Binding of colicins A and E1 to purified TolA domains

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Colicins are divided into two groups according to the proteins required for their import into sensitive bacteria. The Tol and TonB pathways are involved in import of group A and group B colicins respectively. Because previous analyses have shown that colicin E1 and colicin A (two group A colicins) interact *in vitro* with the C-terminal domain of TolA (TolAIII) while colicin B (group B colicin) does not, attention was focused on these interactions with purified proteins. TolA has been described as a three-domain protein with an N-terminal inner-membrane anchor and a long periplasmic region formed by two domains (TolAII and TolAIII). TolAIII, TolAII and TolAII–III soluble domains with an N-terminal hexa-histidine extension were purified. The interactions of colicins with the purified TolA domains were analysed by overlay Western blotting, which indicated that both N-terminal domains of colicins A and E1 interacted with TolAIII, while a gel shift procedure detected no interaction with colicin E1. The binding kinetic values of the N-terminal domains of colicins A and E1 to TolAIII were estimated by surface plasmon resonance and were shown to be similar.

Keywords: Tol proteins, colicins, protein interactions

INTRODUCTION

The Tol and TonB systems are involved in macromolecule import into *Escherichia coli*. Colicins and phage DNA have parasitized these protein complexes to penetrate into bacterial cells. In order to reach their cellular targets, colicins follow a three-step process. First, they bind to bacterial receptors; second, they translocate through the outer membrane; and third, they act either by forming a pore into the inner membrane or by exerting nuclease activity in the cell cytoplasm (for reviews see James *et al.*, 1996; Lazdunski, 1995). The TonB system is required for active transport of vitamin B₁₂ and siderophores (Postle, 1993), while the function of the Tol system is not yet understood, although it is required for the integrity of the cell envelope (Webster, 1991; Fognini-Lefebvre *et al.*, 1987; Lazzaroni & Portalier, 1992). The TolA/B/Q/R/Pal and Orf2 proteins form the Tol system. The topologies of the inner-membrane-anchored TolA, TolQ and TolR proteins have been determined (Levengood & Webster,

1989; Kampfenkel & Braun, 1993; Vianney *et al.*, 1994; Müller *et al.*, 1993). TolB and Orf2 have been found to be periplasmic proteins (Isnard *et al.*, 1994; Vianney *et al.*, 1996). TolA, TolQ and TolR form an inner-membrane Tol-protein complex, with the membrane domain of TolA interacting with TolQ and TolR (Derouiche *et al.*, 1995), while the membrane anchor of TolR was shown to interact with the third membrane segment of TolQ (Lazzaroni *et al.*, 1995). An outer-membrane protein complex consisting of the periplasmic TolB protein and the lipoprotein Pal was also found (Bouveret *et al.*, 1995). Furthermore, cell-fractionation experiments have revealed that TolQ, -R, -A and -B form a protein complex of defined stoichiometry (Guihard *et al.*, 1994). TolA has been described as a three-domain protein (Levengood *et al.*, 1991), corresponding to a membrane anchor, a central domain and a C-terminal domain (domains I, II and III, respectively). *In vitro*, the TolA central domain (domain II) interacts with outer-membrane trimeric porins and its function has been suggested to be related to the assembly process of outer-membrane porins requiring LPS (Derouiche *et al.*, 1996). When expressed and exported to the periplasm of wild-type cells, TolA

Abbreviation: SPR, surface plasmon resonance.

domain III causes the same phenotypic effects as a *tol* mutation; moreover, domain III alone competes with TolA for group A colicin import (Levengood-Freyermuth *et al.*, 1993). We previously demonstrated, using crude cell extracts, that TolAIII interacted with colicins and that these interactions might be relevant to a step in the colicin import process through the outer membrane (Bénédicti *et al.*, 1991). Recently, TolB was shown to interact with the colicin E3 N-terminal domain and, thus, might also play a role in the translocation step of colicins (Bouveret *et al.*, 1997). In this paper, the interactions of purified TolA periplasmic domains with two pore-forming colicins and their translocation domain were analysed by using overlay Western blotting, gel shift assays and surface plasmon resonance (SPR) techniques.

METHODS

Plasmid constructs. Plasmid pAR3 (Perez-Perez & Gutierrez, 1995) contains the chloramphenicol-resistance gene and *ori* from pACY184 with the *araC* gene and the *araB* promoter followed by the multiple cloning site from pAra12 (Cagnon *et al.*, 1991). An additional transcriptional terminator formed by an 81 bp DNA fragment from the crystal protein of *Bacillus thuringiensis* was inserted into the *Bgl*III restriction site, giving plasmid pART3 (J. Gutierrez, unpublished).

First, we inserted into the unique *Pst*I site of pART3 a synthetic DNA fragment (5'-CATCATCATCATCACC GCATGCAAGCGGCCGCAAGGCCT-3') encoding six histidines, giving vector pARTHis. The six histidine codons are followed by three unique restriction sites, *Sph*I, *Not*I (or *Eag*I) and *Stu*I (underlined).

The plasmids encoding the TolA domain II–III and domain III were obtained by inserting the 1146 bp *Sph*I–*Hpa*I and the 447 bp *Eag*I–*Hpa*I DNA fragments of pTPS306 (Sun & Webster, 1986) into pARTHis digested by *Sph*I–*Stu*I and *Eag*I–*Stu*I, respectively. The plasmid encoding TolAII was obtained in two steps. First, the unique *Esp*I site of pART3 was destroyed after being filled with Klenow polymerase and ligated. The *Sph*I–*Hpa*I DNA fragment encoding TolAII–III was inserted into this plasmid. Then, a synthetic DNA fragment containing two stop codons (5'-TAAGAGATCTG-TAATGAACCATGGAA-3') was inserted into the unique *Esp*I site of the *tolAII–III* gene. These three plasmids were called pARTolAII–III, pARTolAIII and pARTolAII.

Plasmid pHS was obtained from plasmid pBRE1SX in which two restriction sites were introduced between the three domains of the colicin E1 gene (Frenette *et al.*, 1991). The *Spe*I–*Afl*II DNA fragment was deleted and thus plasmid pHS encodes the N-terminal domain of colicin E1 (HS domain).

Purification of TolA derivatives and N-terminal domains of colicins A and E1. The plasmids encoding TolAII–III and TolAIII were transformed into the recipient MC1061 cells (Cagnon *et al.*, 1991). TolAII, which was poorly induced in MC1061 (this might be due to the *Esp*I mutation in the *araC* gene), was expressed in C600 cells. Briefly, cultures were grown in LB (containing 30 µg chloramphenicol ml⁻¹) to OD₆₀₀ 1.0. TolA expression was induced with 0.5 mg arabinose ml⁻¹ for 2 h. Cells were collected by centrifugation, lysed and further sonicated. After centrifugation at 100 000 g, supernatants were directly incubated with nickel or cobalt beads (Nb or Cb, respectively) for 1 h. All buffers used for

purification with Nb (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.5 mM PMSF) and Cb (20 mM Tris/HCl pH 8.0, 100 mM NaCl, 0.5 mM PMSF) are described by Qiagen (Quiaexpressionist) and Clontech (TALON metal affinity resin), respectively. After washing, the TolA derivatives were eluted with between 50 and 150 mM imidazole buffer. After dialysis against 20 mM Tris/HCl pH 7.6, 20 mM NaCl buffer, the second step of purification was carried out using MonoS cation-exchange chromatography (Pharmacia). TolAII–III and TolAIII were eluted with between 0.1 and 0.3 M NaCl; cation-exchange chromatography was not performed for TolAII.

AT1 and HS (N-terminal domains of colicins A and E1, respectively) were purified from C600 cell supernatants after induction with mitomycin C. Briefly, after ammonium sulphate precipitation and dialysis against 50 mM phosphate buffer pH 6.8, the N-terminal domains were purified by MonoS chromatography as described for AT1 by Knibiehler *et al.* (1989). Colicin A was purified as described by Baty *et al.* (1987), while colicin E1 was a gift from W. A. Cramer (Purdue University, Ind., USA).

Amino acid compositions and N-terminal protein sequences.

Amino acid composition determinations performed on purified TolA derivatives, AT1 and HS proteins gave the expected ratios. From two amino acid compositions, the ϵ_{280} values of TolAII–III and TolAIII were calculated to be 10300 and 6900 M⁻¹ cm⁻¹, respectively. N-terminal sequences of TolAIII and TolAII–III were checked and the results indicated that the N-terminal formylmethionine was removed. Since TolAII does not have many aromatic residues (no W, no Y and only one F residue), the ϵ_{280} was not estimated.

Western blot analysis. Overlay techniques have been previously described by Bénédicti *et al.* (1991). Briefly, TolA derivatives were heat-denatured with reducing agent in sample buffer before SDS-PAGE. After Western blotting of purified TolA derivatives, colicins were first incubated and immunodetected with their respective antibodies: monoclonal antibodies (mAbs) 1C11 or 3D6 for colicin A (Cavard *et al.*, 1986), or the polyclonal antibody (pAb) raised against the N-terminal domain of colicin E1 (anti-HS antibody), or a pAb raised against colicin E1 or colicin B. Then, immunodetection of colicin at the corresponding migration mobility of the TolA derivative confirmed that colicin interacted with purified TolA domains. The antibodies were previously checked for the absence of cross-reactivity with TolA.

SDS-PAGE shift assay. Protein complexes formed between TolA derivatives and either colicin A (or AT1), or colicin E1 (or HS) were analysed by SDS-PAGE. The incubations (15 to 30 min) of TolA with colicin derivatives were carried out in 10 mM Tris/HCl pH 8.0, 100 mM NaCl. Samples were directly applied with the addition of sample buffer (devoid of SDS, dye and reducing agent) on SDS-PAGE without any denaturing step and the electrophoreses run between 10 and 20 V cm⁻¹. Purified proteins were analysed by Coomassie blue staining.

Peptide synthesis. Two peptides encompassing the putative N-terminal 'TolA box' sequence (Pilsel & Braun, 1995) were synthesized and further purified by reverse-phase chromatography to perform competition-binding to TolAIII with their respective N-terminal domains. The sequences correspond to NH₂-G₂₇TPDGS₂₈SGGG-COOH (G11G) and NH₂-G₈K-GDGTGWSSE-COOH (G11E). The putative TolA boxes are underlined, with the position of the first residue of colicin E1 and colicin A, respectively, indicated.

SPR (BIAcore). The BIAcore system and reagents for interaction analysis including CM5 sensor chips, surfactant P20, *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were obtained from Pharmacia Biosensor. Interaction analysis was performed in 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005 % surfactant P20, pH 7.4 (HBS buffer).

TolA derivatives diluted (about 10-fold) to 100 μ M in 10 mM acetate pH 5.0 were immobilized on CM5 sensor chips through primary amino groups according to the standard EDC/NHS procedure (Johnsson & Löfås, 1991). Concentrations ranging from 0.2 to 2 μ M of colicins A, AT1, colicin E1 and HS in HBS buffer were used. To suppress the interaction observed when colicins A and E1 were injected onto the dextran matrix of the sensor chip, the colicins were diluted in HBS with 1 mg carboxymethyl dextran ml^{-1} (Fluka). Colicin B was used as a negative control. Regeneration steps were optimized for each protein to recover at least 90 % of the initial binding after many (10 to 20) cycles. Reciprocal interactions with immobilized colicins A and E1 and TolA derivatives injected into the buffer flow were also analysed. Experimental data were obtained in the form of sensorgrams, which show the amount of interacting molecules (expressed as resonance units, RU) as a function of time. A signal of 1000 RU corresponds to fixation of 1 ng protein mm^{-2} .

Kinetic data were interpreted with BIAevaluation 2.1 software. The association (k_a) and the dissociation (k_d) rate constants were calculated from the injection and post-injection phases of the interaction curve by a nonlinear regression method (O'Shannessy *et al.*, 1993).

RESULTS

Plasmid constructs and protein purification

Large amounts of TolA derivatives were obtained using the tightly regulated *araB* promoter from pART3 (see Methods). This plasmid contained the *araC* gene encoding the arabinose repressor acting on the *araB* promoter. To facilitate protein purification, a synthetic DNA fragment encoding an N-terminal six-histidine tagging sequence was added. Three plasmids, pARTolAII, pARTolAIII and pARTolAII–III, were constructed which expressed TolAII, TolAIII and TolAII–III proteins corresponding to 236 residues of the central domain, 132 residues of the C-terminal domain and 355 residues of the two soluble domains, respectively (Fig. 1). Soluble TolA derivatives were overproduced after 2 h of arabinose induction and were recovered after sonication of the cell pellet and ultracentrifugation. The first step of purification was obtained by chelating the histidine-tagged recombinant proteins on NTA-nickel beads (Nb) and eluting them by a batch procedure using increasing concentrations of imidazole buffer. By this approach, in addition to TolA derivatives, a protein was eluted between 20 and 40 mM imidazole. The N-terminal sequence of this protein was determined after being transferred onto a PVDF membrane. The first 10 residues, MTIFDNYEVN, were found to correspond to *E. coli* arabinose isomerase (which contains 21 histidines among the 500 residues). Cationic exchange chromatography was performed for TolAIII and TolAII–III purification to eliminate contaminants. TolA deri-

vatives, colicins A and E1 and their N-terminal domains are shown in Fig. 2 and represent at least 90 % purified proteins.

Overlay immunodetection of colicin binding to Western-blotted TolA derivatives

The antibodies were checked for their cross-reactivity with TolA derivatives before overlay immunodetection. A low background interaction was observed using the anti-HS antibody due to cross-reaction with TolA domain II; all the other antibodies, including the anti-colicin E1 antibody, did not cross-react with TolA. The overlay assays were performed according to the immunodetection of bound colicin on Western-blotted TolA domains. By this approach using anti-colicin E1 and anti-HS antibodies colicin E1 was clearly detected on Western-blotted TolAIII while its N-terminal domain (HS) was poorly immunodetected. To avoid any steric hindrance that might occur between the antibody recognition sequence and the region interacting with TolAIII, we used the hybrid colicin E1AA, which comprises the N-terminal domain of colicin E1 (identical to that of the HS protein) and the central and C-terminal domain of colicin A (Frenette *et al.*, 1991). Its binding to TolA was immunodetected by using mAb 3D6 (Cavard *et al.*, 1986) (a mAb directed against an epitope previously shown to contain residues 372 to 388 of colicin A; Bénédicti *et al.*, 1991). This experiment demonstrated that the N-terminal domain of colicin E1 was able to interact with the C-terminal domain of TolA because the two colicin A domains present in E1AA did not interact with TolA (as previously shown by Bénédicti *et al.*, 1991). In conclusion, these results indicated that the N-terminal domain of colicins A and E1 interacted with TolAIII but not with TolAII (Table 1).

Gel shift assays of colicins binding to TolA

When colicin A and AT1 were incubated with TolAII–III or TolAIII without denaturing agent and further analysed by SDS-PAGE shift assay, high-molecular-mass bands appeared after Coomassie blue staining (Fig. 3). Western blot immunodetections indicated that these bands corresponded to complexes formed by TolA and colicin A (not shown). According to the molecular masses of these complexes, they might correspond to a heterodimer formed by colicin A (or AT1) and TolAIII (or TolAII–III). Upon heat denaturation the monomeric form of each protein was recovered. No protein complex was observed between TolAII and colicin A, and no TolA derivative interacted with colicin E1 and HS to form a band shift under the same conditions (Fig. 3). As a negative control, when colicin B or the C-terminal domain of colicin A was used, no complex was detected (not shown).

Estimation of the kinetic values of colicin binding to immobilized TolAIII by SPR measurements

The biosensor monitors interactions between pairs of molecules when one partner (the ligand) is immobilized

Fig. 1. Amino acid sequences of TolA derivatives. The two glycine clusters corresponding to the domain boundaries of TolA are indicated (residues 43 to 47 and 311 to 313). The inner-membrane-spanning segment is indicated by a white box. The N-terminal-tagged TolA derivatives are shown with their hexa-histidine extension. Additional amino acids added during the construction are indicated. Numbers correspond to the residue positions of wild-type TolA.

Fig. 2. Purified colicins and TolA domains used for the binding analyses. Coomassie blue-stained purified proteins used for surface plasmon experiments (the amounts of proteins ranging between 1 and 4 µg) were separated on 12.5% SDS-PAGE.

on the surface of the sensor and the other (the analyte) is in solution. TolAII-III and TolAIII were immobilized on CMS sensor chips through primary amino groups. Their interactions with colicins A, E1 and their N-terminal domains were clearly detected, whereas no interaction was observed using colicin B, the C-terminal domain of colicin A, and the purified colicin A derivative deleted of residues 32 to 172 (Bénédetti *et al.*, 1991) (not shown). BIAcore experiments clearly showed the interaction between HS or AT1 with TolAII-III and TolAIII domains, while no interaction occurred with TolAII. Fig. 4 shows the superimposition of two sensorgrams obtained with the N-terminal domains of colicins A (AT1) and E1 (HS) injected at the same concentration onto immobilized TolAIII. Some differences between HS and AT1 on TolAIII were observed, with a stronger association and higher dissociation rate for HS. To characterize the colicin-TolA interaction, we also measured kinetic constants. Under normal experimental conditions, and with a homogeneous binding partner, the association is expected to be pseudo-first order, as the analyte is continuously replenished in the flow and so remains at a constant concentration during the injection. Concentrations of colicins (A, E1) and their N-terminal domains (AT1 and

Table 1. Western blot immunodetection of colicin binding to purified histidine-tagged TolA domains

Purified TolA derivatives were separated by Western blotting, then colicins or their N-terminal domains interacting with TolA were immunodetected with the specified antibodies (polyclonal or monoclonal indicated by pAb or mAb, respectively). For each test, immunodetection was first performed with the antibody alone to check that no background interaction occurred.

	Antibody	TolAII-III	TolAII	TolAIII
Colicin A	mAb 1C11	+	—	+
Colicin E1	pAb HS	NS	NS	+
Colicin E1	pAb colicin E1	+	—	+
Colicin B	pAb colicin B	—	—	—
AT1	mAb 1C11	+	—	+
HS	pAb HS	NS	NS	+
E1AA	mAb 3D6	+	—	+

NS, Signal was not specific because the antibody reacts weakly with the TolA domain.

Fig. 3. Gel shift assay of colicin A and E1 and their N-terminal domains interacting with TolA derivatives (stained with Coomassie blue). The samples were incubated for 20 min and heat-denatured where indicated (H) prior to loading. TolA derivatives, colicins, N-terminal domains (AT1 and HS) and complexes (*) are indicated.

Fig. 4. Sensorgrams from the BIAcore showing the association and dissociation of AT1 and HS at the same concentration (1.6 μ M) on immobilized TolAIII. Arrow A indicates the beginning of protein injection, while arrow B corresponds to protein arrest in the flow buffer.

HS, respectively) were determined by an average of two amino acid compositions per protein. The homogeneity of each interactant was checked by SDS-PAGE (Fig. 2)

and was at least 90%. The analytical centrifugation experiments indicated the oligomeric state of the proteins. TolAIII and TolAII were monomeric in solution (data not shown), as was colicin A (Cavard *et al.*, 1988) and its N-terminal domain (Knibiehler *et al.*, 1989). Two sets of four sensorgrams for each colicin fragment (concentrations ranging from 0.2 to 2.0 μ M) were analysed using a pseudo-first-order kinetic model ($A + B \leftrightarrow AB$). The experimental sensorgrams were poorly described by that homogeneous model, as indicated by the large magnitude of χ^2 (> 10) associated with the best-fit description obtained by nonlinear regression analysis. Instead, a second exponential term in the integrated rate equation is required to describe the binding data more accurately, which involves a second, slower-binding phase. Apparent kinetic constants values were deduced using a more complex model: $A + B_1 + B_2 \leftrightarrow AB_1 + AB_2$, where the response upon time is described by two exponential terms. In this case the quality of the fit was better, as indicated by the decreased magnitude of χ^2 values (below 5 for HS and colicin E1 and generally below 10 for colicins A and AT1). Apparent values of k_a and k_d estimated for AT1 and HS on immobilized TolAIII and TolAII-III are shown in Table 2. The K_{d1} was calculated as the ratio k_{d1}/k_{a1} . K_{d1}

Table 2. Apparent kinetic constants for AT1 and HS on immobilized TolAIII and TolAII-III

Data were obtained with about 1300 and 2000 RU of immobilized TolAIII and TolAII-III, respectively. Experiments were done at least twice. Equal amounts of complexes 1 and 2 were evaluated (from the model $A + B_1 + B_2 \leftrightarrow AB_1 + AB_2$) except for HS-TolAIII, where complex 1 was predominant (80% of complexes formed).

Analyte	Immobilized protein	$10^{-4} \times k_{a1}$ ($M^{-1} s^{-1}$)	$10^{-5} \times k_{a2}$ ($M^{-1} s^{-1}$)	$10^3 \times k_{d1}$ (s^{-1})	$10^2 \times k_{d2}$ (s^{-1})
AT1	TolAIII	1.8 ± 0.4	1.2 ± 0.7	4.3 ± 0.2	7.2 ± 0.2
AT1	TolAII-III	3.7 ± 0.6	2.8 ± 1.0	6.0 ± 1.8	8.1 ± 0.1
HS	TolAIII	2.8 ± 2.2	10.0 ± 9.5	11.0 ± 2.0	3.8 ± 0.1
HS	TolAII-III	3.0 ± 2.2	1.6 ± 1.0	8.6 ± 1.0	2.4 ± 0.3

and K_{d2} values were estimated to be 0.2 and 0.6 μM for AT1 and 0.4 and 0.04 μM for HS, respectively. However, the K_{d2} value obtained with HS was relevant for only 20 % of complexes, while it represents 50 % for AT1.

A putative TolA box formed with the pentapeptide DGSG(S/W) was suggested for the colicins E1, E2 and E3 as well as for colicin A (sequence DGTGW) (Pilsel & Braun, 1995). We checked if such a peptide might compete with the interaction of the N-terminal domain of colicin A and E1 (fixed concentration of 0.2 μM) with immobilized TolAIII. Two 11-mer peptides containing the specific pentapeptide and the surrounding sequence of colicin A and E1 were synthesized (see Methods). Using 1, 10, 100 and 1000 molar ratio of G11G peptide to HS protein, no decrease in the signal response was measured, compared to the results obtained in the absence of peptide. This result was the same when using an increasing ratio of G11E peptide with AT1 protein. According to the RU signal, which is related to the molecular mass of the interactant, we might expect a decrease in the RU signal if the peptide interacted with TolAIII. Our data indicated that no competition occurred with the two peptides containing the putative TolA boxes of colicins A and E1.

DISCUSSION

This study describes the purification of TolA soluble derivatives with six-histidine tagging extensions, and their interaction with the translocation domain of colicins A and E1. We have previously shown, using crude cell extracts and overlay immunodetections, that TolAIII interacts with colicin A and E1 and that this interaction might be relevant to the *in vivo* translocation of colicins (Bndetti *et al.*, 1991). Here, with purified proteins, different approaches were used to analyse these interactions *in vitro*.

The six-histidine tagging system and the tightly regulated arabinose promoter-operator vector were useful for the purification of TolA derivatives since initial attempts of TolA purification using the T7 expression system without any tagging yielded very low amounts of soluble TolA derivatives (not shown). The first step in affinity purification was performed by a batch procedure (using either Nb or Cb), then a second step of purification by MonoS chromatography was necessary to remove contaminants. Using this approach, the arabinose isomerase was purified after elution with a low imidazole concentration. Optimization of production expression and the purification procedures is now in progress.

For the first time, we have demonstrated that the N-terminal domain of colicin E1 (HS), like that of colicin A (AT1), can interact with the C-terminal domain of TolA (TolAIII). Overlay analyses were performed using various antibodies. Because we observed that the antibody directed against the N-terminal domain of colicin E1 (HS) cross-reacted with TolAII, we checked for the homology of HS against a protein database by using the

NCBI BLAST network service. A sequence of 123 residues within TolAII protein was found with 37 % identity to that of HS (while no significant homology was found with AT1), which might explain the cross-reactivity of this antibody. The homologous regions corresponded to residue 43 to 156 and 113 to 226 of colicin E1 and TolA, respectively. The overlay assay using HS or the hybrid colicin E1AA followed by immunodetection using the anti-HS antibody (which does not cross-react with TolAIII) or the monoclonal antibody directed against colicin A demonstrated that TolAIII-HS interaction did occur. Mobility shift assays using SDS-PAGE confirmed the interaction of AT1 with TolAIII when the incubation was performed in the absence of SDS. According to the migration mobilities, the TolA-colicin complexes corresponded to dimers. However, by the same approach (SDS-PAGE followed by Coomassie blue staining), no complex was detected using colicin E1 or HS. This might indicate that the colicin E1-TolA interaction dissociated more rapidly during electrophoreses in the presence of SDS.

The last set of *in vitro* analyses was performed using SPR techniques. The first attempts at immobilization were obtained by amine coupling. Since the proteins contain numerous basic residues their coupling should generate some heterogeneity of presentation of the proteins on the chip. Another procedure using NTA coupling and further Ni^{2+} -chelating (Gershon & Khilko, 1995) was performed but did not work for the TolA-immobilization step, although the histidine extension was still detected by Western blotting. This can probably be explained by a faster dissociation of the chelated TolA under SPR conditions. However, by the amine coupling approach, the qualitative results indicating that the N-terminal domains of colicin A and E1 specifically interacted with TolAIII were confirmed. We also checked that TolAIII interacted with immobilized colicins A and E1. Quantitative data obtained with the BIAcore enabled us to estimate only the apparent kinetic parameters, since sensorgram interpretations deviate from pseudo-first-order kinetics. Several factors are likely to cause this kind of deviation, such as heterogeneity of ligand sites within the matrix, restricted access of the analyte to the ligand sites because of mass transport limitation or parking problems resulting from possible masking of potential sites by bound analytes (O'Shannessy & Winzor, 1996). The most common source, which was probably responsible in our experiments, is the heterogeneity of immobilized ligand sites, as the amine coupling chemistry used does not provide homogeneous presentation of the TolA molecules. Under these conditions, and knowing that the TolAIII protein was monomeric in solution and that the protein complex might correspond to a dimer (see above), the dissociation constants were found to be between 0.2 and 0.6 μM for the N-terminal domains of colicins A and E1 binding to immobilized TolAIII. Elementary tests for self-consistency of kinetic analysis obtained with biosensor instruments have been recently proposed but they are inappropriate in this analysis as they assumed a

simple binding model to be valid (Schuck & Minton, 1996). However, due to the relative deviation values, no significant differences were found between TolAIII and TolAII–III. On the other hand, competition experiments using two peptides containing the putative TolA boxes located within the N-terminal sequences of colicins A and E1 (Pilsel & Braun, 1995) were checked in competition assays with their respective colicin. The peptide containing residues 8 to 19 of colicin A did not interfere with AT1 binding on TolAIII. This is in accordance with the result obtained by Bénédetti *et al.* (1991) showing that the colicin A derivative deleted of residues 32 to 172 did not interact with TolA in overlay experiments, and suggests that residues 8 to 19 of colicin A do not correspond to a TolA box. However, we did not find any competition with the peptide of colicin E1 on HS binding either. Because we do not have a positive control, such experiments using SPR will be useful to check for a peptide sequence inhibiting colicin interaction to give a clear conclusion. New experiments using the isothermal titration calorimetry (ITC) technique have been described to analyse the interactions between colicin N, its OmpF receptor and other porins (Evans *et al.*, 1996). Such experiments using the ITC or SPR techniques with colicins, their receptors and Tol proteins might provide useful thermodynamic and kinetic information about the translocation step of colicins through the outer membrane.

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