JOURNAL OF BACTERIOLOGY, Aug. 1982, p. 983-988
0021-9193/82/080983-06$02.00/0

Vol. 151, No. 2

Colicin A Receptor: Role of Two *Escherichia coli* Outer Membrane Proteins (OmpF Protein and *btuB* Gene Product) and Lipopolysaccharide

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Received 19 November 1981/Accepted 5 April 1982

ompF cells were completely resistant to colicin A, whereas *btuB* cells were partially resistant. The OmpF protein, in the presence of added lipopolysaccharide, inactivated colicin A. This inactivation was enhanced by added *btuB* gene product. *btuB* gene product with lipopolysaccharide did not inactivate colicin A. These data, together with the observation that vitamin B_{12} protected *btuB*$^+$ cells from the killing effect of colicin A, suggest that the colicin A receptor in *Escherichia coli* K-12 is composed of the OmpF protein, the *btuB* gene product, and lipopolysaccharide.

Colicins are a class of protein antibiotics which kill sensitive cells after initially adsorbing to a specific receptor located on the cell surface. Where colicin receptors have been identified in gram-negative bacteria, they are associated with the outer membrane. They are formed, at least in part, by outer membrane protein (22, 31). Certain colicin receptors participate in other cellular functions, such as the uptake of nutrients such as iron complexes (4, 28), vitamin B_{12} (9), and nucleosides (16). In addition, certain colicin receptors, such as the receptors for colicins E, K, and M, also serve as receptors for specific bacteriophages (for reviews see references 18 and 19). Thus, colicin receptors may participate in the binding of up to three distinct classes of biologically active materials.

The receptor for colicin A has not been identified, although *coa* (colicin A resistance) mutants have been identified (20). *coa* mutants are allelic with *ompF* mutants and lack the OmpF outer membrane protein (Foulds, unpublished data). Colicin A is a group A colicin which kills sensitive cells by a mechanism which is at least superficially similar to the killing action of colicin E1. The primary biochemical effect of colicin E1, and presumably of colicin A, is an interruption of energy metabolism after the collapse of membrane potential (15).

The *Escherichia coli* outer membrane serves as a partial permeability barrier which excludes potentially harmful agents, such as bile salts and antibiotics, while allowing the passage of small hydrophilic molecules (24) (molecular weight, up to about 650). The membrane is composed of proteins, phospholipids, and lipopolysaccharides (LPSs). The protein composition of the *E. coli* outer membrane is such that only a few (three to five) proteins account for about 70% of the protein associated with this membrane. These abundant proteins are called the major proteins and include the OmpF protein (23, 33, 34). The OmpF protein has also been called protein Ia, b, and O-9.

The OmpF protein is a peptidoglycan-associated protein with a molecular weight of about 36,000 (30). The OmpF protein is one of the major outer membrane proteins that functions as a general pore to facilitate the diffusion of small hydrophilic molecules across the outer membrane (24). It forms at least part of the receptor for bacteriophages TuIa (9) and T2 (17), and purified OmpF protein can inactivate bacteriophage TuIa. This inactivation requires added LPS and is not enhanced by the addition or omission of phospholipid (8). The inactivation of bacteriophage TuIa is specific for the OmpF protein. A similar preparation of the OmpA protein can inactivate bacteriophage K3 but not bacteriophage TuIa (36). The inactivation of specific bacteriophages by purified outer membrane proteins such as the OmpA protein (8), the OmpC protein (8), and the PhoE protein (7) also requires added LPS. These results, obtained in vitro, strongly suggest that these proteins interact with LPS in vivo.

E. coli mutants lacking demonstrable amounts of the OmpF protein in the outer membrane can be isolated, for example, by selection for resistance to bacteriophage TuIa (8) or colicin L (12). In addition, since the OmpF protein functions to form a pore, mutants lacking this protein can be

TABLE 1. *E. coli* K-12 strains used

Strain	Description[a]	Source or reference
JF404	HfrH *thi-1 thyA*	
JF568	*proC25 proA357 his-53 purE41 ilv-277 met-75 lacY29 xyl-14 rpsL97 cycA1 cycB2? tsx-63* λ^-	7
JF703	JF568 *aroA*$^+$ *ompF254*	
JF733	JF568 *aroA*$^+$ *ompA252 ompC262*	13
JF752	JF733 *btuB451?*	This paper
JF777	JF703 *btuB451?*	This paper
RK4662	Hfr KL96 *lacZ*(Am) *nalA rpsL supD43,74*(Ts) *btuB451*(Am)	R. Kadner
JF385	O:6H16, colicin A production	P. Fredericq

[a] Gene symbols are those proposed by Bachmann and Low (1).

identified by their altered permeability of nucleotides (3), metal ions (21), or antibiotics such as chloramphenicol and tetracyclines (29).

The *ompF* gene (formerly called *tolF*) is the structural gene for the OmpF protein (32). The *ompF* gene is allelic with certain *cmlB* and *cry* mutants (11).

We have purified the OmpF protein, and we show here that in the presence of added LPS, this protein inactivated colicin A. This inactivation was enhanced by added *btuB* gene product. The *btuB* gene product is a minor outer membrane protein which binds vitamin B_{12} and functions in the energy-independent phase of vitamin B_{12} uptake (2). It also serves as part of the receptor for type E colicins and bacteriophage BF23 (9).

The data presented here suggest that at least three outer membrane components, i.e., the OmpF protein, the *btuB* gene product, and LPS, interact to form the colicin A receptor in the *E. coli* outer membrane.

MATERIALS AND METHODS

Strains and media. The *E. coli* strains used in this study are described in Table 1. These strains were grown in a rich medium (proteose peptone-beef extract medium) or in Vogel-Bonner minimal medium supplemented with 2 g of glucose per liter and appropriate levels of growth requirements. These media and growth conditions have been described previously (11).

Preparation of cell envelopes. The cell envelope fraction was prepared as previously described (6). Briefly, cells were grown in proteose peptone-beef extract medium, collected by centrifugation, washed, and disrupted by passage through a French pressure cell. The cell envelope fraction was purified by differential centrifugation.

Solubilization and separation of outer membrane protein fraction. Differential solubilization of cytoplasmic and outer membranes with Triton X-100–EDTA was accomplished as described by Schnaitman (34).

Preparation of LPS. LPS was prepared from strain JF568 as described by Galanos et al. (14).

Purification of the OmpF protein. The OmpF protein was isolated from strain JF752. This strain does not contain detectable amounts of the OmpA or OmpC outer membrane proteins. It also carries an amber mutation in the *btuB* gene and presumably lacks this gene product. The Triton X-100–EDTA-solubilized cell envelope fraction prepared from strain JF752 contained primarily the OmpF protein. The OmpF protein was purified by chromatography of this fraction on a DEAE-cellulose column (2.5 by 100 cm) as described by Schnaitman (34). The protein composition of selected fractions was determined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Fractions containing the OmpF protein were pooled, and the protein was precipitated with 2 volumes of ethanol. To remove other contaminants, primarily LPS and phospholipids, the ethanol-precipitated material was extracted first with acetone-water (5:1, vol/vol) and then with chloroform-methanol (2:1, vol/vol). These extractions were repeated three times in the order given. This procedure yielded an electrophoretically homogeneous preparation of the OmpF protein containing a low level of LPS (0.2% by weight).

Preparation of crude colicin A. Crude colicin A was prepared from strain JF385 as previously described (12). The crude colicin A preparation contained 6.4×10^5 arbitrary units of colicin A per ml. The colicin activity was estimated by the critical dilution method (10).

Purification of colicin A. A sample of crude colicin A containing about 4.6 mg of protein and 5×10^5 arbitrary units of colicin A activity was applied to a DEAE-Sephadex column (0.9 by 30 cm) which had been previously equilibrated with 0.01 M Tris-hydrochloride (pH 7.8) containing 1 mM disodium EDTA and 1 mM β-mercaptoethanol. Chromatographic separation was accomplished under the initial conditions by washing the column with the equilibration buffer. A distinct band of material remained at the top of the column, and all of the colicin A activity eluted was found in the earliest fractions which adsorbed light at 280 nm. No additional colicin activity was eluted from the column by a 0 to 1 M NaCl gradient. About 25% of the colicin A activity added to the column was recovered in a single peak. No other colicin active on the indicator strain was detected. Fractions containing colicin A activity were pooled, and 1- to 2-ml samples were stored frozen at −20°C. Electrophoresis of an unheated sample (20 μg of protein) in a polyacrylamide gel containing sodium dodecyl sulfate revealed, after staining with Coomassie brilliant blue G, a predominant band (molecular weight, ca. 56,000). This is likely to be colicin A (5). At least eight additional minor bands were also seen on the gel.

Genetic techniques for preparation of strains JF752 and JF777. Preparation of bacteriophage P1 *vir* lysates and transduction techniques were as described by Signer (35). A lysate prepared on strain RK4662 was used to transduce strain JF703 or JF733. After centrifugation to remove unadsorbed bacteriophage, the cells

TABLE 2. Killing effect of colicin A

Strain	Outer membrane protein present				Colicin A[a]	% Survival ± SD[b]
	OmpF	OmpC	OmpA	BtuB		
JF568	+	+	+	+	1.0	14 ± 3
JF733	+			+	1.0	15 ± 5
JF752	+				50.0	15 ± 1
JF703		+	+	+	320	>99

[a] In arbitrary units per milliliter. Crude or purified colicin was added with no significant difference in survival level. The arbitrary units of colicin A were determined by using strain JF568 as the indicator strain.

[b] The survival was measured by mixing freshly grown cells (5×10^8/ml) with colicin and determining the number of survivors after a 10-min incubation.

were diluted 40-fold into PPBE medium lacking added Ca^{2+} and containing citrate. Next, the cells were grown for 2 h and spread onto proteose peptone-beef extract agar together with 10^4 arbitrary units of colicin E2. Several survivors were purified by successive single-colony streaks and checked for resistance to colicins E1 and E2 and bacteriophage BF23. All colonies tested (16 of 16 from each transduction) were resistant to bacteriophage BF23 and to both colicins E1 and E2; these were presumed to be *btuB* mutants. Although we do not know whether JF752 and JF777 contain the *btuB451* allele of strain RK4662, the number of survivors after the transduction mixture was plated with colicin E2 was increased 40-fold compared with a control transduction with a bacteriophage P1 lysate prepared on strain JF568.

Determination of colicin A adsorption and inactivation. The adsorption and killing of sensitive *E. coli* cells by low doses of colicin A is a single-hit process. Freshly grown sensitive cells (5×10^8 cells per ml) were mixed with an equal volume of colicin A, and the mixture was incubated for 10 min at 25°C. At the end of the incubation period, adsorption of colicin was considered to be complete, and the number of survivors was determined by spreading an appropriate dilution onto plates containing proteose peptone-beef extract agar. For inactivation experiments, strain JF568 was used as the colicin A-sensitive strain, whereas for adsorption experiments, strain JF404 was used. The use of strain JF404 allowed the enumeration of JF404 cells in the presence of JF568 cells.

Adsorption of colicin A by intact JF568 cells was determined by an estimation of the colicin which remained in the supernatant fraction.

Effect of vitamin B_{12} on colicin A activity. Freshly grown cells were washed once with saline and suspended at 4×10^8 cells per ml in minimal medium. A 0.8-ml portion of this suspension was mixed with 0.1 ml of saline or a solution of vitamin B_{12}, and the mixture was incubated at 37°C for 5 min. A 0.1-ml portion of saline or colicin A was then added, and after the mixture was incubated at 37°C for 10 min, the viable count was determined.

In vitro inactivation of colicin A. Purified OmpF protein prepared from strain JF752 (*ompF*$^+$ *btuB*) was mixed with solubilized outer membrane protein prepared from strain JF703 (*ompF btuB*$^+$) and LPS. The solubilized outer membrane protein was the Triton X-100–EDTA-solubilized material described by Schnaitman (34). This material contains 10 to 20% LPS by weight. The mixture was incubated at 37°C for 15 min in 0.05 M Tris-hydrochloride buffer (pH 7.8), and 2.0 arbitrary units of colicin A was added. The incubation was continued for 15 min at 37°C. Next, an equal volume of a freshly grown early-log-phase culture of colicin A-sensitive cells was added so that the final concentration of cells was about 3×10^3 cells per ml. The mixture was incubated for 10 min and plated directly to determine the number of surviving cells.

RESULTS

Comparison of crude and partially purified colicin A. Colicin A killed *ompF*$^+$ cells but had no effect on *ompF* cells. The killing of sensitive cells by low levels of colicin A can be used to estimate the amount of colicin A remaining after adsorption or inactivation. A killing curve with strain JF568 as the indicator (data not shown) showed that 1 arbitrary unit of colicin A contained approximately 2×10^9 killing units. A comparison of the killing effect of crude and purified colicin A on various strains showed that the two preparations were equivalent. Strain JF703 (*ompF*) was completely resistant to colicin A, whereas strain JF752 required about 50-fold more colicin to exhibit the same decrease in survival as *ompF*$^+$ *btuB*$^+$ strains JF568 and JF733 (Table 2).

Adsorption of colicin A. *ompF*$^+$ cells adsorbed colicin A, whereas no adsorption could be detected when *ompF* cells were used (Fig. 1). The most striking alteration in the composition of the outer membrane of *ompF* cells was the absence of the OmpF protein. The adsorption of colicin A by the *ompF*$^+$ *btuB* strain JF752 was similar

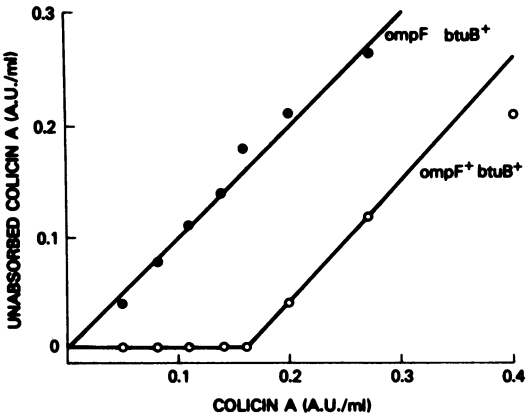

FIG. 1. Adsorption of colicin A. The adsorption of colicin by freshly grown JF703 (*ompF btuB*$^+$) or JF568 (*ompF*$^+$ *btuB*$^+$) cells was measured as described in the text. A. U., Arbitrary units.

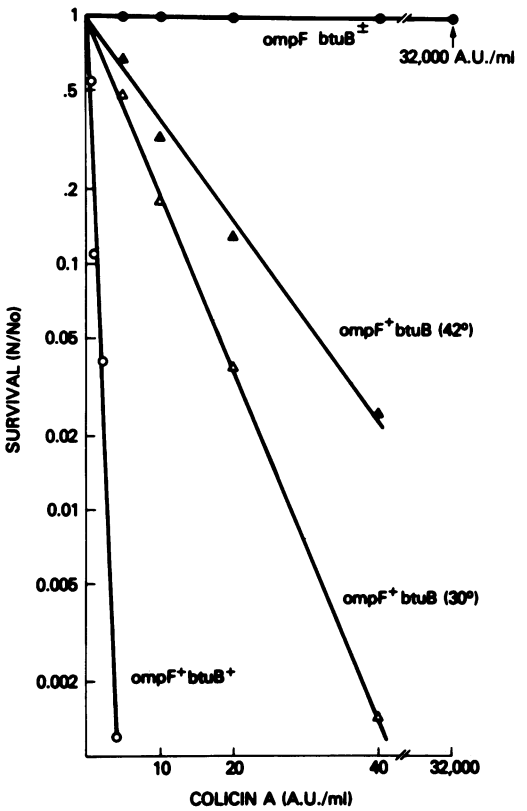

FIG. 2. Effect of the *btuB* mutation on colicin sensitivity. Various amounts of colicin A were mixed with freshly grown cells (5×10^8/ml), and after the mixture was incubated for 10 min, the viable count was determined. Strains JF568 (*ompF*$^+$ *btuB*$^+$) and JF703 (*ompF btuB*$^+$) were grown at 37°C, whereas strain RK4662 [*ompF*$^+$ *btuB*(Am)] was grown at either 30 or 42°C. Strain JF777 [*ompF btuB*(Am)] was grown at 30°C. A. U., Arbitrary units.

to the adsorption by the *ompF*$^+$ *btuB*$^+$ strain JF568 (data not shown).

Role of *btuB*$^+$ gene product in colicin A sensitivity. Figure 2 shows the killing of cells which contain a *btuB*(Am) allele and a *supD*(Ts) allele. When cells were grown under nonsuppressing conditions (42°C), strain RK4662 showed a demonstrable, albeit markedly reduced, sensitivity to colicin A. When these cells were grown at 30°C, a temperature at which the *supD*(Ts) allele permits a low-efficiency expression of an amber mutation (27), the colicin A sensitivity was somewhat increased. These data indicate that a cell is sensitive to colicin A in the absence of *btuB* gene product and that this sensitivity is enchanced by increased levels of this protein. *ompF* cells were completely resistant to even 32,000 arbitrary units of colicin A, regardless of whether the strain was *btuB*$^+$ or *btuB* (Fig. 2).

Effect of vitamin B_{12} on colicin A killing. The killing of sensitive cells by colicin E1 is completely inhibited by sufficient amounts of vitamin B_{12} (9). We confirmed that strain JF733, after preincubation in the presence of 1.0 μg of vitamin B_{12} per ml, was completely insensitive to the killing effect of 100 arbitrary units of colicin E1 per ml (data not shown). The killing effect of colicin A on *ompF*$^+$ *btuB*$^+$ cells was also largely prevented by pretreatment of cells with vitamin B_{12} (Table 3). Although this reversal was not complete at 100 arbitrary units of colicin A per ml, these data suggest a role of the *btuB* gene product in colicin A killing. Vitamin B_{12} had no effect on the killing of *btuB* cells by either crude or partially purified colicin A (Table 3).

In vitro inactivation of colicin A. Purified OmpF protein was able to inactivate colicin A. This inactivation was enhanced by LPS. All of the added colicin A was inactivated by a mixture of purified OmpF protein and LPS (Table 4). The inactivation of colicin A by OmpF protein and LPS was enhanced by Triton X-100–EDTA-solubilized material prepared from an *ompF btuB*$^+$ strain (Table 5). A similar preparation from an *ompF btuB* strain had no effect, indicating that the *btuB*$^+$ gene product was an active component.

DISCUSSION

Colicin A has a mode of action similar to that of colicins such as E1, Ia, and K. These colicins depolarize the cytoplasmic membrane, resulting in the formation of "ion channels" (15). For this

TABLE 3. Inhibition of colicin A killing effect by vitamin B_{12}

Vitamin B_{12} (μg/ml)	Colicin A[a]	% Survival ± SD[b]	
		JF733	JF752
0	0	100	100
0.1	0	97	
1	0	105	113
0	1	7 ± 1	60 ± 5
0.1	1	30 ± 5	
1	1	100 ± 8	65 ± 1
0	10	0.001	14 ± 4
0.1	10	8 ± 2	
1	10	78 ± 3	16 ± 5
0	100[c]	0.001	0.05
0.1	100	1	
1	100	17	0.05

[a] In arbitrary units per milliliter.
[b] Survival was measured by mixing freshly grown cells with vitamin B_{12} and, after a 5-min incubation, adding colicin A. After a 10-min incubation, the number of survivors was determined.
[c] Only a single experiment was done at this colicin concentration.

interaction with the cytoplasmic membrane to occur, at least a portion of the colicin molecule must breach the barrier formed by the outer membrane. This is accomplished after an interaction of the colicin molecule with a specific receptor located on the outer membrane, which may be followed by the translocation of the colicin molecule or a portion of it through the outer membrane to a site of action on the cytoplasmic membrane.

A number of colicin receptors located in the outer membrane have been described for *E. coli* (19). For several of these, specific outer membrane proteins have been identified which serve in three distinct but related roles: colicin reception, phage reception, and transport of hydrophilic molecules across the outer membrane barrier. The common role that the receptor plays is to assist in the transport of materials across the outer membrane.

E. coli porin proteins participate in the formation of water-filled holes or pores in the outer membrane, which facilitate the uptake of small hydrophilic molecules (24, 25). The data presented here suggest that a specific porin protein, the OmpF protein, interacts with colicin A and that the colicin A receptor may be a complex which involves this protein, the *btuB* gene product, and LPS. The *btuB* gene product is a receptor for bacteriophage BF23 and group E colicins and participates in the uptake of vitamin B_{12} (9). The role of the *btuB* gene product in the activation of colicins A and E in vitro, together with the observation that *btuB* and *ompF* cells are tolerant to colicin A, suggests that these two proteins may be located close together in the *E. coli* outer membrane. The *btuB* gene product may stabilize the colicin A-OmpF protein complex or facilitate the translocation of the colicin A molecule across the outer membrane by forming a specific pore.

TABLE 4. Role of LPS in inactivation of colicin A by purified OmpF protein

OmpF protein (μg)[a]	LPS (μg)	% Colicin A inactivated[b]
0	20	0
18	0	40
2	20	60
6	20	75
18	20	100

[a] OmpF protein was purified from strain JF752 by DEAE-Sephadex chromatography. This preparation contained approximately 2% LPS by weight, but did not contain the BtuB protein. LPS by itself did not kill the sensitive bacteria or inactivate colicin A.

[b] Colicin A was added at a final concentration of 1 arbitrary unit per ml.

TABLE 5. Role of *btuB* gene product in inactivation of colicin A

Outer membrane prepn (μg)[b]			% Colicin A inactivated[a]
ompF$^+$ *btuB*	*ompF btuB*$^+$	*ompF btuB*	
1			25
2			60
20			80
1	20		80
2	20		100
20	20		100
1		20	30
2		20	50
20		20	75

[a] Colicin A was added at a final concentration of 1 arbitrary unit per ml.

[b] Triton X-100–EDTA-solubilized fraction. This material contained 10 to 20% LPS by weight. The following strains were used for these preparations: *ompF*$^+$ *btuB*, JF7252; *ompF btuB*$^+$, JF703; *ompF btuB*, JF777.

We suggest that the association of these two outer membrane proteins in the formation of the colicin A receptor may reflect a similar association in the physiological function of the *btuB* gene product. For example, a minor outer membrane protein (the *btuB* gene product) may interact with a major outer membrane protein (the OmpF protein) to form a specific pore which, in this case, would be involved in the uptake of vitamin B_{12}.

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