Isolation and Characterization of Tetracycline Resistance Proteins from *Staphylococcus aureus* and *Escherichia coli*

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Immunoglobulin (adsorbed resistance antiserum) reacting specifically with antigens from tetracycline (Tc)-resistant *Staphylococcus aureus* or *Escherichia coli* was produced by adsorbing immunoglobulin against cell envelopes of resistant strains with envelope extracts from the respective isogenic susceptible strains. Adsorbed resistance antiserum against *S. aureus* reacted with envelope extracts from 32 Tc-resistant strains and failed to react with similar extracts from 76 Tc-susceptible strains of *S. aureus*. An antigen (Tc resistance antigen [TRA]) found only in Tc-resistant strains was produced by adsorbing envelope extracts from these strains with immunoglobulin against envelopes from isogenic Tc-susceptible strains. On immunodiffusion no cross-reactivity between TRAs from *S. aureus* and *E. coli* was observed. The TRAs behaved as proteins. The molecular weight of TRA from *S. aureus* was determined to be 32,000 and from *E. coli* to be 50,000. Data obtained by preliminary amino acid analysis of the TRAs are presented.

We have previously demonstrated particular antigens connected with tetracycline (Tc) resistance in *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli* (4) and *Staphylococcus aureus* (3, 15). These antigens were demonstrated in envelope fractions of *E. coli* and *P. aeruginosa* as well as in cytoplasmic extracts of *P. aeruginosa*, *S. typhi*, and *S. aureus*. It was later found that more potent antigens could be obtained by extraction of *S. aureus* envelopes with sodium deoxycholate or Triton X-100. Specific antisera against these antigens were obtained by immunization of rabbits with the appropriate fraction of the resistant culture and adsorbing the immunoglobulin obtained with a homologous fraction from an isogenic susceptible culture.

We shall now present further details on the isolation of the Tc resistance antigens and some information about their chemical structure.

MATERIALS AND METHODS

E. coli strains and Tc R factor. *E. coli* K-12, W 1485, *met*⁻, *Flac*⁺, as well as the R factor carrying Tc^R from *S. typhi* have been described previously (11).

S. aureus strains, phages, and Tc resistance genes. Two Tc-susceptible strains were chosen for the preparation of antigens and as recipients of Tc resistance genes by transduction, strain 3 (phage pattern 52/52A) isolated from clinical material and strain 1540 obtained from R. P. Novick, The Public Health Research Institute of the City of New York, Inc. Donors of Tc resistance genes were strains 111

(13) and V738 (12), both previously characterized. The program for transductions is indicated in Table 1. The phages were grown in the donor strains in one to three cycles until a titer of 10⁹ plaque-forming units per ml was obtained. The phage suspension was then sterile filtrated and portions of it were irradiated for various time periods with a Sterisol F 1140 Original Hanau ultraviolet lamp. The phage was added to an exponential growing culture of the recipient strain in nutrient broth (NB) supplemented with CaCl₂, 0.15 µg/ml (37 C, slight aeration). After 30 min, 0.5% sodium citrate was added and the bacteria were sedimented, washed with NB supplemented with citrate, and resuspended in this medium containing 0.1 µg of Tc per ml for induction of Tc resistance in the transductants. After 2 h at 37 C, portions of the culture were plated on Casitone agar with the selective drug additions indicated in Table 1. Colonies that appeared in 48 h were analyzed for phage patterns and resistance levels. Quantitative details of the transduction and the influence of ultraviolet irradiation will be reported separately (manuscript in preparation, J. Krausz and D. Sompolsky).

For a survey of the correlation between Tc resistance and presence of the Tc resistance antigen (TRA) to be described, we also used strain 649 T from R. W. Lacey, Bristol Medical School, U.K. (5), 75 clinical isolates, and 32 propagation strains for typing phages. Cultures that grew on nutrient agar (NA) plus 1.5 µg of Tc per ml were considered resistant; these strains gave an inhibition zone of ≤22 mm with 30-µg Tc sensitivity disks from Baltimore Biological Laboratories (Cockeysville, Md.).

Nutrient media. NB and NA were from Difco Laboratories, Detroit, Mich. Casitone agar con-

TABLE 1. Program for transduction of Tc resistance in *Staphylococcus aureus*

Recipient strain	Donor	Phage	Tc concn for induction of resistance ($\mu\text{g/ml}$)	Drugs for selection of transductants ^a ($\mu\text{g/ml}$)	Designation of transductants
3	111	52	0.1	Tc 4.0	3 (111)
3	V738	29	0.1	Minoc 0.4	3 (738)
3 (738)	111	52	0.1	Oxytc 160	3 (111, 738)
1540	111	52	0.1	Tc 4.0	1540 (111)
1540	3 (738)	52	0.1	Minoc 0.4	1540 (738)
1540 (738)	111	52	0.1	Oxytc 160	1540 (111, 738)

^a Abbreviations: Minoc, Minocycline; Oxytc, oxytetracycline.

tained per 1 liter: Casitone (Difco), 25 g; NaCl, 8 g; agar powder, 15 g (pH 7.4).

Buffers and solutions. One solution was veronal buffer (sodium barbitone, 50 mM, pH 8.6)-phosphate-buffered saline (PBS) (0.15 M Na_2HPO_4 , 61.1 ml; 0.15 M KH_2PO_4 , 38.9 ml; 0.8% NaCl, 100 ml). Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride and sodium phosphate buffers (PB) were used at various molarities and pH values as will be indicated. Color and washing solutions were as described (3, 15).

Envelope preparations. The preparation of envelope extracts from susceptible and resistant *E. coli* has been described (4). The *S. aureus* strains were grown in plain NB (susceptible strains) or NB plus 1 μg of Tc per ml (resistant strains); 1-liter portions of the cultures in the exponential growth phase were cooled, sedimented by centrifugation, washed twice with Tris-hydrochloride (10 mM, pH 7.5), and resuspended in 10 ml of Tris-hydrochloride. The cocci were thereafter crushed in a French press (20,000 lb/in²) and, after addition of 10 μg of deoxyribonuclease (DNase) per ml and 45 mM Mg acetate, incubated at 4 C for 15 min. Residual whole cells were separated by centrifugation (15 min at $5,000 \times g$) and cell envelopes were sedimented at $40,000 \times g$ and washed three times with Tris-hydrochloride. Finally, the $40,000 \times g$ sediment was cleared by dialysis against several changes of Tris-hydrochloride. The protein content in the dialysis bag was determined according to Lowry et al. (10). The envelope preparations were checked as described for gram-negative organisms (4). On electron micrographs, membrane vesicles with rather little cell wall material were observed.

This procedure was followed for strains 3 and 1540 and their resistant derivatives. For the survey of correlation between resistance and presence of TRA, the method was simplified in the following ways. All strains, susceptible and resistant, were grown in NB without Tc; overnight cultures were used rather than cocci from the exponential growth phase; DNase after French press and dialysis of the $40,000 \times g$ sediment were omitted.

Immunization of rabbits. Young rabbits were given weekly intramuscular injections of envelope emulsions (3 mg of protein) in 1 ml of Freund complete adjuvant. Satisfactory antisera giving strong precipitation lines on double immunodiffusion against extracts of the antigens were obtained only

after prolonged immunization (3 to 4 months). At this stage, the rabbits were exsanguinated and immunoglobulin was prepared (3).

Envelope extracts. For electrophoresis and immunodiffusion, 35 mM MgCl and 2% Triton X-100 (final concentration) were added to the envelope suspension in Tris. After 1 h at 37 C and 18 h at 4 C, the suspension was centrifuged at $40,000 \times g$ (20 min) and the supernatant fluid (envelope extract) was stored at -18 C in small portions. Generally, *S. aureus* envelopes were extracted with Triton X-100 and *E. coli* envelopes with sodium deoxycholate (4), but identical results were obtained with both organisms when the alternative detergents were used.

Ouchterlony double immunodiffusion. Immunoelectrophoresis. The techniques were as described earlier (3, 4).

Radial immunodiffusion. A solution of immunoglobulin (25 mg of protein/ml) was incorporated into an equal volume of liquified Noble agar (Difco) in saline at 45 to 50 C. The mixture was poured onto a glass plate (20 by 10 cm) with a dry underlayer of agar. Three-microliter amounts of antigen were inserted into pits of 2-mm diameter. Reading of precipitation rings followed after 48 h.

Electrophoresis in polyacrylamide gels. This was performed by modification of procedures described by others (1, 17). For gel electrophoresis of crude extracts of cell envelopes, the gel was composed of two layers, the lower part (8 cm) was prepared by polymerization of 15% acrylamide with the addition of 0.1% sodium dodecyl sulfate (SDS), and the upper part (2 mm) contained 0.1% SDS plus 3% acrylamide plus 0.5% agarose. After insertion of the comb into the upper mixture, the apparatus was left at 45 to 50 C for polymerization of the acrylamide and thereafter at ambient temperatures until solidification of the agarose. Samples containing 100 μg of protein per 10 μl were heated for 2 min, cooled, and separated at ambient temperatures during 4 h (200 V, 150 mA). The buffer contained 0.1% SDS, 25 mM Tris, and 192 mM glycine, pH 8.3. For determination of molecular weight, 10% polyacrylamide slabs were used with samples of about 3 μg of protein each (2 mA per disk, 8 h).

Isolation of TRAs. (i) One-hundred milliliters of a 2.5% solution of immunoglobulin from rabbits immunized with envelopes from Tc-susceptible cul-

tures was mixed with 50 ml of a 1% carboxymethyl-cellulose solution. (ii) A 250-mg amount of dicyclohexyl-carbodiimide was dissolved in 3 ml of tetrahydrofuran and added to the globulin carboxymethyl-cellulose mixture. The pH was adjusted to 4.5 and the solution was kept at 45 to 50 C for 30 min. Thereafter the pH was lowered to 3.8, 30 ml of 2.5% agarose solution was added, and incubation was continued for 2 h at 45 to 50 C and at 2 to 4 C until solidification. (iii) The gel was cut into small pieces and washed in several portions of PBS till no traces of protein could be demonstrated in the washing solution. The gel was then dried at 40 C, carefully powdered, and then packed onto a 300-mesh steel sieve. The part that did not pass the sieve was packed into a wide column (50 by 100 mm). PBS was passed through the column until zero light absorption at 280 nm. (iv) Extracts of envelopes from a resistant culture were dialyzed against several portions of PBS. An amount corresponding to 30 mg of protein was left on the column for 90 min at 37 C. Thereafter, PBS was passed through the column and 1-ml fractions were collected. All protein-containing fractions were mixed, concentrated over polyvinylpyrrolidone, and used for immunodiffusion and immunoelectrophoresis. This material showed one distinct protein band with only traces of impurities (TRA) on disk electropherograms in 10% polyacrylamide.

Influence of enzymes on TRA. An amount of TRA corresponding to 100 μ g of protein was dissolved in 1 ml of PB (20 mM, pH 7.6) and 1.5 μ g of a proteolytic enzyme (trypsin, chymotrypsin, or Pronase) was added. After incubation at 37 C for 1 h, the material was concentrated over polyvinylpyrrolidone. TRA treated in the same way with heat-inactivated enzyme served as a control. For assay of lipase, 10 μ g of enzyme (type I lipase, Sigma Chemical Co., St. Louis, Mo.) was added to 100 μ g of TRA in PB (20 mM, pH 6.0). For ribonuclease (RNase) and DNase, 10- μ g amounts were used with 100 μ g of TRA, and 20 mM magnesium sulfate was added. Incubation was for 30 min before the immunodiffusion assay.

Influence of heat (100 C) on TRA. Five hundred micrograms of protein of TRA in 1 ml of PB (20 mM, pH 7.6) was divided into seven portions which were boiled for 0, 2, 10, 20, 30, 45, and 60 min, respectively, and thereafter cooled immediately. Conservation of antigenicity was checked by immunodiffusion.

Amino acid analysis. A portion of 0.5 to 2 mg of TRA protein was hydrolyzed at 110 C under vacuum by 6 N HCl for 30 h. The analysis was performed with a Beckman automatic amino acid analyzer in the Department of Chemical Immunology, Weizman Institute of Science, Rehovot, through the courtesy of D. Givol. Samples were also assayed on a newly purchased Technicon System amino acid auto analyzer in the Department of Biology, Bar Ilan University, Ramat Gan. The results obtained by both analyzers were entirely comparable; only the data obtained at the Weizman Institute will be presented. No attempt was made to determine tryptophan or to analyze cystine-cysteine after conversion to cysteic acid.

Preparation of immunoglobulin specific for

TRA. Immunoglobulin against envelopes from a resistant culture was titrated against extracts of envelopes from isogenic susceptible cultures by the technique of Dean and Webb (6). A solution of immunoglobulin was then added to an envelope extract in the relation corresponding to the zone of equivalence and the mixture was incubated for 1 h at 45 C and 18 h at 4 C. The precipitate formed was sedimented by centrifugation and small portions of the supernatant were stored at -18 C until use. This immunoglobulin will be designated as adsorbed resistance antiserum (ATRA).

RESULTS

Nonadsorbed antigens and immunoglobulins. Disk electropherograms of 70- μ g protein samples of envelope extracts from *S. aureus* 3 and *S. aureus* 3 (111, 738) in SDS-polyacrylamide revealed a large number of protein bands. The only difference demonstrable between extracts from the susceptible and the resistant organism was a distinct band obtained from the extract of 3 (111, 738) but not of 3. This protein band migrated in the gel to the same position as isolated TRA (Fig. 1). With increasing sample size the difference between the extracts was blurred due to the appearance of more and wider protein bands.

Immunodiffusion and immunoelectrophoresis of nonadsorbed antisera against envelope extracts from susceptible and resistant strains showed a large number of precipitation bands. We were unable by these methods to clearly define any difference between susceptible and resistant strains.

Adsorbed immunoglobulin reacting specifically with extracts of Tc-resistant strains. The adsorption of *E. coli* Tc^R envelope antiserum with envelope extract of the isogenic susceptible strain gave an antiserum that reacted with extracts from Tc-resistant *E. coli*, *P. aeruginosa*, and *S. typhi* but not with extracts from susceptible strains (4). This adsorbed serum (ATRA) has so far not been tested with a large collection of susceptible and resistant strains.

Immunoglobulin against envelopes from *S. aureus* 3 (111, 738) that was adsorbed with envelope extracts from the susceptible strain 3 reacted in immunodiffusion with envelope extracts from the resistant transductants 3 (111), 3 (738), 3 (111, 738), 1540 (111), 1540 (738), and 1540 (111, 738), as well as from the resistant donor strains 111 and 38; ATRA did not react with the susceptible 3 and 1540 (Fig. 2). Similar results were obtained by immunoelectrophoresis (Fig. 3). Neither of these methods enabled us to decide whether the double transductants 3 (111, 738) and 1540 (111, 738) contained more of the resistance antigen than the transductants

FIG. 1. Polyacrylamide disk gel electrophoresis of 5 μ g of protein of TRA from *S. aureus* 3 (111, 738) in comparison with 70 μ g of membrane extracts from *S. aureus* 3 (A) and *S. aureus* 3 (111, 738) (B). N, Negative electrode; P, positive electrode.

with a single resistance gene. In these experiments, antigens were produced from cocci harvested in the exponential growth phase; however, extracts from overnight cultures reacted as well with the adsorbed immunoglobulin. Likewise, growth of the resistant culture with induction (NB plus 1 μ g of Tc per ml) or in plain NB gave identical results.

The efficacy of our method to extract resistance antigens from envelopes was also tested. Envelopes that were extracted as usual with Tris-Triton X-100 were sedimented at $40,000 \times g$ and reextracted in the same way. The procedure was repeated six times. Figure 4 shows that the first three extracts gave fairly strong precipitation bands with ATRA, the fourth extract gave only a weak band, and later extracts did not react at all.

ATRA was also tested with envelope extracts from the Tc-resistant strain 649 T obtained from Lacey (5) and extracts from about 100

FIG. 2. Ouchterlony double immunodiffusion in agar. A, Envelope extract from the susceptible *S. aureus* 3. The other extracts were from the following resistant strains: B, 3 (111); C, 111; D, V738; E, 3 (738); F, 3 (111, 738).

FIG. 3. Immunoelectrophoresis of staphylococcal envelope extracts. In the wells are envelope extracts from the following strains: A, *S. aureus* 3; B, 3 (111); C, 111; D, V738; E, 3 (738); F, 3 (111, 738).

other strains of *S. aureus*, i.e., clinical isolates and phage propagation strains. All these envelope extracts were tested both by radial immunodiffusion (Fig. 5) and by Ouchterlony immunodiffusion. Table 2 shows that all the 32 extracts from resistant strains, but none of the 76 extracts from susceptible strains, were precipitated by ATRA.

FIG. 4. Double immunodiffusion in agar. In the peripheral wells successive extracts (1 to 6) of an envelope preparation of 3 (111, 738) with Tris containing 2% Triton X-100. Strong precipitation lines were obtained with the supernatant fluid from the first three extractions, a weak one (scarcely reproducible on photograph) with the fourth extract, and no precipitation with numbers 5 and 6.

Specific TRA from *S. aureus*. TRA was prepared by adsorption of an envelope extract of 3 (111, 738) with solidified immunoglobulin against envelopes of 3. TRA reacted on double immunodiffusion (Fig. 6) and immunoelectrophoresis with immunoglobulin against envelopes from 3 (111, 738) but not with envelope immunoglobulin against envelopes from 3. Figure 1 shows a disk electropherogram of 5 μ g of protein of TRA in comparison with 70 and 150 μ g of protein samples of envelope extracts of 3 and 3 (111, 738).

The molecular weight of TRA was estimated to be about 32,000 by comparison with commercial proteins of known molecular weight on electrophoresis in 10% polyacrylamide with 0.1% SDS (Fig. 7 and 8). Trypsin, chymotrypsin, and Pronase treatment destroyed the antigenicity of TRA; lipase, DNase, and RNase had no influence. Heating for 2 min to 100 C had seemingly no influence on antigenicity but 5 min was detrimental.

Table 3 records the data of an amino acid analysis of TRA from *S. aureus* as obtained with a Beckman automatic amino acid analyzer. The acid hydrolysis time was 24 h and no attempt was made at this stage to correct for possible residual valine and leucine peptides. Likewise, cysteine and tryptophan have not yet been assayed by other methods.

TRA from *E. coli*. Envelope extract of *E. coli* Tc^R that was passed through a column of solidified *E. coli* Tc^S envelope immunoglobulin gave a single protein band on an SDS-polyacrylamide electropherogram with only minor traces of impurities. In Ouchterlony double immunodiffusion assay, TRA reacted with immunoglobulin against envelopes of the Tc^R strain,

FIG. 5. Radial immunodiffusion in agar containing ATRA, immunoglobulin to TRA. In the pits, envelope extracts (3 μ g of protein) from 32 resistant and 76 susceptible strains of *S. aureus*. A ring precipitation around the pits indicate a positive reaction.

but not with immunoglobulin against envelopes of the Tc^s strain. Comparison with known proteins gave an estimated molecular weight of 50,000 (Fig. 7 and 8), a result entirely in concert with the findings of Levy et al. (8, 9).

The result of an amino acid analysis of TRA from *E. coli* is indicated in Table 3. The data are by and large similar to those obtained with TRA from *S. aureus*. Also in this case, the results should be considered as preliminary since no particular analysis of cysteine and tryptophan was performed.

Cross antigenicity between TRA from *S. aureus* and *E. coli*. Figure 9 shows no identity in antigenicity of TRA from staphylococci and *E. coli*. We have earlier reported that ATRA from *E. coli* showed identity reaction between TRAs from *E. coli*, *S. typhi*, and *P. aeruginosa* even when their R plasmids were different (4).

DISCUSSION

We have produced an immunoglobulin specific for Tc resistance (ATRA) in *S. aureus* and

TABLE 2. Correlation between Tc resistance and precipitation of envelope extracts with ATRA^a

Strains	Precipitation with ATRA	
	+	-
No. susceptible ^b	0	76
No. resistant ^c	32	0

^a Identical results were obtained by Ouchterlony double immunodiffusion and radial immunodiffusion assays.

^b No growth on NA + 1.5 µg of Tc per ml.

^c Growth on NA + 1.5 µg of Tc per ml.

E. coli by immunizing rabbits with emulsions of envelopes from resistant strains in Freund complete adjuvant and adsorbing the immunoglobulin with envelope extracts from the respective isogenic susceptible strains.

ATRA reacted with extracts from noninduced *S. aureus* strains of which some are known to be of inducible Tc resistance. We have previously shown that the difference in Tc accumulation between Tc-susceptible and Tc-resistant strains is pronounced only during the exponential growth phase (3, 16). Nevertheless, enve-

TABLE 3. Amino acid composition of TRAs from *Staphylococcus aureus* and *Escherichia coli*

Amino acids	<i>S. aureus</i>		<i>E. coli</i>	
	No./mol ^a	%	No./mol ^b	%
Lysine	20.3	7.8	21.2	5.2
Histidine	6.8	2.6	8.1	2.0
Arginine	18.2	7.0	17.0	4.2
Aspartic acid	27.1	10.3	38.9	9.7
Threonine	12.0	4.6	21.8	5.4
Serine	18.7	7.2	21.2	5.3
Glutamic acid	32.7	12.5	45.8	11.4
Proline	14.2	5.4	11.7	2.9
Glycine	44.9	17.2	41.7	10.3
Alanine	25.0	9.6	52.8	13.1
Half-cystine	1.4	0.5	0.9	0.2
Valine	9.8	3.8	28.2	7.2
Methionine	3.2	1.2	14.1	3.5
Isoleucine	7.4	2.8	20.5	5.1
Leucine	12.2	4.7	33.8	8.4
Tyrosine	3.2	1.2	9.7	2.4
Phenylalanine	4.3	1.6	13.1	3.3
Hexosamine	0.9	0.3		

^a Calculated on the basis of a molecular weight of 32,000.

^b Calculated on the basis of a molecular weight of 50,000.

FIG. 6. Immunodiffusion reactions of TRA from *S. aureus* with immunoglobulin to an envelope extract of the resistant (R) *S. aureus* 3 (111, 738) and the susceptible (S) *S. aureus* 3.

FIG. 7. Determination of molecular weights of TRA from *S. aureus* (2) and *E. coli* (7) by electrophoresis in 10% polyacrylamide with 0.1% SDS. As reference proteins we used the following commercial enzymes: (1) trypsin (Sigma Chemical Co.), molecular weight 24,000; (3) pepsin (Sigma), molecular weight 35,000; (4) rennin (Serva, Heidelberg), molecular weight 40,000; (5) chymopapain (Sigma), molecular weight 45,000; (6) α -amylase from *Bacillus subtilis* (Serva), molecular weight 48,000; (8) radish peroxidase (Sigma), molecular weight 55,000.

FIG. 8. Plot of relative mobility in SDS-polyacrylamide electrophoresis against molecular weights of known commercial proteins (see legend to Fig. 7) and TRA of *S. aureus* (2) and *E. coli* (7).

FIG. 9. Double immunodiffusion in agar. STRA, TRA from *S. aureus*; ETRA, the same from *E. coli*. SATRA and EATRA contained the respective immunoglobulins against tetracycline resistance antigens. Clearcut crossing of the precipitation lines was observed.

lope extracts of resistant strains grown overnight in NB reacted quite well with ATRA. It should, however, be recalled that only large variations in the amount of resistance antigens are likely to be revealed by such procedures as immunodiffusion and immunoelectrophoresis. Furthermore, what appears as induction of resistance might be due to the synthesis of special lipids (7) binding the resistance protein to a strategically important site (the cell membrane); also, the resistance material might be particularly active during the exponential growth phase due to the need for cofactors or energy.

Specific TRAs were produced from resistant *S. aureus* and *E. coli* by passing an envelope extract of resistant strains through a column of agarose-bound immunoglobulin against envelopes of the respective isogenic susceptible strains. On electrophoresis in SDS-polyacrylamide, these TRAs proved to be quite pure. Both TRAs stained as protein; TRA from *S. aureus* was shown to be susceptible to heat and proteolytic enzymes and resistant to DNase, RNase, and lipase. The molecular weight of TRA from *S. aureus* was calculated to be about 32,000 and from *E. coli* to be about 50,000; the *E. coli* TRA isolated by us might be identical with Tc resistance protein from *E. coli* minicells isolated by Levy et al. at Tuft's University (9). Despite the considerable difference in molecular weight and in antigenicity, the amino acid composition of the staphylococcal and the gram-

negative TRA show a certain degree of resemblance.

Although R plasmid containing strains of *E. coli* has been reported to synthesize several membrane-bound proteins absent in the R⁻variant (8, 9), and this might also be the case in *S. aureus*, we have been able to demonstrate only one major protein fraction after precipitation of our cell wall extracts with immunoglobulin to cell walls of sensitive strains (see Fig. 1 and 7). Also, repeated extraction of cell walls of resistant *S. aureus* did not reveal more than one precipitation line with ATRA (see Fig. 4). The molecular weight of TRA from *E. coli* (50,000) might indicate that this material is identical to the protein that is induced in minicells during incubation with Tc (8). Probably, this material is of functional importance for resistance. For *S. aureus* the correlation between positive reaction for TRA and Tc resistance in a large collection of clinical isolates (Table 2) likewise suggests that this protein is of importance for Tc resistance rather than for another plasmid function, since the susceptible strains probably contained a multitude of other plasmids and the resistant strains might include some with chromosomal resistance (2). However, this essential question needs a direct investigation, and this is currently being undertaken in our laboratory.

The results of this study encourage further efforts to unravel the still obscure mechanism of bacterial resistance to Tc. At this stage it might be most profitable to attack such problems as the exact biochemical set-up of the TRAs, including assay for possible association with cations (14) and their in vitro interaction with Tc (if any).

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