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Integration of the Overproduced Bacteriophage T5 Receptor Protein in the Outer Membrane of *Escherichia coli*

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The *tonA* gene codes for an outer membrane protein, a receptor of phage T5, the TonA protein. Strains harboring pLG513, a multicopy plasmid in which the *tonA* gene has been cloned, overproduced TonA protein, which appeared in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell envelope proteins as a 78,000-molecular-weight protein. Identical results have been observed by Plastow et al. (FEBS Lett. **131**:262–264, 1981) with plasmid pLC19-19, in which the *tonA* gene has also been cloned. The activity of the TonA protein, measured by its capacity to inactivate phage T5, increased by five- to sixfold in purified envelopes of cells harboring pLG513 compared with cells lacking the plasmid. Solubilization of the cytoplasmic membrane by Triton-Mg^{2+} treatment did not increase this activity. However, partial solubilization of outer membrane proteins by Triton-EDTA unmasked further T5 receptor activity, resulting in a final increase of around 50-fold, a value more consistent with the expected gene dosage effect. Treatment of whole cells by trypsin in conditions in which trypsin is allowed to enter the outer membrane revealed that part of the overproduced T5 receptors were embedded in the outer membrane and masked by a trypsin-sensitive protein. In addition, no T5 receptor activity was detected in either the periplasmic space or the cytoplasm. These results suggest that all of the overproduced TonA molecules were synthesized in an active form and integrated in the outer membrane, but only a small fraction could be reached or recognized by phage T5 in vivo.

Protein TonA, a minor *Escherichia coli* outer membrane protein, is involved in ferrichrome uptake and used as a specific receptor by phages T1, T5, and φ80 and by colicin M (6, 35). Hancock et al. (15) identified the TonA protein as a 78,000-(78K) molecular-weight polypeptide by electrophoresis of proteins from *E. coli* $tonA^+$ and *E. coli tonA* strains. Dubertret and Legault-Demare showed that TonA protein is present as only 400 molecules per cell in *E. coli* K-12 (10); in contrast to most of the outer membrane proteins mediating iron transport, this protein is only slightly induced by iron starvation (5, 21): Hantke (16) has measured, after induction, a TonA protein level increased two- to threefold over that of the control. To overcome this difficulty, Plastow et al. (26), in elegant experiments, showed the synthesis of a large quantity of 78K-molecular-weight polypeptide to be directed by the pLC19-19 plasmid (7) in which the *tonA* gene is encoded. This polypeptide, undetectable in the parent strain, was then identified as TonA protein.

In this work we used a multicopy plasmid overproducing TonA protein to answer the questions of (i) whether all or only some of the overproduced receptors were located in a physiological site at the outer membrane surface where they can be reached by the phage; and (ii) if not at the outer membrane surface, where could the overproduced TonA proteins be located in the cell?

MATERIALS AND METHODS

Strains and phage. Bacteriophage T5sto was used in all experiments, and *E. coli* F was the indicator strain for plating phage (10). Plasmid pLG513 (Tc^r Cm^r) was prepared from an *E. coli recA* strain (Table 1) according to Guerry et al. (12). It was purified by dye-buoyant density centrifugation in cesium chloride gradients containing ethidium bromide (8) and then analyzed after digestion by restriction enzyme *Pst*I (Boehringer, Mannheim, West Germany) used as recommended by the manufacturer. The ability of cells transformed by pLG513 to acquire T5 sensitivity was tested on recombinants of *E. coli* GC375(5), a resistant mutant isolated in our laboratory. The recombinants were screened for tetracycline and chloramphenicol resistance and tested for their sensitivity to phage T5. All recombinants tested were T5 sensitive. The presence of the initial plasmid in the recombinants was verified by plasmid extraction and analysis of *Pst*I DNA digest by agarose gel electrophoresis, according to the method of Birnboim and Doly (3). *E. coli*

TABLE 1. *E. coli* strains

Strain	Genotype/phenotype	Origin
Rec-35-I (pLG513)	*thr leu his ile arg thi thyA lac tonA tsx recA* Str^r Tc^r Cm^r	M. Jackson
GC375 (= AB2480)	*uvrA recA pro lac gal* Str^r	R. d'Ari
GC375/5	From GC375, $T5^r$	
GC375 (pLG513)	From GC375/5, Tc^r Cm^r	
JE5505	F^- *lpo ppo his proA argE Thi gal lac xyl mtl tsx*	Suzuki et al. (33)
JE5505 (pLG513)	From JE5505, Tc^r Cm^r	

JE5505 was also transformed by pLG513. Strains used are reported in Table 1.

Culture conditions. Bacteria were grown in complete medium (0.8% nutrient broth, 0.4% tryptone, 50 mM Tris-hydrochloride [pH 7.4], 50 mM NaCl). Cells were harvested in exponential phase at 6×10^8 cells per ml. When needed, tetracycline and chloramphenicol were added at final concentrations of 10 and 25 μg/ml, respectively.

Preparation and fractionation of outer membrane. Outer membranes were prepared as described by Schnaitman (29) with slight modifications. Cells were grown in complete medium, harvested at 6×10^8 cells per ml, washed in buffer A (50 mM Tris-hydrochloride [pH 7.4], 50 mM NaCl), and then concentrated (1/20 initial volume) in buffer A; 20 μg of DNase per ml was added with $MgSO_4$ (10 mM final concentration). Cells were disrupted at 4°C by two passages through a French press cell at 20,000 lb/in^2.

The cell extract was incubated for 15 min at 37°C, to complete DNase action, and then envelopes were concentrated over a 70% sucrose cushion by centrifugation at 4°C (25,000 rpm, 16 h; SW27 rotor). The band of envelopes was harvested and diluted in buffer A (1/8 initial volume) and concentrated by centrifugation; then the envelopes were treated with 2% Triton X-100–50 mM $MgSO_4$ to solubilize cytoplasmic membrane (30). After centrifugation at 4°C (50,000 rpm, 1 h; Spinco rotor type 65 Ti), the pellet was resuspended in 2% Triton X-100–50 mM EDTA and incubated for 15 min at room temperature. At this step, lipopolysaccharides and some proteins of the outer membrane were solubilized.

Treatment of whole cells. Cells harvested at 6×10^8 per ml were washed with buffer A (1/10 initial volume) and incubated for 10 min at 37°C with 2% Triton X-100–2 mM EDTA and then centrifuged in an Eppendorf centrifuge to eliminate cell debris.

Phage T5 adsorption capacity of outer membrane and cell extracts of whole cells. For a final incubation volume of 200 μl, 100 μl of phage T5 suspension (10^{12} PFU/ml) was incubated with decreasing quantities of membrane or cell extract for 1 h at room temperature and then diluted, and the lytic unadsorbed phages were titrated. The number of inactivated phages was calculated by difference. Quantities of membrane fraction were expressed in cell-equivalents. The number of T5 receptors was determined from the plateau of saturation curves. In the stock of phages used here, lytic phages amounted only to 50% of the total population, whereas the other half consisted of killer phages able to adsorb to the host cell but not to form plaques (19). To determine the total number of T5 receptors in a cell, the number of receptors for lytic phages was multiplied by 2.

In experiments with whole cells, bacteria were first centrifuged and then washed with buffer A; incubation with T5 phages was performed for 30 min in the presence of 300 μg of spectinomycin per ml to stop protein synthesis. Bacteria were then centrifuged, and free T5 phages were measured in the supernatant. The number of T5 receptors per cell was determined as previously described.

Protein assays. Proteins were estimated by the colorimetric method of the Bio-Rad protein assay, as described by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

Protein fractionation by sequential ammonium sulfate precipitation. Triton X-100 was first removed from samples of outer membrane proteins by treatment with SM_2 Bio-beads (Bio-Rad Laboratories, Richmond, Calif.), as described by Holloway (18); Bio-beads were eliminated by decantation, and proteins of the supernatants were precipitated by 10, 20, and 35% ammonium sulfate saturation. Pellets were solubilized by the initial volume of 2% Triton X-100–buffer A, and each solution was tested for its capacity to inactivate T5 phage.

Formation of spheroplasts and protoplasts. *E. coli* protoplasts were prepared according to Weiss (36); spheroplasts were obtained by the procedure described by Osborn et al. (25).

Digestion with trypsin. Cells were harvested at a concentration of 5×10^8 cells per ml, washed in 10 mM Tris-hydrochloride, pH 7.4, and then incubated with or without 10 mM $Mg(CH_3COO)_2$ and 50 μg of trypsin per ml for 45 min at 4°C, as described by Halegoua and Inouye (13). Cells were then washed with 10 mM Tris-hydrochloride (pH 7.9), resuspended in 1/30 of the initial volume of buffer A, and tested for their T5 receptor activity as previously described.

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis of proteins was performed according to Laemmli (22). When required, Triton X-100 was removed from protein samples by treatment with SM_2 Bio-beads, and then samples were concentrated by precipitation with 10% trichloroacetic acid and denatured by standing for 1.5 min at 100°C in the presence of 1% SDS–1% mercaptoethanol; 10% acrylamide gels were used.

Electrophoretic analysis of DNA was carried out in 0.7% agarose gels in borate buffer (0.1 M Tris-hydrochloride, 0.1 M sodium borate, 3 mM EDTA).

RESULTS

Increase of T5 receptor activity due to presence of plasmid pLG513 in the cell. (i) Unmasking of activity by fractionation of purified envelopes. Multicopy plasmids in which the *E. coli tonA* gene has been cloned provided a useful tool for overproducing this protein.

The plasmid used in this work was a generous gift from Maria Jackson and J. Pratt. This multi-

TABLE 2. Total T5 receptor sites per cell-equivalent or per cell

Expt		Receptor sites/cell-equivalent with strain:		Amplification factor	Receptor sites/cell with strain:		Amplification factor
		JE5505	JE5505 (pLG513)		GC375	GC375 (pLG513)	
Whole cells		174	630	3.6	124	750	6
Triton-EDTA-treated whole cells	Supernatant	554	7,920	14.6	160	2,300	14.3
	pellet	ND[a]	ND		80	500	
Purified envelopes		470	2,520	5.3	390	2,380	6.1
Triton X-100–Mg^{2+}-treated envelopes	Supernatant	ND	ND		ND	ND	
	pellet	470	2,500		390	2,480	
Triton X-100–EDTA-treated Triton-Mg^{2+} pellet	Supernatant	484	23,800	49	531	22,400	53
	pellet	ND	ND				

[a] ND, Not detectable.

copy plasmid, pLG513, derived from pBR325, contains a duplicate *PstI* fragment encoding "*tonA*" cloned from pLC19-19 (34; B. Holland, personal communication). We have introduced this plasmid by transformation into *E. coli* JE5505, an *lpo* mutant from which the T5 receptor has been shown to be efficiently extracted (11), and also into *E. coli* GC375, a *uvrA* mutant able to function as a maxicell in a semi-in vitro system (27).

(ii) Whole cells and purified envelopes. The number of T5 phage receptors per cell for whole cells from four strains was determined as described in Materials and Methods. Results are summarized in Table 2. Around 100 to 200 receptors per cell were measured in cells without plasmid, and 600 to 750 were measured in cells harboring plasmid pLG513. Due to the presence of the plasmid, the receptor activity in whole cells was increased by 3.5- to 6-fold.

Receptors per cell-equivalent were then measured in the purified envelopes of the four strains. The number of T5 receptors per cell-equivalent was 400 to 500 in cells without plasmid and 2,400 to 2,500 in cells with plasmid, giving an amplification factor of five- to sixfold, as for whole cells (Table 2).

If we make the assumption that all TonA molecules are synthesized in a physiologically active form, the increase in receptor activity due to the presence of pLG513 is expected to be determined by a gene dosage effect. Since plasmid pLG513 is derived from pBR325, a plasmid copy number of around 20 to 50 per cell can be expected (9); since the *tonA* gene is duplicated in pLG513, the number of active T5 receptor would be 40 to 100 times higher than in cells without plasmid. The experimental value (5 to 6) was thus very small compared with that expected. Two possible explanations are proposed: either a proportion of the overproduced TonA molecules are inactive, or any active TonA molecules in excess are so located in the envelopes as to be inaccessible to the phage. In the latter case, analysis of different fractions of the envelopes would allow us to unmask additional receptor activity.

(iii) Fractions of purified envelopes. The separation of outer and inner membrane from purified envelopes was performed according to the method of Schnaitman (29) as described in Materials and Methods.

Treatment of purified envelopes by Triton X-100 in the presence of Mg^{2+} solubilizes cytoplasmic membrane proteins (30); the four strains studied (with and without plasmid) were submitted to this treatment. After centrifugation, no activity was detected in the supernatants; in contrast, 90 to 100% of the activity previously measured in purified envelopes was associated with the resuspended pellets (Table 2). These pellets consist of outer membrane proteins and peptidoglycan. Their treatment by Triton X-100 in the presence of EDTA solubilizes some outer membrane proteins (31). The four Triton-Mg^{2+} pellets were then submitted to this treatment and centrifuged. The receptor activity was found in the soluble fraction in all cases (Table 2). Quantitative results revealed a striking difference between experiments concerning cells with and those without plasmid. In the case of cells without plasmid, around 100% of the activity measured in the Triton X-100–Mg^{2+} pellet was recovered in the soluble fraction. In the case of cells with plasmid, the solubilized activity is 9- to 10-fold that measured in the corresponding Triton-Mg^{2+} pellet. Figure 1 illustrates the experiments performed with *E. coli* JE5505 and JE5505(pLG513). Identical results were obtained with GC375 and GC375(pLG513) (data not shown). Consequently, due to the presence of plasmid, T5 receptor activity is enhanced around 50-fold, in good agreement with the anticipated gene dosage effect. These results

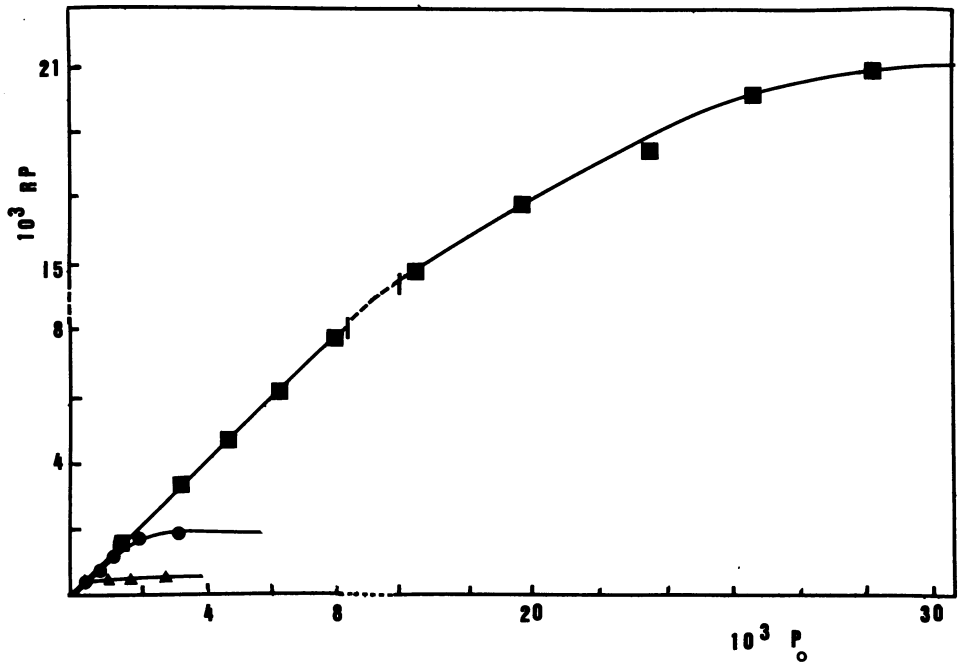

FIG. 1. Determination of the number of T5 receptor sites per cell-equivalent in *E. coli* JE5505- and *E. coli* JE5505(pLG 513)-purified and Triton-EDTA-treated envelopes. Symbols: ▲, purified envelopes and Triton-EDTA-treated envelopes of JE5505; ●, purified envelopes of JE5505(pLG513); ■, Triton-EDTA-treated envelopes of JE5505(pLG513). RP, Number of receptor sites per cell; P_o, phage input per cell.

provide evidence that TonA proteins produced in excess are present in the outer membrane, but that their activity is revealed only after solubilization of outer membrane proteins. To explain these observations, the role of steric hindrance due to phage heads in whole cells was examined.

Role of steric hindrance in the expression of receptor activity in whole cells. The low activity of T5 receptors could be an effect of steric hindrance due to the phage heads. Schwartz has demonstrated that such an effect is responsible for the limited number of λ phages absorbed by bacteria in which LamB protein has been induced (32). Thus, we tested whether the limitation of the number of active T5 receptors in vivo was also an effect of steric hindrance.

The theoretical maximum number of T5 receptors able to express their activity in a cell can be calculated approximately by using a schematic representation in which the phage head is assumed to be a sphere and the cell is assumed to be a cylinder with hemispherical ends. The number of receptors available at the cell surface is given approximately by the ratio of an enveloping cell surface located at a distance $(d/2) + l$ from the surface (d = phage head diameter; l = phage tail length) to the surface of the phage head section. A simple arithmetic calculation led to a value of 1,280 receptors per cell, accessible to phages at the cell surface. In bacteria lacking the plasmid, in which the total number of receptors is low (about 500 per cell), only 120 to 175 receptors per cell were measured in whole cells, and it seems reasonable to conclude that steric hindrance cannot be responsible for this limitation. In addition, the number of receptors measured per whole cell harboring the plasmid was only 600 to 750, although the total extracted activity amounted to as many as 22,000 receptors per cell. This suggests that steric hindrance is not the principal limiting factor for receptor expression.

This conclusion is supported by comparison of the activity in whole cells harboring plasmid and that of their purified envelopes. The number of T5 receptors per cell-equivalent in purified envelopes of these strains is only three to four times higher than that in intact cells (Table 2). Recently, Bayer et al. (1) showed that disruption of bacteria by French pressure cell (the first step in our envelope procedure) produces spherical outer membrane vesicles of 0.05 to 0.1 μm in diameter. Using the same arithmetical calculation already applied to whole cells, we can easily determine that 172 phages can be adsorbed to the surface of one 0.1-μm-diameter vesicle, and

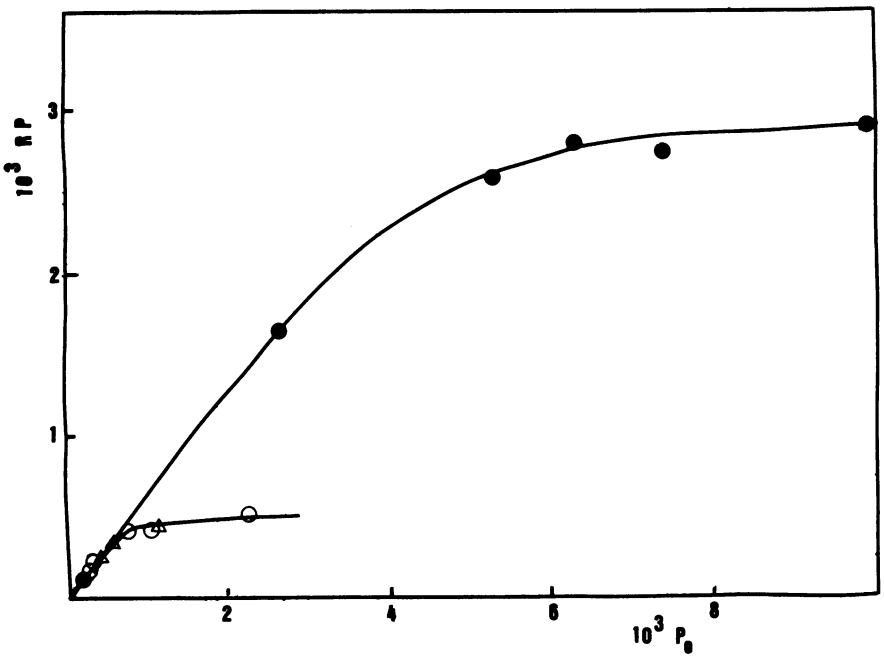

FIG. 2. T5 receptor activity of whole *E. coli* JE5505(pLG513) cells in the presence of trypsin. RP, Number of receptor sites per cell; P_o, phage input per cell. Symbols: ●, with 40 μg of trypsin per ml; △, without trypsin; ○, with 40 μg of trypsin per ml, 10^{-2} M $Mg(CH_3COO)_2$.

one bacterium can be converted into 110 vesicles, able to adsorb a total of 19,000 T5 phages. This would give 14 times more receptors per cell in purified envelopes than in whole cells, in contrast to the experimentally determined increase of three- to fourfold.

Finally, to further test the real role of steric hindrance, an experiment was carried out in which receptor activity was measured in large outer membranes vesicles compared with smaller ones. Spheroplasts from the four strains were prepared by the method of Birdsell and Cota-Robles (2) in which outer membranes are liberated as large vesicles. To eliminate errors due to differences in spheroplast yield, receptor activity was expressed as the number of T5 receptors per microgram of protein; about 8×10^9 receptors per μg of protein were measured in the strains without plasmid and 5×10^{10} were found in the strains with plasmid. The large vesicles were then broken down by passage through a French pressure cell at 20,000 lb/in^2. This treatment led to the production of smaller membrane vesicles (28). A drastic change in the turbidity of the suspension was observed after passage through the French press, demonstrating the efficiency of this treatment. The same number of T5 receptors was found in the two kinds of vesicles. This result confirms that in intact cells factors other than steric hindrance limit T5 phage adsorption. For example, part of overproduced TonA proteins could be embedded in the outer membrane; attempts were made to locate these overproduced proteins in the outer membrane.

T5 receptor activity in whole cells. (i) Trypsin-treated whole cells. Halegoua and Inouye (13) showed that in the absence of Mg^{2+} ions trypsin is able to penetrate the cell up to the outer surface of the inner membrane, whereas in the presence of Mg^{2+} ions, it digests only proteins exposed at the cell surface. Whole cells were digested by trypsin as described in Materials and Methods. When submitted to trypsin action in the presence of Mg^{2+}, *E. coli* JE5505(pLG513) whole cells retained their T5 receptor activity. Thus, even though isolated TonA protein is partially sensitive to trypsin (6), this result shows that, in vivo, the exposed part of the TonA protein does not contain accessible arginine or lysine. More surprising is the fact that, when treated by trypsin in the absence of Mg^{2+}, the same intact cells showed an increase in their receptor number: from 600 to 2,600 receptors per cell (Fig. 2). This value is high compared with the theoretical value allowed by steric hindrance. This increase above the theoretical number can be explained by a solubilization of

TonA molecules during the trypsin treatment. To test this hypothesis, we performed the following experiment.

Each sample of trypsin-treated cells was divided into two parts: one was tested for anti-T5 activity, and the other was incubated in the presence of the same buffer, but without T5 phages, and finally centrifuged.

About 1,000 receptors per cell-equivalent were found in the supernatant. Thus, among 2,600 receptors per cell measured after trypsin treatment, 1,600 remained attached to the whole cell. As 600 receptors were accessible to the phage in the intact cells, about 1,000 sites are unmasked by trypsin.

In whole cells without plasmid, trypsin treatment did not enhance T5 receptor activity (data not shown). This suggests that at least a proportion of the overproduced TonA proteins embedded in the outer membrane are covered by trypsin-sensitive proteins.

(ii) Triton-EDTA-treated whole cells. Experiments were performed to see whether all T5 receptor activity extracted from isolated outer membranes could be extracted directly from intact cells treated with Triton-EDTA. Receptor activities solubilized from outer membrane (Table 2) were taken as 100% of total receptor activity. We deduce that Triton-EDTA treatment of whole cells lacking the plasmid resulted in the solubilization of 110% of total activity for *E. coli* JE5505 (*lpo* mutant) and 30% for *E. coli* GC375. In the case of whole cells harboring plasmid, only 33% of the total activity for JE5505(pLG513) and 10% for GC375(pLG513) were extracted. Although the reason for the discrepancy between the yields of T5 activity extracted from the two strains is not clear, we suggest that it could be an effect of the presence of an *lpo* mutation in JE5505. The solubilization of overproduced T5 receptor activity from cells was not as efficient as from outer membranes of purified envelopes; this suggests that most of the overproduced TonA molecules are embedded deeply in the outer membrane, from which they cannot be liberated except by its total disruption.

(iii) Research of T5 activity in cytoplasm, periplasmic space, and culture medium. The recovered receptor activity after Triton-EDTA treatment of purified membrane of cells harboring pLG513 was in agreement with the expected value from a gene dosage effect. So no consistent receptor activity was expected to be found in culture medium, cytoplasm, or periplasmic space. This was verified in the following ways: 1 ml of a semilogarithmic culture of *E. coli* JE5505(pLG513) was centrifuged for 10 min at 4°C and 5,000 rpm; no detectable receptor activity was found in the supernatant, although in this case, elimination by centrifugation of aggregates of TonA proteins released by cells cannot be ruled out.

Purified envelopes were prepared from cell extracts centrifuged to a 70% sucrose cushion (as described in Materials and Methods). The thin band of envelopes floating on the cushion was removed. The remaining fractions of the centrifuge tube contained cytoplasmic proteins. In the case of *E. coli* JE5505(pLG513) and GC375(pLG513), the fractions were tested for their T5 receptor activity, which amounted to less than 60 to 70 receptors per cell-equivalent (around 3% of the total activity of purified envelopes).

Jacobson et al. (20) showed that only typical periplasmic enzymes are released into the soluble fraction during spheroplast preparation and that, in contrast, cell plasmolysis by osmotic shock liberates some cytoplasmic proteins in addition to periplasmic ones. Therefore, we chose the first method to look for T5 receptor activity in the periplasmic space. Protoplasts of *E. coli* JE5505(pLG513) and GC375(pLG513) were produced according to Weiss (36). This procedure led to a yield of 95 to 100% protoplasts. Periplasmic proteins and large fragments of outer membrane are released into the medium, and the latter were pelleted by centrifugation in a J21 Beckman centrifuge at 15,000 rpm for 20 min at 4°C. The number of T5 receptors measured in the supernatant was about 30 receptors per cell-equivalent. A second centrifugation in a Beckman Airfuge Ultracentrifuge, 1 h at 30 lb/in^2, was performed to eliminate any smaller outer membrane residues and membrane vesicles still present in the first supernatant. The same number of receptors as previously determined was detected in the second supernatant.

Partial purification of TonA protein. To correlate the increase of T5 receptor activity measured in the purified envelopes with their actual content of TonA protein, analysis of proteins was performed by SDS-polyacrylamide gel electrophoresis. Figure 3 shows the results obtained for *E. coli* GC375, GC375(5), and GC375(pLG513). Each analyzed sample corresponded to 5×10^9 cells. No polypeptide in the range of 78K molecular weight appeared either in GC375(5) (lane 2)- or GC375 (lane 3)-purified envelopes. In the GC375(pLG513) envelope (lane 1) two new polypeptides were present in large quantities: polypeptide 1 (78K) and polypeptide 2 (74K). From studies of Covarrubias et al. (9), the synthesis of polypeptides in this molecular weight range is not directed by the parent plasmid pBR325. So, it seems likely that these two polypeptides were synthesized from *E. coli* DNA cloned in pBR325.

To show unequivocally which of these pro-

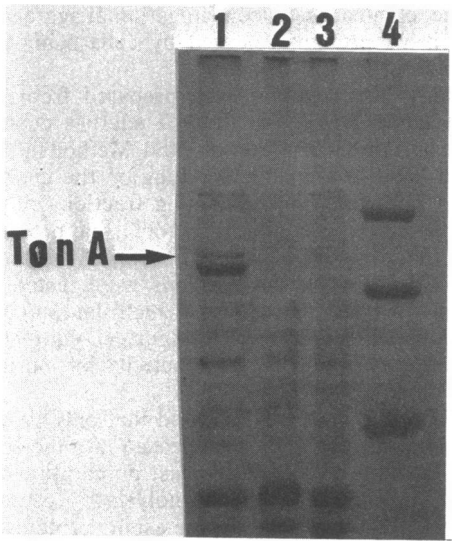

FIG. 3. SDS-polyacrylamide gel electrophoresis of proteins from purified envelopes. Gels are 0.2% SDS–10% polyacrylamide. Lane 1, From *E. coli* GC375(pLG513) envelopes; lane 2, from *E. coli* GC375(5) envelope; lane 3, from *E. coli* GC375 envelope; lane 4, molecular weight markers (from the top): phosphorylase b, 94K; serum albumin, 67K; ovalbumin, 43K.

teins is the T5 receptor, a Triton-EDTA-soluble fraction of the outer membrane was treated to remove Triton and then submitted to sequential precipitation by ammonium sulfate. After centrifugation of the different fractions, 95% of the total receptor activity was found in the pellet of the 35% ammonium sulfate saturation fraction. Analysis of proteins by SDS-polyacrylamide gel electrophoresis showed that removal of Triton did not result in any change in the analysis profile of proteins (Fig. 4). In the active fraction precipitated by 35% ammonium sulfate, the 78K- but not the 74K-molecular-weight protein was present. These results confirm that the 78K-molecular-weight protein is the TonA protein responsible for phage T5 inactivation. These experiments constituted the first step in TonA protein purification, to be published elsewhere (A. Buu and B. Menichi, manuscript in preparation).

DISCUSSION

The outer membrane provides for the cell both a barrier against the environment and a permeation system allowing ions and nutrients to pass through the bacterial envelope. This latter role is played in most of the cases by proteins, both major and minor species. Because of their importance, the regulation of these proteins has been extensively studied not only at the level of their biosynthesis (4, 23, 24), but also by their integration in the outer membrane (4, 13). Regulation of the number of major proteins in the outer membrane has been demonstrated by genetic experiments, indicating, for example, that relative quantities of OmpA and matrix proteins are correlated (21, 31). This suggests that the total amount of these proteins in the outer membrane is in some way limited. We were interested in the number of overproduced minor proteins able to be integrated at a physiological site of the outer membrane; for this purpose we have taken advantage of the T5 receptor activity of the TonA protein and of the easy amplification system provided by cells harboring the pLG513 plasmid.

In the presence of plasmid pLG513 the total receptor activity is amplified 50-fold compared with cells lacking the plasmid. This could be due to a simple gene dosage effect (within a factor of 2); in the absence of accurate copy number data for pLG513, a limitation of the number of TonA molecules by competition with other membrane proteins cannot be ruled out. All receptor activity was located in the outer membrane, and the overproduced TonA molecules were in a mature form (78K molecular weight).

In intact bacteria, only about 3% of the TonA molecules made in excess were accessible to the phages. This limitation of expression of the receptor activity could be an effect of steric hindrance due to the phage heads, but this hypothesis was shown to be unlikely. Rather, it is suggested that 3% of TonA protein molecules were inserted in physiological sites at the cell

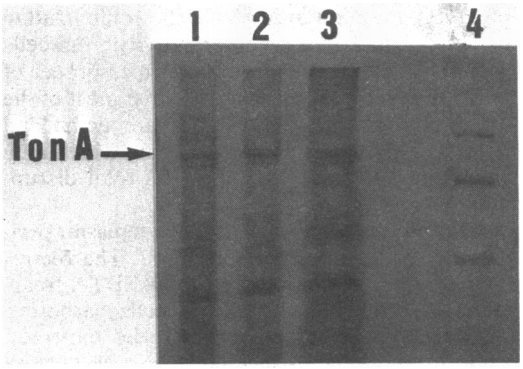

FIG. 4. SDS-polyacrylamide gel electrophoresis of proteins from Triton-EDTA-treated outer membrane. Gels are 0.2% SDS–10% polyacrylamide. Lanes 1 and 3, Triton-EDTA extract from *E. coli* GC375(pLG513) deprived of Triton by SM_2 beads; lane 2, 35% ammonium sulfate-precipitated fraction from extract analyzed in lane 1; lane 4, molecular weight markers are as in Fig. 3.

surface, and the others were embedded deeper in the outer membrane.

Evidence in favor of this hypothesis was provided by the fact that trypsin treatment of amplified cells unmasked a proportion of the T5 receptor activity. This also suggests that, in vivo, at least some TonA proteins in excess are covered by trypsin-sensitive proteins. However, it is not possible to know how many receptors are really unmasked by trypsin since the steric hindrance does not allow us to measure a number of sites higher than the one determined here.

In conclusion, all of the TonA molecules were found in the outer membrane, and only a small proportion of them were accessible to phage T5. We suggest that the total number of outer membrane proteins at the cell surface is approximately constant and genetically determined to keep the cell size; the assembly of these proteins would be directed by a simple competition between different molecular species.

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