: 758-768.
3. Arima, K. and Izaki, K. (1963) Nature (London) 200: 192-193.
4. Izaki, K., Kiuchi, K., and Arima, K. (1966) J. Bact. 91: 628-633.
5. Franklin, T. J. (1967) Biochem. J. 105: 371-378.
6. Sompolinsky, D., Krawitz, T., Zaidenzaig, Y., and Abramora, N. (1970) J. Gen. Micro. 62: 341-349.
7. Adler, H. I., Fisher, W. D., Cohen, A., and Hardigree, A. A. (1967) Proc. Nat. Acad. Sci. (USA) 57: 321-326.
8. Levy, S. B. and Norman, P. (1970) Nature (London) 227: 606-607.
9. Levy, S. B. (1971) J. Bact. 108: 300-308.
10. Levy, S. B., (manuscript in preparation).
11. Levy, S. B. (1973) Bact. Proc. (Abstr., Amer. Soc. Micro.) p. 62.
12. Datta, N. and Hedges, R. W. (1971) Nature (London) 234: 222-223.
13. Hedges, R. W. and Datta, N. (1972) J. Gen. Micro. 71: 403-405.
14. Studier, W. F., and Maizel, J. V. (1969) Virology 39: 575-586.
15. Clowes, R. C. (1972) Bacteriol. Rev. 36: 361-405.
16. Franklin, T. J. and Rownd, R. (1973) J. Bact. 115: 235-242.
17. Van Embden, J., and Cohen, S. N. (1973) J. Bact. 116: 699-709.