Plasmid-Determined Resistance to Serum Bactericidal Activity: a Major Outer Membrane Protein, the *traT* Gene Product, Is Responsible for Plasmid-Specified Serum Resistance in *Escherichia coli*

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Resistance to the bactericidal activity of serum appears to be an important virulence property of invasive bacteria. The conjugative multiple-antibiotic-resistance plasmid R6-5 was found to confer upon *Escherichia coli* host bacteria increased resistance against rabbit serum. Gene-cloning techniques were used to localize the serum resistance determinant of R6-5 to a segment of the plasmid that encodes conjugal transfer functions, and a pACYC184 hybrid plasmid, designated pKT107, that contains this segment was constructed. The generation and analysis of deletion and insertion mutant derivatives of the pKT107 plasmid that no longer specify serum resistance permitted precise localization of the serum-resistance cistron on the R6-5 map and demonstrated that this locus is coincident with that of *traT*, one of the two surface exclusion genes of R6-5. Examination of the proteins synthesized in *E. coli* minicells of pKT107 and its serum-sensitive mutant derivative plasmids confirmed that the serum-resistance gene product of R6-5 is the *traT* protein and showed that this protein is a major structural component (about 21,000 copies per cell) of the bacterial outer membrane.

Animal serum contains substances that are lethal for most bacteria and that constitute important host defenses against bacterial infection. The major bactericidal activity of serum is the combined lytic action of the complement system and lysozyme on the bacterial surface (for recent review, see M. M. Frank in H. Smith, J. J. Skehel, and M. J. Turner, ed., *The Molecular Basis of Microbial Pathogenicity*, in press). Although nonpathogenic and noninvasive pathogenic bacteria are usually sensitive to serum, invasive pathogens are frequently highly resistant (8, 18, 22, 24). These findings, and studies with experimental animal models (5, 11), indicate that the serum-resistance property of invasive bacteria is an important aspect of their pathogenicity.

Considerable interest has developed in recent years in the mechanisms whereby bacteria may be resistant to serum, although the experiments published so far are quite inconclusive (25, 26). Elucidation of these mechanisms will clearly require detailed studies of bacterial genetic determinants responsible for serum resistance and biochemical and physiological investigations of their gene products.

Recent reports that some plasmids substan-

tially increase serum-resistance levels of their host bacteria (13, 19, 27) indicated to us that the mechanism of at least one type of serum resistance would be readily amenable to analysis by the powerful genetic and molecular approaches, including gene-cloning techniques, that have been developed for the analysis of plasmid functions (31). We report here experiments which show that a serum-resistance factor encoded by the FII incompatibility group conjugative antibiotic-resistance plasmid R6-5 is the *traT* gene product, a major outer membrane protein that mediates plasmid surface exclusion.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* K-12 strains CR34nal, JC3272, and M2141 have been described previously (6, 28). *E. coli* 59 was isolated from the feces of a healthy child and is moderately sensitive to serum; its serotype has, thus far, not been determined. *E. coli* 59rif is a spontaneous mutant that is resistant to 100 µg of rifampin per ml. R6-5 (30) is a conjugative antibiotic-resistance plasmid that confers upon host bacteria resistance to chloramphenicol, kanamycin, streptomycin, sulfonamide, and mercury salts (Fig. 1), and R1-19 is a closely related plasmid that additionally carries the ampicillin transposon Tn3. Plasmid pFC011 is a ColE1 hybrid containing the *EcoRI* fragment E-7 of R6-5 (30). Plasmid pACYC184 is a vector plasmid that encodes resistance to chlor-

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amphenicol and tetracycline and that contains a unique *EcoRI* cleavage site within the chloramphenicol-resistance gene (10). Plasmid deoxyribonucleic acid (DNA) was isolated as previously described (30) and transferred into new host bacteria by either transformation (4) or conjugation (29).

Media and buffers. The general media used for growth of bacteria were L-broth for liquid cultures and antibiotic medium no. 3 (Difco Laboratories) solidified with 1.5% agar (Difco) for plates. Antibiotics were used in media at the following concentrations: ampicillin, 20 $\mu\text{g}/\text{ml}$; chloramphenicol, 50 $\mu\text{g}/\text{ml}$; and tetracycline, 10 $\mu\text{g}/\text{ml}$. Peptone-glucose broth contained 1% peptone (Difco) and 1% glucose. Peptone-glucose-bromothymol blue broth contained additionally 0.5% of a stock solution of 1.5% bromothymol blue (E. Merck) in ethanol. Phosphate-buffered saline contained 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4 , and 0.2 g of KH_2PO_4 , and was adjusted to pH 7.3 prior to autoclaving.

Blood was obtained from healthy adult rabbits, allowed to clot at room temperature for 60 min, and centrifuged at 4°C for 10 min. The serum thus obtained was stored in small quantities in liquid nitrogen until required for use, at which time it was allowed to thaw slowly on ice.

Determination of serum resistance. In order to carry out a genetic analysis of serum resistance, it is necessary to obtain a series of serum-sensitive mutants from an appropriate serum-resistant bacterial strain. This, in turn, requires the availability of a rapid screening method for testing the serum-resistance levels of a large number of bacterial clones. The most reliable method that has thus far been described requires the construction of survival curves of bacteria exposed to serum (e.g., see 18). This method is extremely slow and laborious and quite unsuitable for screening large numbers of clones. We have therefore developed a rapid colorimetric serum-sensitivity assay that is quantitative and that makes it possible to test up to 100 strains of bacteria per day (17). The test has the further advantage that it can be scaled down in volume to a qualitative microtest, which can be performed in microtiter plates, thus allowing up to several thousand clones per day to be screened. The viable-count method and the newly developed colorimetric and microcolorimetric assays for the determination of bacterial resistance to serum have been described in detail elsewhere (17), but in essence consist of mixing serum with washed cells from an exponentially growing bacterial culture and determining the viability of the cells after a 90- or 120-min incubation period. This is carried out either by plating dilutions of the cell suspension on antibiotic medium no. 3 plates (viable-count method) or by measuring the absorbance of the suspension (colorimetric test). In the latter method, the cell-serum suspension medium is peptone-glucose broth containing the pH indicator bromothymol blue. If the bacteria are killed, the medium remains green, whereas if they survive and grow, they produce acid from the glucose and the medium turns yellow (17). The degree of survival of the bacteria is reflected in the rapidity of acid production/color change and is monitored spectrophotometrically as the change in culture absorbance at 500 nm. In the microassay,

bacterial clones to be tested are transferred from agar plates to wells of a microtiter plate containing L-broth. When the bacteria reach the exponential phase of growth, they are transferred with a multipoint inoculator to two microtiter plates containing peptone-glucose-bromothymol blue broth, one with and one without serum. The microtiter plates are incubated for about 3 h, and the serum sensitivity or resistance of each clone is determined by comparing the color change in the wells of the plus and minus serum plates (17). The microassay is particularly useful for the preliminary screening of a large number of strains or a large number of derivatives from a mutated strain.

Analysis of protein expression in minicells. Methods used for the isolation of minicells from cultures of plasmid-carrying derivatives of strain M2141, their labeling with [^{35}S]methionine, and the analysis of their labeled proteins will be described in detail elsewhere (P. A. Manning, B. Kusecek, G. Morelli, and C. Fisseau, submitted for publication). Briefly, the minicells were purified by sucrose gradient centrifugation and labeled with [^{35}S]methionine. Samples of 10 and 5 μl of labeled cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were stained overnight at room temperature in a solution of Coomassie blue G250 (Serva Feinbiochemica; 0.06% in 3.5% perchloric acid), followed by destaining in 5% acetic acid. Radioactive plasmid-encoded proteins were visualized by autoradiography of the dried gel at room temperature for 2 to 8 days on Kodak XR-5 X-ray film.

Other procedures. Bacterial envelopes were prepared as described previously (3), as were outer cell membrane preparations, which consisted of the Triton X-100-insoluble fractions of cell envelopes (3, 21). Restriction endonuclease cleavage of plasmid preparations, agarose gel electrophoretic analysis of the DNA fragments thereby generated, and gene-cloning procedures were carried out as described (4, 30).

RESULTS

Cloning and mapping of the R6-5 serum-resistance determinant. Reynard and Beck reported that the antibiotic-resistance plasmid R100 confers upon *E. coli* K-12 host bacteria resistance to low levels of serum (19). Taylor and Hughes later extended this finding by demonstrating that plasmid NR1 (NR1 is an alternative designation for R100) confers resistance to high levels of serum (75%) upon a smooth *E. coli* strain isolated from river water (27). Like R100/NR1, plasmid R6-5 is a large (100 kilobase), low-copy-number, conjugative, multiple-resistance plasmid (Fig. 1) that belongs to the FII plasmid incompatibility group. Electron microscope heteroduplex studies have shown that R100 and R6-5 share extensive sequence homology (23), and we therefore expected to find that the latter would encode the R100-type serum resistance. The genetic and physical structure of R6-5 has been extensively investigated, and its *EcoRI* and *HindIII* fragments have been cloned

FIG. 1. Physical and genetic map of plasmid R6-5. The R6-5 map (top) is that of Timmis et al. (30); cross bars indicate *EcoRI* cleavage sites. The genetic map of the R6-5 *tra* region (expansion, center) is that of Achtman et al. (2); the bars above the map indicate *EcoRI* cleavage sites whereas bars below the map indicate *HindIII* cleavage sites. The detailed restriction map of the R6-5 *EcoRI* fragment E-7 (bottom) was derived from data shown in Fig. 3 and 4. E, H, P, and B numbers indicate restriction endonuclease fragments generated by *EcoRI*, *HindIII*, *PstI*, and *BstEII*, respectively. The *PstI* and *BstEII* fragments of the E-7 fragment are numbered according to size as they exist in the pKT107 hybrid plasmid, for ease of recognition in Fig. 3, 4, and 5, and not according to size as they exist in R6-5. Abbreviations: *Cm*^r, *Km*^r, *Sm*^r, *Su*^r, and *Hg*^r, resistance to chloramphenicol, kanamycin, streptomycin, sulfonamide, and mercury salts, respectively; Rep and IS1, replication functions and insertion sequence 1; RTF, R-det, and tra, resistance transfer factor, resistance determinant, and transfer functions, respectively.

on the high-copy-number vectors ColE1 and pML21, respectively (30). This plasmid is therefore an attractive system for genetic and molecular studies of plasmid-specified serum resistance.

To determine whether R6-5 encodes a serum-resistance factor, we introduced it by conjugation into wild (59rif) and laboratory (K-12 CR34nal) strains of *E. coli* and compared the serum-resistance levels of plasmid-containing and plasmid-lacking cells. As can be seen in Fig. 2, *E. coli* 59rif bacteria tolerated serum levels up

to 4% but were rapidly killed by serum at concentrations of 10 to 20%. In contrast, *E. coli* 59rif bacteria containing plasmid R6-5 were quite resistant to serum at these concentrations. The "rough" laboratory *E. coli* K-12 strain was highly sensitive to serum, and K-12 bacteria were rapidly killed by 2 to 3% serum. Again, R6-5-carrying bacteria were quite resistant to these low levels of serum, although they were killed at higher concentrations. Because of the parallel responses to serum of the wild and K-12 strains of *E. coli*, and the ease of conducting genetic

and structural analyses with the latter, we subsequently analyzed R6-5-mediated serum resistance, using the response of the K-12 strain to 3% rabbit serum.

FIG. 2. Resistance to the lethal activity of serum conferred upon host bacteria by R6-5 and pKT107. Exponentially growing bacteria were washed in phosphate-buffered saline and mixed with rabbit serum at the indicated concentrations, and survivors were determined after incubation for 90 min as described in Materials and Methods and reference 17. Open and filled squares represent plasmid-negative and plasmid R6-5-carrying bacteria, respectively, whereas filled circles represent pKT107-carrying bacteria. (A) *E. coli* 59rif; (B) *E. coli* K-12 CR34nal.

To locate the approximate position of the R6-5 serum-resistance determinant, we measured the sensitivity/resistance to 3% rabbit serum of derivatives of CR34nal bacteria carrying individual ColE1 and pML21 hybrid plasmids that contain the various cloned *EcoRI* and *HindIII* fragments of R6-5. Two hybrid plasmids, namely pFC011 and pKT001, were found to specify serum resistance (Fig. 2). Plasmid pFC011 carries R6-5 *EcoRI* fragment E-7, whereas plasmid pKT001 carries R6-5 *HindIII* fragment H-1; fragment E-7 is entirely contained within fragment H-1 (Fig. 1 and reference 30). The serum-resistance determinant has therefore been shown to be located on a 6-kilobase DNA segment having termini with R6-5 coordinates 81.5 and 87.5 kilobases. This segment of DNA contains the *traS* and *traT* genes (surface exclusion genes) and *traD* genes of the transfer region of R6-5, i.e., the region responsible for its conjugal proficiency (2).

The ColE1 vector plasmid specifies only colicin E1 immunity as a selection function and is inconvenient for a variety of genetic manipulations. In order to have the E-7 fragment on a more easily selected vector plasmid, it was transferred into the tetracycline-resistance pACYC184 vector plasmid (10) by standard cloning procedures. One pACYC184/E-7 hybrid plasmid, designated pKT107, was selected for further studies and shown by the colorimetric and the viable-count serum-resistance tests to confer upon host bacteria resistance to serum. This hybrid plasmid was subsequently analyzed

FIG. 3. Mapping of restriction endonuclease cleavage sites in the E-7 fragment of R6-5. Preparations of pACYC184 (10) and pKT107 (pACYC184 + R6-5 E-7 fragment) were cleaved with the indicated restriction endonucleases using conditions recommended by the suppliers, and the fragments generated were analyzed by electrophoresis through a tris(hydroxymethyl)aminomethane-borate-buffered 1% agarose gel as described previously (4). (a) pKT107/PstI; (b) pKT107/PstI + *EcoRI*; (c) pACYC184/*EcoRI*; (d) pKT107/HpaI + *EcoRI*; (e) pKT107/PstI + HpaI + *EcoRI*; (f) pACYC184/BamHI + *EcoRI*; (g) pKT107/PstI + BamHI + *EcoRI*; (h) pKT107/PstI + HpaI + BamHI + *EcoRI*; (i) pKT107/BstEII + BamHI + *EcoRI*; (j) pKT107/BstEII + *EcoRI*; (k) pKT107/BamHI + *EcoRI*; (l) pKT107/BamHI + HpaI + *EcoRI*; (m) pACYC184/PvuII + *EcoRI*; (n) pKT107/PvuII + *EcoRI*; (o) pACYC184/PvuII + BamHI + *EcoRI*; (p) pKT107/PvuII + BamHI + *EcoRI*; (q) pKT107/PvuII + HpaI + *EcoRI*.

by cleavage with a variety of restriction endonucleases (Fig. 3), and a detailed restriction map of the E-7 fragment was obtained (Fig. 1).

Generation of serum-sensitive deletion and insertion mutant derivatives of the pKT107 plasmid. Although there are no *Pst*I sites in the pACYC184 vector plasmid (10), there are six in the R6-5 E-7 fragment (Fig. 1). We were therefore able to generate deletion derivatives of the pKT107 plasmid by in vitro elimination of one or more of the *Pst*I subfragments of the E-7 fragment. pKT107 DNA was partially cleaved with *Pst*I endonuclease, and the fragments thereby generated were treated with DNA ligase. The ligation mixture was used to transform competent cells of *E. coli* K-12 CR34 nal , and a total of 392 transformant clones were obtained. These were then screened with the microcolorimetric serum-sensitivity test described above, and 22 were found to be serum sensitive. Plasmid DNA was prepared from these clones, treated with *Pst*I endonuclease, and analyzed by agarose gel electrophoresis (Fig. 4). All serum-sensitive *Pst*I deletion mutants of pKT107 were found to lack one of the two adjacent *Pst*I fragments, P-4 and P-6, indicating that the serum-resistance determinant is contained on these two fragments.

Although the serum-sensitive deletion derivatives of the pKT107 plasmid provided an approximate localization of the serum-resistance gene of R6-5, the nonrandomness of the deletion

termini; namely, the fact that they are *Pst*I cleavage sites, prevents precise localization of the gene. To identify more exactly the region encoding serum resistance, we isolated and analyzed serum-sensitive insertion mutants of plasmid pKT107. To obtain these, we introduced plasmid R1-19, which carries the ampicillin resistance transposon Tn3, into *E. coli* K-12 CR34 nal carrying plasmid pKT107 by conjugation (29). Four bacterial clones carrying both plasmids were purified and cultured for a further 25 generations. Plasmid DNAs were then prepared from cultures of these clones and used to transform cells of CR34 nal . We obtained 309 transformants that were resistant to ampicillin and tetracycline, but not to chloramphenicol or streptomycin (i.e., contained pKT107 derivatives into which the Tn3 element of R1-19 had transposed). These transformants were then tested by the microcolorimetric test for serum resistance, and 12 were tentatively identified as being serum sensitive. The serum sensitivity of the 12 colonies was subsequently confirmed by the quantitative colorimetric test. Plasmid DNA was then isolated from the clones and characterized by cleavage analysis with restriction endonucleases *Bst*EII and *Pst*I. As can be seen in Fig. 5, none of the insertion mutants retained the small *Bst*EII fragment of pKT107; i.e., the insertions were all located within this *Bst*EII fragment. *Pst*I digestion of the insertion mutants showed that three of the insertions were located in *Pst*I fragment P-4, seven in fragment P-6, and two in fragment P-5, thus confirming the results obtained with the serum-sensitive plasmid deletion mutants. The serum-resistance determinant of R6-5 is therefore located on a short DNA sequence about 600 base pairs in length that includes the small *Bst*EII DNA fragment of pKT107; careful genetic mapping experiments with derivatives of plasmid R100 have recently placed the surface exclusion gene *traT* in this region (B. Hansen, P. A. Manning, and M. Achtman, in preparation).

Identification of the product of the R6-5 serum-resistance gene product, *traT*p, as the surface exclusion protein. To identify the serum-resistance gene product and to determine whether it is the *traT* protein, we examined the proteins encoded by the pKT107 plasmid and its serum-sensitive insertion and deletion mutant derivatives. Minicells of plasmid-carrying derivatives of strain M2141 were purified and labeled with [35 S]methionine, and the cell proteins were analyzed by electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel. A photograph of the stained gel, showing total cellular proteins, and an autoradiograph of the dried gel, showing the plasmid-specific proteins, are pre-

FIG. 4. Restriction endonuclease cleavage analysis of serum-sensitive plasmid deletion derivatives of pKT107. *Pst*I deletion mutants of plasmid pKT107 were isolated as described in Results and analyzed by cleavage with the *Pst*I endonuclease followed by electrophoresis through a 1% agarose gel. Track e: pKT107. Tracks a-d and f-i: pKT107 deletion mutants. (a) pKT108; (b) pKT109; (c) pKT110; (d) pKT111; (f) pKT112; (g) pKT113; (h) pKT114; (i) pKT115.

FIG. 5. Localization of the *Tn3* insertions in serum-sensitive *Tn3* insertion derivatives of pKT107. Plasmids in tracks a-f and h-m are different pKT107 insertion mutants and are identical to those in tracks n-s and u-z, respectively. The plasmid in tracks g and t is pKT107. DNA preparations in tracks a-m are digested with *BstEII*, whereas those in tracks n-z are digested with *PstI*. (a, n) pKT116; (b, o) pKT117; (c, p) pKT118; (d, q) pKT119; (e, r) pKT120; (f, s) pKT121; (h, u) pKT122; (i, v) pKT123; (j, w) pKT124; (k, x) pKT125; (l, y) pKT126; (m, z) pKT127.

sented in Fig. 6A. From the stained gel, it can be seen that a 25,000-dalton protein species, which corresponds to the *traT* gene product, is a major component of bacteria carrying pKT107 (Fig. 6A, track a) but is lacking from bacteria carrying serum-sensitive insertion derivatives of this plasmid (tracks b-f). This result is confirmed by the autoradiogram which shows the 25,000-dalton *traT* protein to be the major pKT107 plasmid-specified product. This species is completely lacking in minicells carrying serum-sensitive plasmids. It should be noted that a slightly smaller, less prominent species that could be a breakdown product of the *traT* protein is also missing in the serum-sensitive derivatives. None of the *Tn3* insertions which cause loss of serum resistance and *traT* protein cause loss of either the *traD* or the *traS* protein, which have been

identified recently (B. Hansen, P. A. Manning, and M. Achtman, in preparation) and are known to be encoded by genes located adjacent to *traT*. Insertion of *Tn3* into the serum-resistance gene of pKT107 results in the synthesis of several new proteins. Two major proteins, which have molecular weights of 26,000 and 27,000, correspond to products of the β -lactamase gene of *Tn3*, whereas several minor products may represent fusion peptides created by the insertions.

The *traT* proteins of several F-like sex factors are closely related to the *traT* protein of F (P. A. Manning and M. Achtman, unpublished data), which is an outer membrane protein exposed on the cell surface (1, 16a). We therefore examined the outer membranes (Triton X-100-insoluble component of the cell envelopes) for the presence of *traT* protein (Fig. 6B). As anticipated from the minicell experiments, no *traT* protein was detected in the outer membranes of bacteria carrying serum-sensitive plasmid derivatives (Fig. 6B, tracks b-f), whereas it was detected in amounts corresponding to about 21,000 copies per cell in the outer membranes of pKT107-carrying bacteria (Fig. 6B, track a). The envelopes of bacteria carrying serum-sensitive derivatives of pKT107 deleted for *PstI* fragments P-4 or P-6 also lacked *traT* protein (not shown).

DISCUSSION

Bactericidal and lytic activities in serum are thought to represent important primary host defenses against microbial infection. The major lethal activity of serum is the complement system which attacks the surface layers of invading bacteria. Complement may be activated via the formation of specific antigen-antibody complexes (classical pathway) between the surface antigens of invading microbes and preexisting antibody, or directly by cell envelope lipopolysaccharide structures (alternate pathway) (for recent review, see Frank, in press). Invasive bacterial pathogens are usually highly resistant to the lethal activities of serum (8, 18, 22, 24), and it is assumed that serum resistance is an important virulence property of such strains. Although the precise mechanisms whereby bacteria resist serum are thus far not elucidated (25, 26), it seems likely that they involve cell surface alterations that inhibit the activation or functioning of complement. Such surface alterations could result from the synthesis of new cell envelope structural components that (i) shield surface antigens and prevent their interaction with specific antibodies, thereby hindering activation of complement via the classic pathway, (ii) shield cell surface lipopolysaccharides and prevent their direct activation of complement via the

FIG. 6. Identification of the serum-resistance gene product as the *traT* protein. (A) Analysis of plasmid-encoded proteins synthesized in minicells. Minicells containing pKT107 (a) or its serum-sensitive Tn3 insertion derivatives (b-f) were purified, radioactively labeled with [35 S]methionine, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was subsequently stained (left) and autoradiographed (center). (b) pKT116; (c) pKT117; (d) pKT118; (e) pKT119; (f) pKT120. (B) Analysis of outer membrane proteins of plasmid-carrying bacteria. The outer membranes (Triton X-100-insoluble component of the cell envelope) of cells harboring pKT107 or its serum-sensitive Tn3 insertion derivatives (see A) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining.

alternate pathway, as has been proposed for polysaccharide capsules (J. R. Robin in H. Smith, J. J. Skehel, and M. J. Turner, ed., *The Molecular Basis of Microbial Pathogenicity*, in press), or (iii) interfere with one or more of the steps of complement action on the bacterial surface. Serum resistance presumably could also result from the synthesis of products that directly inactivate complement components. Analysis of serum-resistance mechanisms will require genetic and biochemical analyses of bacterial components involved, including their localization and quantitation, and also characterization of the specific host factors with which they interact.

In this report, we have presented evidence which shows that the conjugative multiple-antibiotic-resistance plasmid R6-5 can confer upon host bacteria substantial resistance to the lethal activity of serum. Because serum resistance and plasmid-mediated bacterial conjugation are both thought to involve specific structural changes of the cell surface, it is perhaps not surprising that the serum resistance determinant was found to be a conjugation cistron. R6-5-mediated resistance to serum results from the activity of the *traT* gene which (with *traS*) is responsible for surface exclusion, the reduced ability of plasmid-carrying bacteria to act as effective recipients in conjugation (1). The *traT* protein, which is present in about 21,000 copies per cell, is a major

structural component of the cell envelope and is almost certainly situated on the outer surface of the outer membrane. It therefore fulfills the expectations for one type of serum-resistance factor. Recent experiments have shown that serum resistance in *Neisseria gonorrhoeae* is also correlated with a specific bacterial outer membrane protein (15).

It should be mentioned that the role of the *traT* protein in bacterial resistance to serum is highly specific. We have recently shown that plasmids containing point mutations in the *traT* gene fail to protect host bacteria from serum, even though such bacteria may contain much greater quantities of a *traT*-like protein in their outer membranes than do bacteria containing the parental plasmid (P. A. Manning, A. Moll, C. Echarti, and K. N. Timmis, in preparation). Thus, the presence of large quantities of a *traT*-like protein in the bacterial outer membrane is, of itself, insufficient for serum resistance. This suggests that the *traT* protein must interact with other bacterial surface structures in a highly specific way in order to provide protection against serum. The finding of Taylor and Hughes that plasmid R100 protects some bacteria but not others from the lethal effects of serum (27) is consistent with this finding.

Fietta et al. reported that a number of different antibiotic-resistance plasmids confer upon host bacteria resistance against the lethal activ-

ity of serum and suggested that several distinct types of plasmid gene product may be responsible for this property (13). Because R6-5 shares extensive polynucleotide sequence homology with other IncFII-group plasmids like R100/NR1 and R1 (23), it is almost certain that the serum resistance encoded by these plasmids (19, 20) is also due to the *traT* gene product. On the other hand, Binns et al. reported recently the mapping of a serum-resistance determinant of ColV,I-K94 at a location outside of the *tra* region of this plasmid (7). Serum resistance mediated by the R6-5 and ColV,I plasmids would therefore appear to involve different gene products, although firm conclusions must await identification of the ColV,I serum-resistance gene and gene product. The cloning of the R6-5 and ColV,I serum resistance genes on high-copy-number vector plasmids now provides specific DNA hybridization probes which will permit their direct comparison and which should be of considerable use for molecular epidemiological studies of the prevalence of these types of serum resistance in invasive bacteria (see 30 and 31 for more detailed discussion of this point).

Two important questions must now be addressed: what is the mechanism of plasmid-determined serum resistance, and what is the contribution of this type of resistance to the virulence of invasive bacteria? To the first question we can presently say very little. Adult rabbit and human sera usually contain specific antibodies against *E. coli* antigens. Our plasmid-negative strains are rapidly killed when mixed with serum at appropriate concentrations, suggesting that they are attacked by complement activated by the classical pathway. On the other hand, bacteria of the C8⁻ strain of Taylor and Hughes are killed only after 60 min of exposure to serum (27), indicating that they are attacked by complement activated by the alternate pathway. In all cases, the bacteria are protected by R6-5/R100. This may indicate that the *traT* protein either shields all reactive structures (specific surface antibody binding sites and deeper lipopolysaccharide structures) on the bacterial surface that activate complement or causes the bacterial membrane in some way to resist lysis by activated complement. To distinguish among these and other possible mechanisms, we will require substantially more experimental data on the mechanism of killing of bacteria by serum. There is also insufficient evidence currently available to answer the second question in a definitive manner. The ColV plasmid is known to be a major virulence determinant of some invasive *E. coli* strains (9). Recently, Binns et al. mapped the virulence and serum-resistance

genes of this plasmid to the same 5.3-kilobase DNA segment and concluded that these two plasmid activities probably result from the expression of a single gene.

It is of course well known that a variety of key bacterial virulence properties, including several toxins, adhesion antigens, and iron-scavenging systems (32), are encoded by plasmids (9). Indeed, plasmids appear to be evolving in such a way as to accumulate multiple virulence functions on single replicons (9). It is known that serum potentiates the bactericidal activity of antibiotics and vice versa (12). The existence of plasmids encoding resistance to both serum and antibiotics may represent an early stage in this evolution. In this connection it is worth noting that epidemiological investigations of enteric infections caused by *Salmonella* species have shown in at least two instances that a change in incidence pattern from one of occasional mild infections to one of epidemics of severe infections was associated with the acquisition of multiple-antibiotic resistance by the causative organisms. Prior to 1970, antibiotic-susceptible *Salmonella wien* strains were responsible for sporadic cases of salmonellosis in France. Since 1970, however, there have been numerous epidemics of salmonellosis caused by multiply resistant derivatives of this serotype. These recent, more virulent forms of *S. wien* carry a plasmid that confers resistance to streptomycin, sulfonamide, ampicillin, tetracycline, chloramphenicol, and kanamycin (16). A similar change in the pattern of typhoid fever infections in Mexico has also been correlated with the acquisition of an antibiotic-resistance plasmid (14). Thus, although no causal relationship between the increase in bacterial virulence responsible for the epidemic spread of the two *Salmonella* serotypes and their acquisition of antibiotic-resistance plasmids has thus far been established, it seems quite likely that the plasmids encode a product which directly contributes to the virulence of these invasive bacteria. A serum-resistance outer membrane protein is one candidate for such a product.

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LITERATURE CITED

1. Achtman, M., N. Kennedy, and R. Skurray. 1977. Cell-cell interactions in conjugating *Escherichia coli*: role of *traT* protein in surface exclusion. *Proc. Natl. Acad. Sci. U.S.A.* 74:5104-5108.

2. Achtman, M., B. Kušček, and K. N. Timmis. 1978. *tra* cistrons and proteins encoded by the *Escherichia coli* antibiotic resistance plasmid R6-5. *Mol. Gen. Genet.* **163**:169-179.
3. Achtman, M., S. Schwuchow, R. Helmuth, G. Morelli, and P. A. Manning. 1978. Cell-cell interactions in conjugating *Escherichia coli*: Con⁻ mutants and stabilization of mating aggregates. *Mol. Gen. Genet.* **164**:171-183.
4. Andrés, I., P. M. Slocombe, F. Cabello, J. K. Timmis, R. Lurz, H. J. Burkhardt, and K. N. Timmis. 1979. Plasmid replication functions. II. Cloning analysis of the *RepA* replication region of antibiotic resistance plasmid R6-5. *Mol. Gen. Genet.* **168**:1-25.
5. Archer, G., and F. R. Fekety. 1976. Experimental endocarditis due to *Pseudomonas aeruginosa*. I. Description of a model. *J. Infect. Dis.* **134**:1-7.
6. Bachmann, B. J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriol. Rev.* **36**:525-557.
7. Binns, M. M., D. L. Davies, and K. G. Hardy. 1979. Cloned fragments of the plasmid ColV, I-K94 specifying virulence and serum resistance. *Nature (London)* **279**:778-781.
8. Björkstén, B., and B. Kaijser. 1978. Interaction of human serum and neutrophils with *Escherichia coli* strains: differences between strains isolated from urine of patients with pyelonephritis or asymptomatic bacteriuria. *Infect. Immun.* **22**:308-311.
9. Cabello, F., and K. N. Timmis. 1979. Plasmids of medical importance, p. 55-69. In K. N. Timmis and A. Pühler (ed.), *Plasmids of medical, environmental and commercial importance*. Elsevier/North Holland, Amsterdam.
10. Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. *J. Bacteriol.* **134**:1141-1156.
11. Durack, D. T., and P. B. Beeson. 1977. Protective role of complement in experimental *Escherichia coli* endocarditis. *Infect. Immun.* **16**:213-217.
12. Dutcher, B. S., A. M. Reynard, M. E. Beck, and R. K. Cunningham. 1978. Potentiation of antibiotic bactericidal activity by normal human serum. *Antimicrob. Agents Chemother.* **13**:820-826.
13. Fietta, A., E. Romero, and A. G. Siccardi. 1977. Effect of some R factors on the sensitivity of rough *Enterobacteriaceae* to human serum. *Infect. Immun.* **18**:278-282.
14. Gangarosa, E. J., J. V. Bennett, C. Wyatt, P. E. Pierce, J. Olarte, P. M. Hernandez, V. Vásquez, and D. Bessudo M. 1972. An epidemic-associated episode? *J. Infect. Dis.* **126**:215-218.
15. Hildebrandt, J. F., L. W. Mayer, S. P. Wang, and T. M. Buchanan. 1978. *Neisseria gonorrhoeae* acquire a new principal outer membrane protein when transformed to resistance to serum bactericidal activity. *Infect. Immun.* **20**:267-273.
16. Le Minor, S. 1972. Apparition en France d'une épidémie à *Salmonella wien*. *Med. Malad. Infect.* **2**:441-448.
- 16a. Manning, P. A., L. Bentin, and M. Achtman. 1980. Outer membrane of *Escherichia coli*: properties of the F sex factor *traT* protein which is involved in surface exclusion. *J. Bacteriol.* **142**:285-294.
17. Moll, A., F. Cabello, and K. N. Timmis. 1979. Rapid assay for the determination of bacterial resistance to the lethal activity of serum. *FEMS Lett.* **6**:273-276.
18. Olling, S. 1977. Sensitivity of Gram-negative bacilli to the serum bactericidal activity: a marker of the host parasite relationship in acute and persisting infections. *Scand. J. Infect. Dis. Suppl.* **10**:1-40.
19. Reynard, A. M., and M. E. Beck. 1976. Plasmid-mediated resistance to the bactericidal effects of normal rabbit serum. *Infect. Immun.* **14**:848-850.
20. Reynard, A. M., M. E. Beck, and R. K. Cunningham. 1978. Effects of antibiotic resistance plasmids on the bactericidal activity of normal rabbit serum. *Infect. Immun.* **19**:861-866.
21. Schnaitman, C. A. 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. *J. Bacteriol.* **108**:545-552.
22. Schoolnik, G. K., T. M. Buchanan, and K. K. Holmes. 1976. Gonococci causing disseminated gonococcal infection are resistant to the bactericidal action of normal human sera. *J. Clin. Invest.* **58**:1163-1173.
23. Sharp, P. A., S. N. Cohen, and N. Davidson. 1973. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. II. Structure of drug resistance (R) factors and F factors. *J. Mol. Biol.* **75**:235-255.
24. Simberkoff, M. S., I. Ricupero, and J. J. Rahal, Jr. 1976. Host resistance to *Serratia marcescens* infection: serum bactericidal activity and phagocytosis by normal blood leucocytes. *J. Lab. Clin. Med.* **87**:206-217.
25. Taylor, P. W. 1975. Genetical studies of serum resistance in *Escherichia coli*. *J. Gen. Microbiol.* **89**:57-66.
26. Taylor, P. W. 1976. Immunochemical investigations on lipopolysaccharides and acidic polysaccharides from serum-sensitive and serum resistant strains of *Escherichia coli* isolated from urinary tract infections. *J. Med. Microbiol.* **9**:405-421.
27. Taylor, P. W., and C. Hughes. 1978. Plasmid carriage and the serum sensitivity of enterobacteria. *Infect. Immun.* **22**:10-17.
28. Thompson, R., and M. Achtman. 1978. The control region of the F sex factor DNA transfer cistrons: restriction mapping and DNA cloning. *Mol. Gen. Genet.* **165**:295-304.
29. Timmis, K. N. 1972. Purification and characterization of colicin D. *J. Bacteriol.* **109**:12-20.
30. Timmis, K. N., F. Cabello, and S. N. Cohen. 1978. Cloning and characterization of *EcoRI* and *HindIII* restriction endonuclease-generated fragments of antibiotic resistance plasmids R6-5 and R6. *Mol. Gen. Genet.* **162**:121-137.
31. Timmis, K. N., S. N. Cohen, and F. Cabello. 1978. DNA cloning and the analysis of plasmid structure and function. *Prog. Mol. Subcell. Biol.* **6**:1-58.
32. Williams, P. H., and H. K. George. 1979. ColV plasmid-mediated iron uptake and the enhanced virulence of invasive strains of *Escherichia coli*, p. 161-172. In K. N. Timmis and A. Pühler (ed.), *Plasmids of medical, environmental and commercial importance*. Elsevier/North Holland, Amsterdam.