Comparison of the Uptake Systems for the Entry of Various BtuB Group Colicins into *Escherichia coli*

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(Received 8 May 1989; revised 11 July 1989; accepted 11 September 1989)

Colicins A, E1, E2 and E3 belong to the BtuB group of colicins. The NH₂-terminal region of colicin A is required for translocation, and defects in this region cannot be overcome by osmotic shock of sensitive cells. In addition to BtuB, colicin A requires OmpF for efficient uptake by sensitive cells. The roles of BtuB and OmpF in translocation and binding to the receptor of the colicins A, E1, E2 and E3 were compared. The results suggest that for colicin A OmpF is used both as a receptor and for translocation across the outer membrane. In contrast, for colicin E1, OmpF is used neither as a receptor nor for translocation. For colicins E2 and E3, the situation is intermediate: only BtuB is used as a receptor but both BtuB and OmpF are involved in the translocation step.

INTRODUCTION

Three consecutive stages can be distinguished in the lethal action of colicins on susceptible cells of *Escherichia coli*: (i) binding onto the outer-membrane receptor protein; (ii) transport across the cell envelope; and (iii) biochemical interaction of the colicin with its target in the cell. Evidence has been put forward that there are three distinct domains along the polypeptide chain, involved in different steps of the lethal action.

The molecular topology of the functional domains of colicin A was determined by Baty *et al.* (1988). The NH₂-terminal region (residues 1 to 172) is involved in the translocation step through the outer membrane. The central region (residues 173 to 336) contains the receptor binding domain. The COOH-terminal domain (residues 389 to 592) carries the pore-forming activity of colicin A.

Colicin A belongs to a class of colicins (A, E1–9) grouped on the basis of their reduced activity against *E. coli* K12 mutants lacking the outer-membrane protein BtuB (Mock & Pugsley, 1982), the receptor for vitamin B12 and for bacteriophage BF23 (Kadner *et al.*, 1979; Males & Stocker, 1980, 1982; Reeves, 1972; Cooper & James, 1984). However, differences in the colicin receptor activity of BtuB for various colicins within this group have previously been noted. Outer membranes of sensitive cells inhibited activity of colicins E1 to E9, while they have no effect on colicin A. Furthermore, the latter colicin could kill only cells which also produced the OmpF porin (Pugsley & Schnaitman, 1979).

In recent years, we have studied the mechanism of action of colicin A (Lazdunski *et al.*, 1988). Its receptor is composed of two proteins, BtuB and OmpF (Cavard & Lazdunski, 1981; Chai *et al.*, 1982). However, so far we have not been able to determine whether these two proteins have a similar role in colicin A uptake.

There have been several studies on the initial steps of colicin uptake (Tilby *et al.*, 1978; Cavard & Lazdunski, 1981; Braun *et al.*, 1980). For many colicins, the product of a number of genes belonging to the *tol* cluster (*tolA*, *tolB*, *tolC*, *tolQ*, *tolR*) are likely to be involved in the second step of colicin uptake across the cell envelope. Recently, more information on the

identity, structure, topology, synthesis and regulation of outer-membrane proteins has become available. In addition, most of the sequences of the Tol proteins [TolA, TolB, TolQ, TolR (Sun & Webster, 1987), TolC (Hackett & Reeves, 1983)] have been deduced from the nucleotide sequence of respective genes and their localization has been determined.

In this study, the roles of BtuB and OmpF in the uptake of colicins A, E1, E2 and E3 have been compared, and the role of the immunity protein in uptake of colicins E2 and E3 has been evaluated.

METHODS

Bacterial strains, growth conditions and plasmids. The bacterial strain MC4100 was used as a host for the various colicinogenic plasmids: pColA9 (Lloubès *et al.*, 1986), colicin A mutant plasmids (pDS1, pBD2, pBE1, pAR1) (Baty *et al.*, 1988), ColE1-K30 (Herschman & Helinski, 1967*b*) ColE2-P9 and ColE3-CA38 (Watson & Visentin, 1980). The MC4100 strain containing the various plasmids was grown in LB medium (Miller, 1972). Colicin synthesis was induced by adding 300 ng mitomycin C (MTC) ml⁻¹ to growing cultures at an OD₆₀₀ of 1.

The strains used for the sensitivity assay under normal or bypass conditions were the wild-type, the OmpF⁻ (OmpF-LacZ) or the BtuB⁻ (BtuB-LacZ) mutants of the MC4100 strain; these have all been previously described (Cavard & Lazdunski, 1981).

Purification of colicins. Purification and genetic constructions were done as described by Baty *et al.* (1988) for colicin A and truncated colicin A derivatives; as described by Herschman & Helinski (1967*a*) for the colicin E2 and E3 complex; and as described by Schwartz & Helinski (1971) for colicin E1.

Colicin E2 and E3 heterodimers were dissociated as described by Sidikaro & Nomura (1974). Gel filtration on a Sepharose 12 column (Pharmacia) was used for separation of the proteins.

Colicin assays under normal conditions. Cells of *E. coli* MC4100 (wild-type, OmpF⁻ or BtuB⁻) were grown in LB medium at 37 °C to an OD₆₀₀ of 0.2. Colicin (10 µl) diluted in sodium phosphate buffer (10 mM-NaH₂PO₄/Na₂HPO₄, pH 6.8) containing 0.1% Triton X-100 was added to 100 µl of this culture and incubated for 20 min at 37 °C with shaking. As a control, sodium phosphate buffer was used instead of a colicin dilution. Subsequently, 1.25 ml of LB medium was added, and the culture OD₆₀₀ measured until the OD₆₀₀ of the control reached 0.4 (i.e. after 1.5 to 2 h incubation). The percentage of surviving cells was estimated from the OD₆₀₀ ratios of the colicin-treated and control cultures.

Colicin assays under bypass conditions. A culture of *E. coli* MC4100 (wild-type, OmpF⁻ or BtuB⁻) was grown in LB medium at 37 °C to an OD₆₀₀ of 0.8. The cells were then washed three times with 10 mM-sodium phosphate buffer, and diluted in this buffer to an OD₆₀₀ of 0.2. The assay was then done on 100 µl of this cell suspension as described above.

RESULTS

Tilby *et al.* (1978) demonstrated that it is possible to distinguish between the receptor binding step and the translocation step. The receptor step can be bypassed in susceptible or resistant cells under conditions of osmotic shock. However, it was observed (Tilby *et al.*, 1978; Braun *et al.*, 1980), that the viability of a large percentage of osmotically shocked cells was seriously compromised. Therefore, a milder treatment (called bypass conditions) that has been described previously (Cavard & Lazdunski, 1981) as having the same effect as classic osmotic shock was also used. By using a K⁺-specific electrode, it was observed that bypassed cells are extensively depleted in their cytoplasmic content of K⁺, but can rapidly recover (data not shown).

Colicin A mutants lacking part of the NH₂-terminal region are inactive under bypass conditions

Baty *et al.* (1988) demonstrated that colicin A mutants lacking the NH₂-terminal or central domain are inactive against sensitive cells. Those altered in the central domain are unable to bind to the receptor while those altered in the NH₂-terminal domain are affected in the translocation step across the cell envelope.

Here, we used the constructs described in Fig. 1A. The DS1 construct lacking the polypeptide region between amino acid residues 172 and 373 was tested on wild-type, BtuB⁻ and OmpF⁻ strains under normal or bypass conditions (Fig. 1B). As expected, the DS1 protein was unable to kill any of the strains (wild-type, BtuB⁻, OmpF⁻) under normal conditions. Under bypass conditions, all strains tested were killed. However, the OmpF⁻ strain was killed much less efficiently. Neither the BD2, AR1 or the BE1 protein (Fig. 1A) was capable of killing wild-type

Fig. 1. Requirement of the NH₂-terminal region of colicin A for translocation across the outer membrane. A, Description of colicin A deletion mutants; the linear organization of the translocation domain (T), receptor binding domain (RB) and pore-forming domain (PF) of colicin A are indicated, as are the various polypeptide regions contained in the deletion mutants used in this study. B, DS1 activity assay under normal or bypass conditions. A volume (10 μ l) of a colicin dilution was added to 100 μ l of OmpF⁻, BtuB⁻ or wild-type cells under normal conditions ($\square$) or to OmpF⁻ ($\blacksquare$), BtuB⁻ ($\blacktriangle$) or wild-type cells ($\bullet$) under bypass conditions. The cells were incubated at 37 $^{\circ}$ C with shaking. As a control, sodium phosphate buffer was used instead of the colicin dilution; LB medium (1.25 ml) was then added and the culture OD₆₀₀ was measured until it reached 0.4 in the control. The percentage of viable cells was determined from the OD₆₀₀ ratios of the colicin-treated and control culture. The dilution factor indicates the dilution of colicin after the addition of 100 μ l of the cell suspension. Each assay was done in triplicate. C, BD2 activity assay under normal and bypass conditions. The assays were done as in B with OmpF⁻, BtuB⁻ or wild-type cells under normal conditions ($\square$), or with OmpF⁻ ($\square$), BtuB⁻ ($\blacktriangle$), or wild-type cells ($\bullet$) under bypass conditions. Assays were done in triplicate. D, AR1 and BE1 activity assay under normal and bypass conditions. The assays were done as described in B with wild-type cells under normal or bypass conditions ($\blacksquare$).

or mutant strains under both normal and bypass conditions (Fig. 1C, D), although cell viability slightly decreased at very high concentrations of BD2 protein under bypass conditions (Fig. 1C). Similar results were obtained using osmotically shocked cells (data not shown). These results demonstrated that, under bypass conditions, mutant colicin A molecules defective in the receptor binding step were fully active while mutant molecules defective in translocation remained inactive. Therefore, under these conditions, the receptor binding step is no longer required for lethal action of colicin A (or mutant DS1) but the translocation step is still essential. The generic term 'translocation' includes crossing the outer membrane and periplasmic space as well as insertion into (with colicins A and E1) or transfer across (with colicins E2 and E3), the cytoplasmic membrane. One cannot rule out that any of these stages (but not all) may be altered under bypass conditions. since individual stages cannot be assayed, one can only conclude that the global translocation across the envelope cannot be bypassed by shifting cells to low ionic strength buffer.

Fig. 2. Role of BtuB and OmpF in colicin A uptake. The assays (in triplicate) were done as described in Fig. 1B with OmpF⁻ (□), BtuB⁻ (△) or wild-type cells (○) under normal conditions or with OmpF⁻ (■) BtuB⁻ (▲) or wild-type (●) under bypass conditions.

Role of BtuB and OmpF in colicin A uptake

The activity of colicin A on wild-type, BtuB⁻ and OmpF⁻ cells was compared under normal and bypass conditions (Fig. 2). As previously reported (Cavard & Lazdunski, 1981), BtuB⁻ cells can be killed although much less efficiently than wild-type cells. However, under bypass conditions, the susceptibility of BtuB⁻ cells was increased by about 100 times as compared to wild-type cells under normal conditions. This suggests that binding to BtuB is a limiting step in uptake of colicin A. The fact that wild-type and BtuB⁻ cells were equally susceptible under bypass conditions suggested that BtuB is not involved in the translocation step, because the presence or absence of this protein makes no difference when the only step in the uptake of colicin A is translocation. A similar result was obtained above with the construct (DS1) lacking the receptor binding domain (Fig. 1B).

Colicin A had no activity on OmpF⁻ cells. Bypass conditions restored to some extent the susceptibility of OmpF⁻ cells, which confirms that OmpF can act as a receptor (Cavard & Lazdunski, 1981; Chai *et al.*, 1982). However, even under bypass conditions, the susceptibility of OmpF⁻ cells was much lower (about 50 to 100 times) than that of BtuB⁻ cells. This strongly suggests that OmpF is also involved in the translocation step. Again, the same type of result was obtained, as mentioned above, with the construct (DS1) lacking the receptor binding domain (Fig. 1B).

Role of BtuB in colicin E1 uptake

The same type of experimental approach, as that described above, was applied to colicin E1. In contrast to the result obtained for colicin A, OmpF⁻ cells were as susceptible to colicin E1 as wild-type cells (Fig. 3), which confirms that OmpF plays no role in colicin E1 binding and uptake. Under bypass conditions, wild-type cells were much less susceptible than normally (Fig. 3). This suggested that interaction of colicin E1 with BtuB was also important for the second step in uptake (translocation). This was confirmed by the marked difference in susceptibility of wild-type and BtuB⁻ cells under bypass conditions (Fig. 3). In fact, hardly any BtuB⁻ cells could be killed. The presence or absence of OmpF in the outer membrane of cells did not make any difference under bypass conditions (Fig. 3). This demonstrates that OmpF is not involved in colicin E1 translocation (OmpF is required neither for binding nor for uptake) and that the opposite result obtained for colicin A does not result from a general defect in osmotically shocked OmpF⁻ cells, but constitutes strong evidence for an involvement of OmpF in colicin A translocation across the outer membrane.

Fig. 3. Role of BtuB in colicin E1 uptake. The assays (in triplicate) were done as described in Fig. 1B with OmpF⁻ (□), BtuB⁻ (△) or wild-type cells (○) under normal conditions or with OmpF⁻ (■), BtuB⁻ (▲) or wild-type cells (●) under bypass conditions.

Fig. 4. Activity assay of colicin E3 under normal and bypass conditions. The assays (in triplicate) were done as described in Fig. 1B with OmpF⁻ (□), BtuB⁻ (△) or wild-type cells (○) under normal conditions or with OmpF⁻ (■), BtuB⁻ (▲) or wild-type cells (●) under bypass conditions. A, assay of colicin E3 immunity complex; B, assay of colicin E3 free of immunity protein.

Role of BtuB and OmpF in the uptake of colicins E2 and E3

Colicins E2 and E3 are very tightly associated with their respective immunity proteins (Jakes & Zinder, 1974). These two colicins are highly homologous except for their catalytic domains (Ohno-Iwashita & Imahori, 1980). Wild-type cells were equally susceptible to the colicin E3 immunity protein complex whether or not they were submitted to a bypass treatment (Fig. 4A). Therefore, in contrast to colicin A, binding of colicin E3 to the receptor is not a limiting step for entry into cells of the colicin E3 complex. Since bypassed BtuB⁻ cells were much less susceptible than bypassed wild-type cells (Fig. 4A), BtuB probably plays a role in the translocation step. A similar result was observed for colicin E3 (free of immunity) (Fig. 4B) and for colicin E2 with (Fig. 5A) or without immunity (Fig. 5B).

BtuB may also play a role in dissociation of the immunity proteins upon receptor binding. BtuB⁻ cells would then be less susceptible either because the complex is hardly taken up for some reason, or because the colicins which enter the cells are still complexed with their immunity proteins and are therefore inactive.

Fig. 5. Activity assay of colicin E2 under normal and bypass conditions. The assays (in triplicate) were done as described in Fig. 1B with OmpF⁻ (□), BtuB⁻ (△) or wild type cells (○) under normal conditions or with OmpF⁻ (■), BtuB⁻ (▲) or wild-type cells (●) under bypass conditions. A, Assay of colicin E2 immunity complex; B, assay of colicin E2 free of immunity protein.

To deal with this problem, colicin E2 and E3 were dissociated from their respective immunity proteins, purified and were then tested (Figs 4B and 5B). Qualitatively the same results were found in the presence or absence of immunity proteins. However, it cannot be ruled out that the low activity observed with colicins E3 and E2, about 1% of that of complete colicins (complexed with immunity proteins), is due to a residual amount of immunity protein still attached to the colicins. Therefore, BtuB may play a role not only in the translocation of E2 and E3, but also in dissociation of the bacteriocin complex. However, recent results from M. Mock (personal communication) suggest that the immunity protein is not required for the uptake of colicin E3.

The OmpF⁻ strain displayed the same susceptibility to colicin E3 complex (Fig. 4A) or to colicin E2 complex (Fig. 5A) regardless of the bypass treatment, which confirmed that OmpF does not play any role in receptor binding. In contrast, under bypass conditions, OmpF⁻ cells were less susceptible (about 10 times) to colicin E2 than were wild-type cells. This suggests that OmpF plays a role in translocation, although to a lesser extent than BtuB, since BtuB⁻ cells are much less sensitive than OmpF⁻ cells under bypass conditions.

DISCUSSION

In this work we took advantage of the fact that in the uptake pathway, the first step, binding of colicins to their receptor protein, and the second step, translocation, can be clearly distinguished. The roles of OmpF and BtuB in the uptake of colicin A, various truncated colicin A polypeptides, and colicins E1, E2 and E3 was investigated.

The results demonstrated that the translocation step of colicin A cannot be bypassed. Such a conclusion has previously been reached using either Tol mutants or colicin A mutants (Bourdineaud *et al.*, 1989). OmpF appeared to be involved both as a receptor protein for colicin A and in the translocation step, while BtuB is only required as a receptor protein for colicin A (Table 1). This conclusion was supported by the fact that OmpF⁻ BtuB⁻ cells cannot be killed even under bypass conditions (Cavard & Lazdunski, 1981).

For colicin E1, in contrast to colicin A, the OmpF protein is used neither as a receptor protein nor for translocation. The BtuB protein has a dual role (Table 1). It might be argued that the different results observed for colicin A with OmpF⁻ and BtuB⁻ cells under bypass conditions could stem from behaviour specific to OmpF⁻ cells under these conditions. This is not the case, since for colicin E1, OmpF⁻ and wild-type cells displayed the same susceptibility under bypass conditions. The OmpF defect therefore is specific to colicin A and not due to some general membrane defect arising during osmotic shock. However, one cannot rule out the hypothesis

Table 1. Requirements for receptor binding and translocation into susceptible cells for colicins A, E1, E2 and E3

Step	OmpF	BtuB
Colicin A translocation	+	—
Colicin A receptor binding	+	+
Colicin E1 receptor binding	—	+
Colicin E2 and E3 translocation	+	+
Colicin E2 and E3 receptor binding	—	+

that OmpC, which is 60% homologous to OmpF, may account for the residual translocation of colicin A under bypass conditions.

For colicins E2 and E3, the situation is intermediate to that for colicin A and colicin E1. Only BtuB is used as a receptor protein, but both BtuB and OmpF are involved in the translocation step (Table 1).

Thus, BtuB and OmpF play different roles in the uptake of colicins. This is not surprising for the OmpF receptor as it has been previously demonstrated that the specificity of OmpF protein to act as a colicin N receptor protein is located within the 80 NH₂-terminal amino acid residues, whereas the specificity of this protein to act as a colicin A receptor protein is located within the 260 COOH-terminal amino acid residues (Tommasen *et al.*, 1984).

The apparent differences in uptake systems with the BtuB colicin group is not correlated with their mode of action since colicin E1 (pore forming) does not require OmpF while colicins E2 and E3 (nucleases) do seem to need OmpF for translocation.

In this study, the role of the immunity protein in uptake of colicins E2 and E3 was also considered. This question has been previously investigated not only with colicin E3 (Ohno *et al.*, 1977) but also with cloacin DF13 which is closely related to colicin E3 (Krone *et al.*, 1986). In the latter case, a detailed investigation indicated that binding of cloacin DF13 to susceptible cells resulted in a specific dissociation of the complex, and the immunity protein was detected in the culture medium. These results suggested that binding to the receptor may cause dissociation. However, it has recently been demonstrated that colicin E3 alone is active on sensitive cells (M. Mock, personal communication). In this paper, we found that dissociation of the colicin E2 and E3 complex prior to receptor binding results in a dramatic decrease in colicin activity. The residual activity is probably related to immunity protein still attached to the colicin, which protects colicin E3 from proteolytic degradation as recently demonstrated (M. Mock, personal communication).

At present, the role of OmpF and BtuB in the translocation step for each specific colicin needs further investigation. The *tolB* mutants are susceptible to colicin E1, whereas they are tolerant to colicins A, E2 and E3 (Nagel De Zwaig & Luria, 1967; Bernstein *et al.*, 1972). It is thus tempting to suggest that OmpF and BtuB may establish a connection with different Tol proteins. Using hybrid colicins, we have obtained indirect evidence that the NH₂-terminal region of colicin A is involved in an interaction with Tol proteins (M. Frenette and H. Benedetti, unpublished results).

We thank Dr D. Cavard for helpful discussions and generous gifts of plasmids ColE1-K30, ColE2-P9, ColE3-CA38 and purified colicin E1 and colicin E2 and E3 heterodimers, and Martine Knibiehler for the purification of the other colicins.

This work was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale and the Fondation pour la Recherche Médicale. M. F. is the holder of a post-doctoral fellowship from the Fonds de la Recherche en Santé du Québec.

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