The cytotoxic domain of colicin E9 is a channel-forming endonuclease

Khédidja Mosbahi[1,6], Christelle Lemaître[1,2,6¶], Anthony H. Keeble[1], Hamid Mobasheri[1,3], Bertrand Morel[4], Richard James[5], Geoffrey R. Moore[4], Edward J.A. Lea[1] and Colin Kleanthous[1]

[1]School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, U.K

[2]Present Address: Laboratoire de Spectrométrie de Masse Bioorganique, Université Louis Pasteur, UMR/ULP CNRS 7509, ECPM 25 rue Becquerel, F-67087 Cedex 2, France.

[3]Present Address: Laboratory of Membrane Biophysics, Institute of Biochemistry and Biophysics, University of Tehran, PO Box 13145-1384, IR Iran.

[4]School of Chemical Sciences, University of East Anglia, Norwich, NR4 7TJ, U.K

[5]Division of Microbiology and Infectious Diseases, University Hospital, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, U.K.

[6]These authors contributed equally to this work.

Address for correspondence: Prof. Colin Kleanthous

School of Biological Sciences

University of East Anglia

Norwich NR4 7TJ, U.K.

Tel: +44-1603-593221

Fax: +44-1603-592250

e-mail: c.kleanthous@uea.ac.uk

SUMMARY

Bacterial toxins commonly translocate cytotoxic enzymes into cells using dedicated channel-forming subunits or domains as conduits. We demonstrate that the small cytotoxic endonuclease domain from the bacterial toxin colicin E9 (the E9 DNase) exhibits non-voltage-gated, channel-forming activity in planar lipid bilayers and that this activity is linked to toxin translocation into cells. A disulfide bond engineered into the DNase abolished channel activity and colicin toxicity but left endonuclease activity unaffected, with NMR experiments suggesting decreased conformational flexibility as the likely reason for these alterations. Concomitant with the reduction of the disulfide bond was the restoration of conformational flexibility, DNase channel activity and colicin toxicity. Our data suggest that endonuclease domains of colicins may mediate their own translocation across the bacterial inner membrane through an intrinsic channel activity that is dependent on structural plasticity in the protein.

Biological membranes present a formidable barrier to the translocation of proteins, a process that often requires large, membrane-bound protein assemblies. This is true of protein translocation whether the protein is in the unfolded state, as in sec-dependent protein translocation, or in the folded state, as occurs in the export of metalloproteins through the Tat pathway of bacteria[1-3]. Bacterial toxins such as anthrax are also examples of proteins that translocate across membranes but here a much scaled-down apparatus is employed involving pore-forming subunits[4]. In the present work we describe the first example of an endonuclease, with widespread structural identity to enzymes in prokaryotes and eukaryotes, that forms ion-conducting channels in membranes and show that this activity is linked to its ability to translocate into bacteria.

Colicins are protein antibiotics released by *Escherichia coli* in order to kill closely related strains during times of stress, their importance in bacterial competition and colonization emphasized by the fact that a significant proportion of enterobacterial species are colicinogenic[5]. Active at nanomolar concentrations and generally induced through the SOS pathway, the mechanism by which colicins translocate into bacterial cells remains poorly understood. Their cytotoxic activities vary as do their cellular sites of action, from those that are cytotoxic in the periplasm, including ionophores that depolarize the cytoplasmic membrane and inhibitors of peptidoglycan synthesis, to those that cross the cytoplasmic membrane to digest DNA or RNA. While disparate in their mode of action, colicins nonetheless appear to use a common mechanism to traverse the outer membrane of Gram-negative bacteria. This involves a centrally-located receptor recognition domain which binds to an outer membrane protein, normally involved in the uptake of nutrients, and an N-terminal translocation domain that contacts proteins in the periplasm, the Tol proteins for group A colicins or the TonB/ExbB/ExbD proteins for group B colicins[5,6]. These multipartite interactions are thought to occur simultaneously and are geared toward bringing the C-terminal cytotoxic domain across the outer membrane.

Colicin E9 forms channels in membranes

Pore-forming colicins such as K, Ia, E1, N and A have been the focus of more than two decades of research and so the mechanism by which their cytotoxic domains form voltage-gated channels in the inner membrane is reasonably well understood[8-10]. By contrast, there have been few studies aimed at understanding how the cytotoxic domains of enzymatic colicins cross the cytoplasmic membrane of bacteria. It is not clear, for example, whether accessory proteins, such as the Tol proteins that are known from *E. coli* mutants to be needed for translocation[7], are involved directly in this process or whether the toxin can translocate across the cytoplasmic membrane unaided. In order to begin addressing these questions we investigated the interactions of the endonuclease toxin colicin E9 with membranes in planar lipid bilayer experiments. E9 (and its homologues E2, E7 and E8) is a group A colicin that kills bacteria through non-specific degradation of chromosomal DNA[11,12]. The hypothesis we intended to test was whether DNase colicins have channel forming domains that might be required for translocation of the DNase into the cytoplasm, by analogy with diphtheria toxin which has a channel-forming domain responsible for translocating its ADP-ribosyl transferase into the cytosol of eukaryotic cells[13].

Colicin E9 is a 60 kDa toxin that is normally released from colicinogenic bacteria in the form of a heterodimeric complex with its 9.5 kDa immunity protein, Im9 (ref. 14). The immunity protein protects the colicin-producing bacterium from the activity of its own toxin but is jettisoned on entry of the colicin into a susceptible cell[15]. Hence, the form of the toxin tested in the bilayer experiments had the immunity protein removed (see Materials and Methods section). Previous work from our laboratory has shown that this form of the toxin retains complete biological activity[14]. Immunity-free colicin E9 (2 nM) was added to the *cis* chamber of a bilayer apparatus in 10 mM Tris/HCl buffer at pH 7.5, containing 0.1 M NaCl and 10 mM $CaCl_2$, and a potential difference (p.d.) applied across the membrane. Random, fluctuating current was observed that showed evidence of opening and closing events with

conductance of the order of ~100 pS, although larger conductance states were also seen (Fig. 1*a*). In order to identify the region(s) of the protein responsible for this activity we analyzed a truncated colicin in which the E9 DNase domain had been deleted[16], but which retained the domains responsible for receptor binding and outer membrane translocation. Surprisingly, this construct did not produce channel activity (Fig. 1*b*) even at micromolar protein concentrations (data not shown). Finally, we analyzed the E9 DNase domain[17] and found that the channel activity, the characteristics of which were very similar to those of the full-length colicin, was associated with this domain (Fig. 1*c*).

Characterization of colicin DNase channels

We have investigated the channel activity of the colicin E9 DNase domain from 7 different protein preparations involving >80 separate membrane experiments with channels evident in every case. The channel data in Fig. 1 demonstrate that the enzyme shows discrete 'open' and 'closed' states, as well as a number of different conducting states with lifetimes that vary over the tens-of-milliseconds time range. In an attempt to evaluate the number, size and frequency of the different conducting states displayed by the E9 DNase domain we collected and analyzed data over a total record time of ~7000 seconds from eight independent bilayer experiments (Fig. 2*a*). The resulting histogram of channel frequency versus channel size revealed that the most frequently observed channels were ~100 pS, although a range of higher conductance states were also observed with a periodicity approximately equivalent to this unit size. This was confirmed when the current distribution for E9 DNase 'open' and 'closed' conductance states was analyzed for a single experiment using an all-points amplitude histogram where the ~100 pS channel is clearly apparent (Fig. 2*b*). The recordings for the E9 DNase have some similarity to those of the pore-forming colicins in that they display a range of conductance states[9]. However, unlike pore-forming colicins, the conductance states of the E9 DNase have much shorter lifetimes (milliseconds compared to

seconds) and display larger conductance states at the single channel level at relatively low salt concentrations. Colicin E1, for example, shows a single conductance state of ~10 pS at pH 6 and 1 M KCl[9].

We analyzed the DNase domains of the other E group colicins E2, E7 and E8 under equivalent conditions. All showed channel activity although the channels varied in size and gating behaviour in each case (data not shown). In a recent paper on the channel-forming toxin colicin A, it was reported that the DNase domain of colicin E2 did not induce channels in bilayers[18]. However, the experimental conditions used in these experiments (where channel events of the order of seconds were recorded) precludes their observation.

Since colicin endonucleases are basic proteins (pI ~9.5) it could be argued that the effects we observe are due to non-specific adsorption of these positively charged proteins to the bilayer resulting in its disruption or destabilisation. This can be discounted on the basis of three observations. Firstly, the discrete nature of the gating events we observe (Fig. 1) imply a specific protein-membrane interaction. Secondly, we investigated the ability of unrelated proteins, some of similarly high pI (cytochrome C, pI 9.5, lysozyme, pI 9.3), and including bovine serum albumin (pI 5.8), in separate bilayer experiments (n = 4) under the same conditions as those reported in Figure 1 and over a range of membrane potentials (±50-100 mV) but could not detect any channel behaviour (data not shown). Finally, we investigated whether an enzymatic toxin from another colicin family could induce channel activity. Colicin E3 is a ribonuclease specific for 16S ribosomal RNA. We added the 11 kDa rRNase domain of colicin E3 (pI 9.7) to bilayers but again failed detect any channel activity (Fig. 2*c*). We conclude that the channel activity of the DNase domain of colicin E9 is specific to colicin endonucleases and not due to non-specific effects of a basic protein interacting with the membrane bilayer.

We determined the voltage-dependence of the E9 DNase channels by collecting single-channel data for each applied voltage over a period of 125 s, analyzing the data in the

form of all-points amplitude histograms. From the peaks of Gaussian curves fitted to these (as in Fig. 2*b*), mean single-channel currents were obtained that were plotted against the applied membrane potential (Fig. 2*d*). The resulting I/V curve showed that the level of current increased linearly with the imposed voltage across positive and negative polarities indicating that, unlike pore-forming colicins, E9 DNase channels are not voltage-dependent. The single channel conductance derived from the regression line fitted to the data was 105 pS.

In order to estimate the relative selectivity of DNase channels for anions and cations, reversal potential (E_{rev}, the potential difference of the *cis* side relative to the *trans* chamber) measurements were determined with 0.1 M salt solution on the *cis* side and 1 M on the *trans* side of the membrane. E_{rev} for three salts, sodium chloride, sodium gluconate and glucosamine chloride, at pH 7.5 were found to be -20 mV, +33 mV and -35 mV, respectively (data not shown). Thus, the order of channel permeability is $Cl^- > Na^+$, $Na^+ > gluconate^-$ and $Cl^- > glucosamine^+$. Since there is significant deviation from the equilibrium potentials of the small ions used (E_{rev} = +58 mV for Na^+ and –58 mV for Cl^-), these data show that the DNase channels are permeable to large ions to a significant degree, similar to findings reported for pore-forming colicins[19-21].

The molecularity of the different E9 DNase conductance states is not known at the present time although the dominance of the ~100 pS channel at 2 nM protein suggests that this is the unit channel conductance for a single DNase molecule. In order to address this question further we analyzed the channel activity at a ten-fold higher protein concentration (Fig. 3). The 100 pS channels were even more prevalent under these conditions, with fewer periods where channels remained closed, which is characteristic of traces at 2 nM, but there did not appear to be any increase in the frequency of larger channel events as might be expected if the channels were oligomeric. However, there were instances of multiple insertions into the membrane as evidenced by ‘ladder-effects’ in bilayer traces (Fig. 3a) with

steps approximately equivalent to ~100 pS. We conclude from these data, and from the experiments at 2 nM, that the E9 DNase channels are likely to be monomeric with a unit conductance of ~100 pS and that the rarer, larger conductances we observe reflect multiple insertions into the bilayer rather than oligomeric assemblies of protein. A unimolecular explanation for this activity would also be consistent with the antibacterial single-hit kinetics displayed by colicins. It is interesting to note that the concentration of one molecule per bacterial cell is 1-2 nM, the concentration at which we readily detect E9 DNase channel activity in planar lipid bilayers (Fig.'s 1 and 2).

Using a bilayer composed of 70% phosphatidyl ethanolamine, 20% phosphatidyl glycerol and 10% cardiolipin, a mixture that more closely mirrors that of the inner membrane of *E. coli*[22], we found that the E9 DNase showed channel activity essentially indistinguishable from that seen using soybean lecithin (data not shown), indicating that membrane composition is not an important factor. We also investigated the effect of transition metal ions, DNA and Im9, all of which bind to the E9 DNase[23-26], on channel activity. Neither metal nor DNA binding affected the ability of the DNase to induce channels whereas Im9, which binds to the E9 DNase with an equilibrium dissociation constant of 10^{-14} M (ref. 25), completely abolished channel activity (data not shown). Hence, Im9 inhibits both the enzymatic activity of the colicin and its ability to form channels in bilayers.

Disulfide bond inhibition of DNase channels

Engineered disulfide bonds have proven to be a useful tool with which to probe the structural reorganisation/unfolding events that accompany the translocation of toxins across membranes[27,28]. Hence, a single disulfide bond was introduced into the 134 amino acid E9 DNase domain between residues 20 (D20C) and 66 (E66C) to assess its influence on channel activity (see Methods section for details; Fig. 4*a*). Although residues 20 and 66 are not optimally positioned for disulfide bond formation (the side-chains are separated by >3.5 Å)[29],

each is part of a highly mobile region of the DNase (see below)[30]. The purified double mutant, E9 DNase D20C/E66C (E9 DNase^{SH_2}), formed a disulfide bond (E9 DNase$^{S\text{-}S}$) when oxidised with diamide that, consistent with its location, did not affect either immunity binding or DNase activity of the domain (Fig. 4*b,c*). However, E9 DNase$^{S\text{-}S}$ failed to produce channels in bilayer experiments but channel activity was restored if the reducing agent dithiothreitol was added to the *cis* chamber, although the resulting channel activity differed to that of the wild type protein in being noisier (Fig. 5*a,b*). Prior alkylation by iodoacetamide of the reduced thiols of E9 DNase^{SH_2} resulted in channel data similar to that of the wild type protein but with smaller channels of ~50 pS also in evidence (Fig. 5*c*). These data demonstrate that the channel forming activity of the E9 DNase is an intrinsic property of the enzyme, and not due to a contaminant associated with its purification, and that a single intramolecular cross-link completely abolishes this activity.

Previous work from our laboratory has shown that the E9 DNase displays extensive conformational dynamics in solution with this flexibility centred on the 20's and 60's regions, where the disulfide bond was engineered, and readily manifest as doubled cross-peaks in ^{1}H-^{15}N HSQC-NMR spectra[30]. It was striking to find that E9 DNase$^{S\text{-}S}$ showed a much-simplified HSQC spectrum with no evidence of chemical exchange cross-peaks (Fig. 6). Nevertheless, there remained strong similarities between the chemical shifts of the wild-type enzyme and E9 DNase$^{S\text{-}S}$ (>80% of the amide resonances had identical chemical shifts) showing that they likely have similar structures. Doubled amide resonances equivalent to those of the wild type protein reappeared on reduction of the disulfide bond (Fig. 6). In addition, we found through temperature denaturation experiments that the stability of the E9 DNase$^{S\text{-}S}$ had increased by 11°C as a result of the covalent crosslink (data not shown). Hence, it is reasonable to assume that the loss of channel activity in E9 DNase$^{S\text{-}S}$ is because of its increased stability and reduced flexibility relative to the wild type DNase.

Colicin E9 cytotoxicity studies

We tested whether the equivalent disulfide bond in colicin E9 (D468C/E514C to generate ColE9$^{S\text{-}S}$) had antibacterial activity. On solid media, wild type toxin showed activity at nanomolar protein concentrations, similar to that reported previously[31], while ColE9$^{S\text{-}S}$ was inactive even at micromolar concentrations (Fig. 7*a*). Indeed, the disulfide-bonded toxin behaved as a previously identified active site mutant, ColE9 H575A, which is completely devoid of cytotoxic activity (Fig. 7*a*)[32]. Unlike conventional active site mutants, however, toxin activity for ColE9$^{S\text{-}S}$ could be recovered on reduction of the disulfide bond with dithiothreitol, although the reduced protein (ColE9^{SH_2}) only showed wild type colicin activity when first alkylated with iodoacetamide suggesting that it may be re-oxidising during import into bacteria. The cytotoxic activity of the thiol-alkylated toxin was confirmed in liquid culture where it showed wild type activity whereas the oxidised protein was again completely inactive (Fig. 7*b*). Our data demonstrate that a disulfide bond in the E9 DNase domain inhibits both channel activity in lipid bilayers and cytotoxic activity, with both activities restored on reduction of the disulfide bond.

DISCUSSION

Enzymatic A-B toxins such as diphtheria, cholera and pertussis kill mammalian cells by translocating their cytotoxic enzymes (the A domain) into the cytosol with the aid of the receptor-binding B domain[33,34]. In the case of diphtheria, it has been shown that the B domain also forms conducting channels (in planar lipid bilayers) that somehow form a conduit for the ADP ribosyl transferase to enter the cytosol[13]. In contrast to this mode of action our data suggest that the endonuclease domain of colicin E9 may act as its own membrane translocator. As well as having important implications for the mechanism by which enzymatic colicins translocate into bacteria, the present study highlights the potential for similar activity in proteins homologous to E9 such as intron and intein-encoded homing endonucleases that initiate recombination events in prokaryotes and eukaryotes[35].

Membrane translocation by DNase colicins

Since all enzymatic E group colicins require the same import apparatus in *E. coli* cells (BtuB/OmpF and the Tol proteins) it has generally been assumed that the mechanism by which the cytoplasmic membrane is breached will also be the same. However, only DNase and not rRNase toxin domains show channel activity in bilayers raising the possibility that their passage across the inner membrane may occur by different mechanisms.

The effects of the intramolecular disulfide bond serves to link colicin E9 cytotoxicity and E9 DNase channel activity and while not proving that the enzyme acts as its own translocator is certainly compelling. Arriving at more direct evidence may prove difficult since unlike diphtheria toxin where the translocating and cytotoxic activities are carried on distinct domains, the channel-forming and cytotoxic DNase activities of colicin E9 are contained within the same 15 kDa domain, functionalities that may not be easily separable.

Channel formation by the E9 DNase almost certainly involves substantial structural changes, and this may lie at the heart of the unusual conformational dynamics exhibited by

the enzyme in solution[30]. Structural rearrangements are also a pre-requisite for channel-formation by the ten helical bundle, pore-forming colicins where a molten globule has been inferred to be the active species[36,37]. In the case of colicin Ia, it has been shown that the domain translocates large segments of polypeptide and even proteins across the bilayer during cytotoxic pore-formation[18,19,38]. While the channel activity of the E9 DNase is associated with the ability of colicin E9 to kill bacterial cells it cannot be the causative agent of cell death. This is inferred from the colicin E9 active site mutant H575A which lacks enzymatic activity and is not cytotoxic (Fig. 7*a*)[32] but is still able to form conducting channels in bilayers (unpublished observations). A key question then is why the DNase channels themselves do not kill the cell during translocation? One way in which cell death could be avoided during channel-mediated translocation of the DNase across the inner membrane is that the endonuclease is proteolytically excised on reaching the cytoplasm, thereby allowing the membrane to re-seal. Indeed, proteolytic cleavage of both rRNase and DNase colicins has been reported where the processing site appears to be in a helical region linking the enzyme to the receptor binding domain of the toxin[39-41].

Concluding Remarks

Bacterial toxins by their nature are compact killing machines that engage in a variety of macromolecular associations with target cells in order to transfer folded protein domains or subunits into them. In general this is accomplished in two stages, receptor-mediated translocation across one or more membranes followed by the expression of a cytotoxic activity in the desired cellular location, with each associated with a specific domain or subunit. The present work has identified a channel-forming activity intrinsic to the cytotoxic endonuclease domain of an enzymatic colicin that is indispensable for cytotoxicity. Endonuclease domains of colicins are therefore remarkable multifunctional proteins capable of binding metal ions, immunity proteins, DNA and lipids, a diversity of macromolecular

associations that underpin the ability of a cytotoxic endonuclease to translocate from the extracellular environment to the cytoplasm of an *E. coli* cell.

METHODS

Proteins Colicin E9 (and the double-mutant ColE9 D468C/E514C) was purified as described by Wallis et al[14], E9 DNase (and the double-mutant E9 DNase D20C/E66C) as described by Garinot-Schneider et al[32] and the colicin T-R deletion construct as described by Penfold et al[16]. Proteins were routinely lyophilized either from water (DNases) or 50 mM potassium phosphate buffer pH 7.5 (colicins). Protein concentrations were obtained by absorbance at 280nm[25,31].

Planar lipid bilayers and single-channel recordings Bilayers were formed principally from Soybean Lecithin (Type II-S, Sigma) using the technique of Montal-Muller[42]. The electrolyte used in the bilayer experiments was 10 mM Tris/HCl, pH 7.5, 0.1 M NaCl, 10 mM $CaCl_2$. Colicin E9, its derivatives and the E9 DNase were added to the *cis* compartment to a final concentration of 2 nM or 20 nM and single-channel measurements made at room temperature as described previously[43,44]. Briefly, using reversible Ag/AgCl electrodes and an applied p.d., membrane current was measured using a bilayer amplifier (HAMK2TC, R.A.P. Montgomery, London, UK) filtered at 1000 Hz with a low pass filter (VBF/3, Kemo Ltd). All quoted p.d.'s are referenced to the *cis* compartment. Recordings were made using a pc with a CED 1401 interface (Cambridge Electronic Design) and data analyzed using patch-clamp software (PAT V7.0)[45]. Records typically lasted ~100 s with 250 ms sections of these records shown in the figures.

To determine the range of channel activities exhibited by the E9 DNase, channel data from 8 separate bilayer experiments were analyzed covering ~7000 s of records. The range of current over which the signal extended was divided into a series of contiguous, equal sized 'bins' and the collective data analyzed as the frequency with which each conductance state was observed against the range of different states detected.

All-points amplitude histograms from single channel records were obtained using the patch-clamp software and the resulting histograms fitted to Gaussian curves to derive mean single channel conductances. An I/V curve was subsequently generated by plotting the mean single-channel currents for the E9 DNase at 2 nM as a function of the applied p.d.

Cation/anion selectivity estimates of E9 DNase channels in bilayers were determined in 10 mM Tris/HCl, pH 7.5, 10 mM $CaCl_2$ with 1 M salt solution on the *trans* side of the membrane and 0.1 M salt in the *cis* compartment. Agar bridges were used in conjunction with Ag/AgCl electrodes to avoid electrode potential problems arising from the different solutions used. The reversal potential (E_{rev}) was estimated for sodium chloride, sodium gluconate and glucosamine chloride as the p.d. applied to the *cis* side of the membrane for which the DNase channel openings changed polarity.

Disulphide bond engineering Plasmid PCR mutagenesis using *Pfu*-Turbo (Stragene) was used to engineer the E9 DNase D20C/E66C double mutant in two rounds of mutagenesis and the presence of the mutations confirmed by DNA sequencing and electrospray ionisation mass spectrometry (ESI-MS) of the purified protein (theoretical MW, 15048 Da; experimental MW: 15048.58±1.70 Da). The E9 DNase D20C/E66C domain, along with the downstream histidine-tagged immunity gene, was also sub-cloned into the complementary *Nco*I-*Xho*I sites of the pCS4 plasmid[32] allowing for purification of intact colicin E9 containing the double mutant in the DNase domain (ColE9 D468C/E514C).

For both E9 DNase D20C/E66C and ColE9 D468C/E514C mutants, proteins were purified in 50 mM Tris pH 7.5 in the presence of 10 mM DTT until disulfide bond formation was induced by oxidation. The E9 DNase D20C/E66C required a 20 min incubation with 1 mM diamide at room temperature whereas the disulfide bond in the intact colicin formed readily on removal of reductant by dialysis (the reasons for this difference in disulfide

stability is unclear). The presence of the disulfide bond was confirmed in both proteins by the differential alkylation of the thiols by iodoacetamide (50 mM for 30 min in the dark at room temperature) with or without prior reduction with DTT, followed by ESI-MS where only monomeric species were detected. In each case, the two thiols could only be alkylated following prior reduction with DTT and all masses were within 1-2 Da of the expected mass. In the case of E9 DNase$^{S\text{-}S}$, the presence of the disulfide bond was also confirmed by the loss of reactivity with the thiol reagent DTNB and by its faster migration on SDS-gels when compared with the reduced protein. Gel-filtration chromatography under native conditions, as described by Wallis et al[14,17], confirmed the monomeric nature of the oxidised forms of E9 DNase$^{S\text{-}S}$ and ColE9$^{S\text{-}S}$.

Biophysical experiments Endonuclease activity was assayed in 50 mM triethanolamine buffer pH 7.5, 10 mM Mg^{2+} at 25°C with 5 μM protein and 2 μM DNA by following the hyperchromic shift resulting from the cleavage of a double-stranded oligonucleotide (after Baldwin et al[46]). A 12 mer dsDNA palindromic sequence 5'-GACGATATCGTC-3' (re-annealed following prior heating to 80°C) was used as substrate and its hydrolysis monitored by time dependent increase of A_{260} after mixing the protein with DNA using a π* stopped-flow CD spectrophotometer in the absorbance mode (Applied Photophysics). Im9 binding studies of the reduced/oxidised E9 DNase D20C/E66C protein were carried out on a Shimadzu RF5000 or Spex Fluoromax essentially as described by Wallis et al[14]. NMR spectra were recorded on a Varian Unity Inova 600 MHz spectrometer with pulse sequences implemented in the Varian 'Protein-pack' suite of experiments using a 2 mM E9 DNase D20C/E66C sample which had been doubly labelled with ^{13}C and ^{15}N, as described by Whittaker et al[30]. HSQC spectra were first recorded for the oxidised protein and then again following reduction of the sample with dithiothreitol (10 mM). Assignments were obtained

from CBCA(CO)NH and HNCACB spectra as described by Boetzel et al[47] and references cited therein.

Biological Activities of Colicins Determination of colicin activity against *E. coli* JM83 using an agar plate assay was as described by Wallis et al[31]. Where alkylated colicin was used, the reduced protein was first treated iodoacetamide as described above. For liquid culture experiments, 3 μg/ml (final concentration) colicin was added to 20 ml culture in LB medium inoculated with an overnight culture of JM83 and growth monitored as change in optical density (OD_{600}) at 30 min intervals.

Word count = 4,253

ACKNOWLEDGMENTS

We thank A. Reilly, C. Moore and N. Cull for expert technical assistance and A. Leech for help with the acquisition of all mass spectrometry data. We also thank the referees of this paper for their helpful comments. This work was supported by The Biotechnology and Biological Sciences Research Council. AHK was supported by a Wellcome Trust Prize Studentship.

REFERENCES

1. Johnson, A.E. & van Waes, M.A. The translocon: A dynamic gateway at the ER membrane. *Annu. Rev. Cell Dev. Biol*. **15**, 799-842 (1999).
2. Manting, E.H. & Driessen, A.J.M. *Escherichia coli* translocase: the unravelling of a molecular machine. *Mol. Microbiol*. **37**, 226-238 (2000).
3. Berks, B.C., Sargent, F. & Palmer, T. The Tat protein export pathway. *Mol. Microbiol*. **35**, 260-274 (2000).
4. Koehler, T.M. & Collier, R.J. Anthrax toxin protective antigen–low pH-induced hydrophobicity and channel formation in liposomes *Mol. Microbiol*. **5**, 1501-1506 (1991).
5. James, R., Lazdunski, C. & Pattus, F (eds) *Bacteriocins, microcins and lantibiotics* (NATO ASI Series H, Springer, Heidelberg; 1992)
6. Lazdunski, C.J. *et al.* Colicin import into *Escherichia coli* cells. *J. Bacteriol.* **180**, 4993-5002 (1998).
7. Webster, R.E. The *tol* gene products and the import of macromolecules into *Escherichia coli* cells. *Mol. Microbiol*. **5**, 1005-1001 (1991).
8. Schein, S.J., Kagan, B.L. & Finkelstein, A. Colicin K acts by forming voltage-dependent channels in phopholipid bilayer membranes. *Nature* **276**, 159-163 (1978).
9. Cramer, W.A. *et al.* Structure-function of the channel-forming colicins. *Annu. Rev. Biophys. Biomol. Struct*. **24**, 611-641 (1995).
10. Gouaux, E. The long and short of colicin action: the molecular basis for the biological activity of channel-forming colicins. *Structure* **5**, 313-317 (1997).
11. James, R., Kleanthous, C. & Moore, J.R. The biology of E colicins: paradigms and paradoxes. *Microbiology* **142**, 1569-1580 (1996).

12. Pommer, A.J., Wallis, R., Moore, R., James, R. & Kleanthous, C. Enzymological characterization of the nuclease domain from the bacterial toxin colicin E9. *Biochem. J.* **334**, 387-392 (1998).

13. Oh, J.K., Senzel, L., Collier, R.J. & Finkelstein, A. Translocation of the catalytic domain of diphtheria toxin across planar phospholipid bilayers by its own T domain. *Proc. Natl. Acad. Sci. USA* **96**, 8467-8470 (1999).

14. Wallis, R. *et al. In vivo* and *in vitro* characterization of overproduced colicin E9 immunity protein. *Eur. J. Biochem*. **207**, 687-695 (1992).

15. Kleanthous, C. & Walker, D. Immunity Proteins: Enzyme inhibitors that avoid the active site. *Trends Biochem. Sci*. **26**, 624-631 (2001).

16. Penfold, C.N. *et al.* A 76 residue polypeptide of colicin E9 confers receptor specificity and inhibits the growth of vitamin B12-dependent *E.coli* 113/3 cells. *Mol. Microbiol*. **38**, 639-649 (2000).

17. Wallis, R. *et al.* Tandem overexpression and characterization of the nuclease domain of colicin E9 and its cognate inhibitor protein Im9. *Eur. J. Biochem*. **220**, 447-454 (1994).

18. Slatin, S.L., Nardi, A., Jakes, K., Baty, D. & Duché, D. Translocation of a functional protein by a voltage-dependent ion channel. *Proc. Natl. Acad. Sci*. *USA* **99**, 1286-1291 (2002).

19. Jakes, K.S., Kienker, P.K. & Finkelstein, A. Channel-forming colicins: translocation (and other deviant behaviour) associated with colicin Ia channel gating. *Quat. Rev. Biophys*. **32**, 189-205 (1999).

20. Raymond, L., Slatin, S.L. & Finkelstein, A. Channels formed by colicin E1 in planar lipid bilayers are large and exhibit pH-dependent selectivity. *J. Membr. Biol*. **84**, 173-181 (1985).

21. Bullock, J.O. & Kolen, E.R. Ion selectivity of colicin E1. *J. Membr. Biol*. **144**, 131-145 (1995).

22. Kadner, R.J. In *Escherichia coli and Salmonella; cellular and molecular biology* 2nd Edition, (ed. Neidhardt, F.C.), Vol. 1, 58-87 (ASM Press, Washington; 1996).

23. Kleanthous, C. *et al.* Structural and mechanistic basis of immunity towards endonuclease colicins. *Nature Struct. Biol.* **6**, 243-252 (1999).

24. Pommer, A.J. *et al.* Homing-in on the role of transition metals in the HNH motif of colicin endonucleases. *J. Biol. Chem*. **274**, 27153-27160 (1999).

25. Wallis, R., Moore, G.R., James, R. & Kleanthous, C. Protein-protein interactions in colicin E9 DNase-immunity protein complexes. Diffusion-controlled association and femtomolar binding for the cognate complex. *Biochemistry* **34**, 13743-13750 (1995).

26. Pommer, A.J. *et al.* Mechanism and cleavage specificity of the H-N-H endonuclease colicin E9. *J. Mol. Biol*. **314**, 735-749 (2001).

27. Falnes, P.Ø., Choe, S., Madshus, I.H., Wilson, B.A. & Olnes, S. Inhibition of membrane translocation of diphtheria toxin A fragment by internal disulfide bridges. *J. Biol. Chem*. **269**, 8402-8407 (1994).

28. Duché, D., Parker, M.W., González-Mañas, J-M., Pattus, F. & Baty, D. Uncoupled steps of the colicin A pore formation demonstrated by disulfide bond engineering. *J. Biol. Chem*. **269**, 6332-6339 (1994).

29. Kühlmann, U.C., Pommer, A.J., Moore, G.R., James, R., & Kleanthous, C. Specificity in protein-protein interactions: The structural basis for dual recognition in colicin endonuclease-immunity protein complexes. *J. Mol. Biol*. **301**, 1163-1178 (2000).

30. Whittaker, S.B.M. *et al.* NMR detection of slow conformational dynamics in an endonuclease toxin. *J. Biomol. NMR* **12**, 145-159 (1998).

31. Wallis, R. *et al.* Protein-protein interactions in colicin E9 DNase-immunity protein complexes. Cognate and noncognate interactions that span the mM-fM affinity range. *Biochemistry* **34**, 13751-13759 (1995).

32. Garinot-Schneider, C., Pommer, A.J., Moore, G.R., Kleanthous, C. & James, R. Identification of putative active-site residues in the DNase domain of colicin E9 by random mutagenesis. *J. Mol. Biol.* **260**, 731-742 (1996).

33. Lacy, B.D. & Stevens, R.C. Unraveling the structures and modes of action of bacterial toxins. *Curr. Op. Struct. Biol*. **8**, 778-784 (1998).

34. Falnes, P.Ø. & Sandvig, K. Penetration of protein toxins into cells. *Curr. Op. Cell Biol*. **12**, 407-413 (2000).

35. Chevalier, B.S. & Stoddard, B.L. Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. *Nucl. Acid. Res*. **29**, 3757-3774 (2001).

36. van der Goot, F.G, González-Mañas, J.M., Lakey, J.H. & Pattus, F. A 'molten-globule' membrane insertion intermediate of the pore-forming domain of colicin A. *Nature* **354**, 408-410 (1991).

37. Zakharov, S.D. *et al.* Membrane-bound state of the colicin E1 channel domain as an extended two-dimensional helical array. *Proc. Natl. Acad. Sci. USA* **95**, 4282-4287 (1998).

38. Slatin, S.L., Qiu, X-Q., Jakes, K.S., & Finkelstein, A. Identification of a translocated protein segment in a voltage-dependent channel. *Nature* **371**, 158-161 (1998).

39. de Zamaroczy, M., Mora, L., Lecuyer, A., Géli, V. & Buckingham, R.H. Cleavage of colicin D is necessary for cell killing and requires the inner membrane peptidase LepB. *Mol. Cell* **8**, 159-168 (2001).

40. Liao, C.C., Hsiao, K.C., Liu, Y.W., Leng, P.H. & Chak, K-F. Processing of DNase domain during translocation of colicin E7 across the membrane of *Escherichia coli*. *Biochem. Biophys. Res. Commun*. **284**, 556-562 (2001).

41. Soelaiman, S., Jakes, K., Wu, N., Li, W. & Shoham, M. Crystal structure of colicin E3: Implications for cell entry and ribosome inactivation. *Mol. Cell* **8**, 1053-1062 (2001).

42. Montal, M. & Muller, P. Formation of bimolecular membranes from monolayers and study of their properties. *Proc. Natl. Acad. Sci. USA* **69**, 3561-3566 (1972).

43. Bishop, N.D. & Lea, E.J.A. Characterization of the porin of *Rhodobacter capsulatus 37b4* in planar lipid bilayers. *FEBS Lett*. **349**, 69-74 (1994).

44. Bishop, N.D., Lea, E.J.A., Mobasheri, H. & Spiro, S. Altered voltage sensitivity of mutant OmpC porin channels. *FEBS Lett* **379**, 295-298(1996).

45. Dempster, J., University of Strathclyde, Glasgow, UK.

46. Baldwin, G.S., Vipond, I.B. & Halford, S.E. Rapid reaction analysis of the catalytic cycle of the EcoRV restriction endonuclease. *Biochemistry* **34**, 705-714 (1995).

47. Boetzel, R. *et al.* Assignment of ^{1}H, ^{13}C and ^{15}N signals of the inhibitor protein Im9 bound to the DNase domain of colicin E9. *J. Biomol. NMR* **12**, 567-568 (1998).

FIGURE LEGENDS

Figure 1. Planar lipid bilayer experiments with colicin E9

Current traces of 2 nM intact colicin E9 and its domains incorporated into soybean lecithin bilayers. The schematic above each set of traces indicates the protein construct used; intact colicin E9 has three domains, the R-domain is the central BtuB-binding domain found in all E group colicins, the T-domain is the translocation domain involved in binding Tol proteins in the periplasm, while the C-terminal DNase domain carries the cytotoxic activity. The dashed and dotted lines in all traces identify zero (also marked by a closed triangle) and 100 pS conductance, respectively. Open triangles identify additional conductance events although not all are marked. ***a***, Current traces for intact colicin. *Top traces* (p.d., +90 mV) show the absence of channel events early in the record followed, after 3 s, by random opening and closing of a 100 pS channel with larger conductances also apparent. After a further 1 s of recording and toward the end of these traces only the 100 pS conductance is seen. Examples of channel opening and closing are indicated. *Bottom traces* (p.d., +80 mV) are from a separate experiment and highlight how conductance at ~100 pS tends to dominate the traces with additional channel events superimposed on this. ***b***, Continuous current traces (p.d., +80 mV) of a truncated form of colicin E9 in which the DNase has been deleted showing the absence of channel activity. No channel activity was evident throughout the entire record of 100 s. ***c***, Continuous current traces (p.d., –80 mV) for the 15 kDa E9 DNase domain. Channels at 100 pS were clearly evident for the E9 DNase domain (not shown), the traces in the figure highlighting the additional conducting states that are also observed.

Figure 2. Characteristics of E9 DNase channels

Data were collected and analyzed as described in the Methods section. ***a***, Histogram showing the range of different conductance states (in 20 pS bins) exhibited by the E9 DNase (x-axis)

and their frequency (y-axis). ***b*** and ***c*** show all-points amplitude histograms for E9 DNase and E3 rRNase domains, respectively, where data were collected over 125 seconds at an applied p.d. of ±80 mV. The data for the E3 rRNase show the absence of any channel activity (zero current) during the entire record while that for the E9 DNase shows two peaks (indicated by arrows) each corresponding to a distinct current level in the recording. The area under each peak represents the proportion of the record spent at that current level; the mean single channel currents obtained from the Gaussian fits to these peaks were 8.5 pA and –11.4 pA, corresponding to conductance states of 106 pS and -142 pS, respectively. ***d***, Current-voltage relationship for single E9 DNase channels showing that they are independent of the applied p.d. The gradient of the regression line fitted to this I/V data is 105 pS.

Figure 3. E9 DNase channels at higher protein concentration

In order to examine the molecularity of the E9 DNase channels, single-channel measurements were made as described in the legend to Fig. 1 but at ten times higher protein concentration (20 nM). The applied p.d. was +150 mV; since the single channel conductance of the E9 DNase is independent of voltage (Fig. 2*d*) the data collected at this p.d. are directly comparable with those in Fig. 1. ***a***, From a state of zero conductance (closed state) a 'ladder effect' is observed as multiple gating events occur to generate a series of 'open' states of differing conductance (the dotted lines correspond to intervals of 100 pS). ***b***, Continuous channel traces later on in the same experiment where a 100 pS channel (which corresponds to the dotted line) is present almost throughout the recording and superimposed on this are other channel events, some of which are identified by an open triangle, that appear largely to be multiples of 100 pS. Note that the timescale in this figure is more compressed than that in Fig. 1.

Figure 4. Engineering a disulfide bond into the E9 DNase

a, Crystal structure of the E9 DNase[29] showing the location of enzyme active site amino acids (labelled *cyan*), the Im9 binding site (labelled *red*) and regions of the enzyme the backbone atoms of which have been shown by NMR[30] to be conformationally mobile (labelled *yellow*). Two residues in these mobile regions of the DNase (Asp20 and Glu66) were chosen for mutation to cysteine in order to generate the disulfide-form of the enzyme, E9 DNase$^{S\text{-}S}$ and ColE9$^{S\text{-}S}$ (the disulfide bond is shown, labelled *green*). ***b***, Comparing Im9 binding data of wild type (open circle), reduced (closed triangle) and oxidised (open triangle) E9 DNase D20C/E66C mutant using tryptophan emission fluorescence spectroscopy, as described by Wallis et al[14]. ***c***, Comparing endonucleolytic digestion of 12mer dsDNA by stopped-flow absorbance at 260 nm in 50 mM triethanolamine pH 7.5 buffer containing 10 mM $MgCl_2$ at 25°C following the change in hyperchromicity. Symbols as for *b*.

Figure 5. Effect of disulfide bond on E9 DNase channel activity

a, E9 DNase$^{S\text{-}S}$ was added to the *cis* chamber of the bilayer apparatus (p.d. ±100 mV). The total record of an experiment is shown (82 s), indicating the absence of channel activity for the oxidised protein. This was confirmed for two protein preparations using a number of different membranes. ***b***, The result of adding 5 mM DTT to the *cis* chamber containing E9 DNase$^{S\text{-}S}$. Recording ceased during the addition of DTT and ~10 s elapsed before it was resumed. Channel activity began to appear midway through the record although was more noisy and less well defined compared to the wild type enzyme. ***c***, Total channel record for E9 DNase^{SH_2} alkylated with iodoacetamide (see Methods section). Sections of the trace are shown in panels *i* and *ii*. Clearer channel events are seen compared to the reduced protein although these still differ to those of the wild type DNase (see text for details).

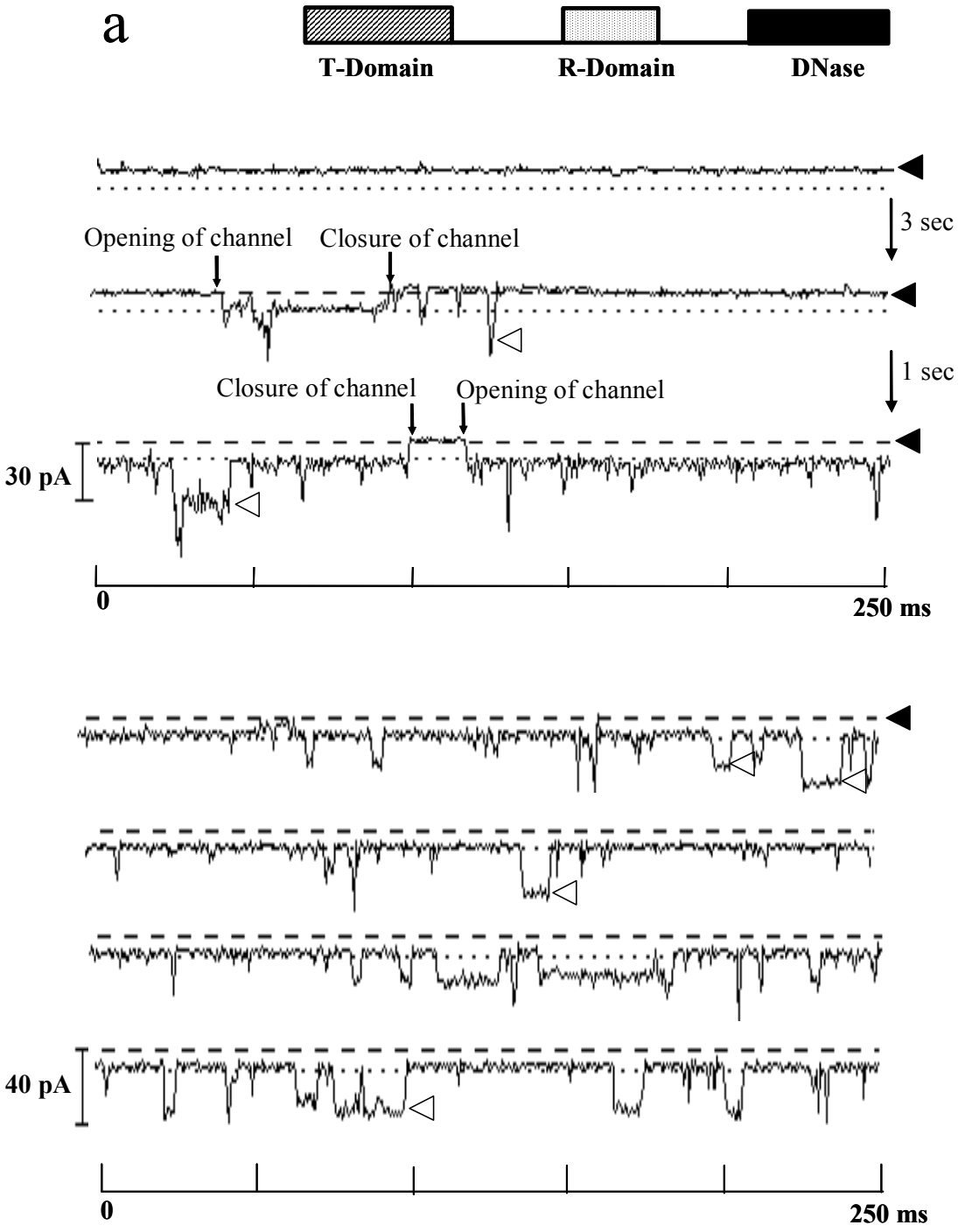
Figure 6. ^{1}H-^{15}N HSQC spectra of E9 DNase$^{S\text{-}S}$ and E9 DNase^{SH_2}

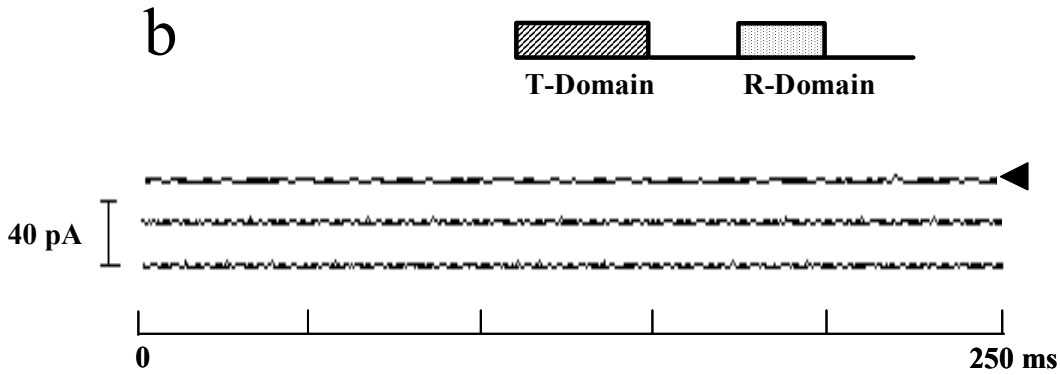
600 MHz ^{1}H-^{15}N HSQC spectra of oxidised (*blue*) and reduced (*red*) E9 DNase D20C/E66C double mutant. The data indicate that the oxidised protein has a single form in solution while the reduced protein, like the wild-type DNase, exists in two forms in an approximately 60:40 molar ratio[30]. The conformational heterogeneity of the wild type protein has been shown to affect the chemical shifts of G15, D36, K63, K69, V121 and T122, and all these peaks are doubled in the spectrum of E9 DNase^{SH_2} (side-panels), while none are doubled in the spectrum of the E9 DNase$^{S\text{-}S}$. The large chemical shift differences between the K69 NH resonances of oxidised and reduced protein likely reflects local conformational effects of Cys66.

Figure 7. Antibacterial activity of oxidised and reduced colicin E9

a, Agar plate assay comparing the biological toxicities of wild type colicin E9 with oxidised, reduced and alkylated colicin E9 D468C/E514C as well as a previously identified DNase active site mutant (H575A), denoted by the asterix. In each lane of the plate has been grown a lawn of *E. coli* JM83 cells onto which was spotted a serial dilution of each of the protein constructs indicated. Zones of clearing indicate cell death. ***b***, Comparing the cytotoxic activities of oxidised and alkylated colicin E9 D468C/E514C against bacterial cells grown in liquid media. The figure shows growth curves of *E. coli* JM83 in the absence of colicin E9 (closed circle), or with the addition of wild type colicin E9 (closed square), ColE9$^{S\text{-}S}$ (open triangle) or reduced and alkylated colicin E9 D468C/E514C (open square).

Figure 1


a
T-Domain
R-Domain
DNase
3 sec
Opening of channel
Closure of channel
1 sec
Closure of channel
Opening of channel
30 pA
0
250 ms
40 pA
0
250 ms


b
T-Domain
R-Domain
40 pA
0
250 ms

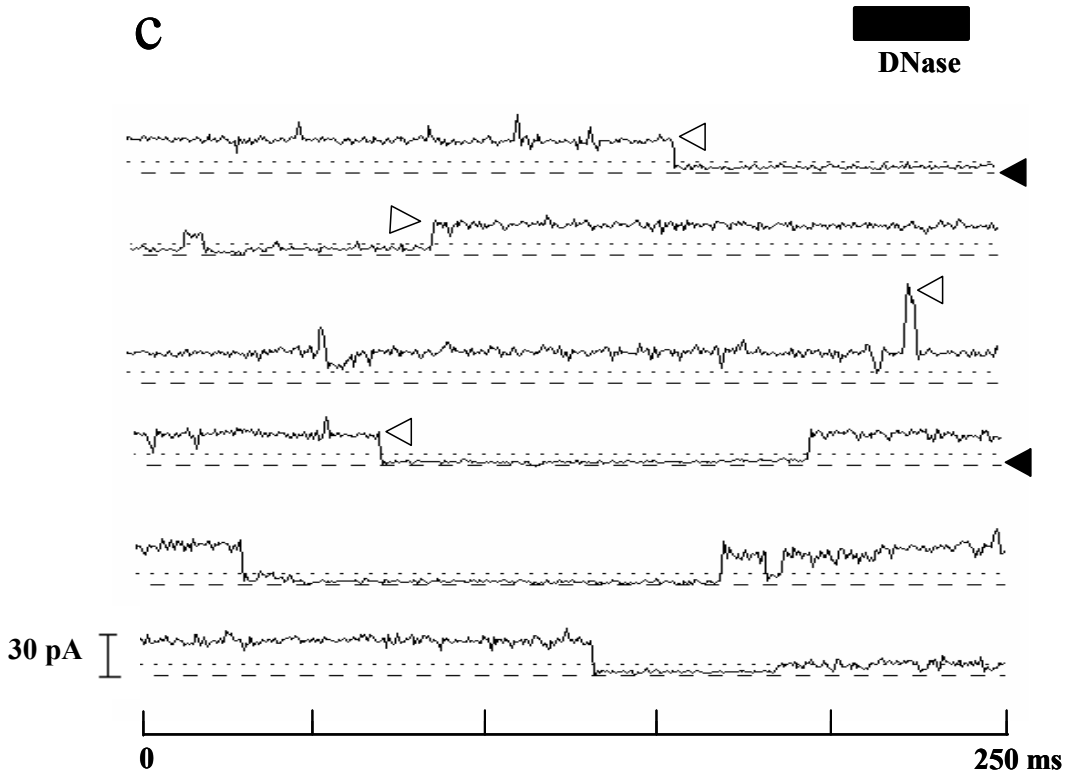

c
DNase
30 pA
0
250 ms

Figure 2

a

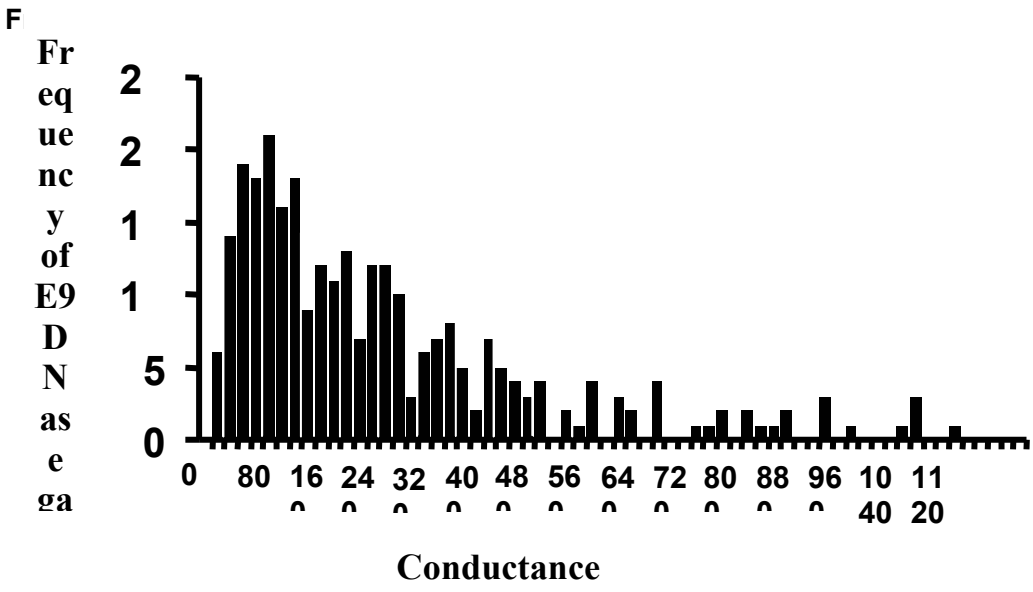
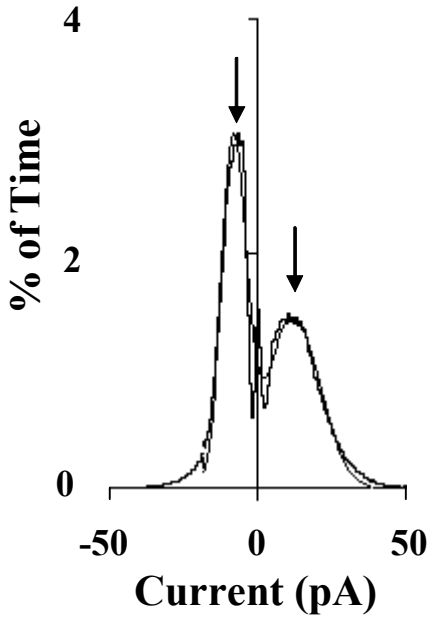
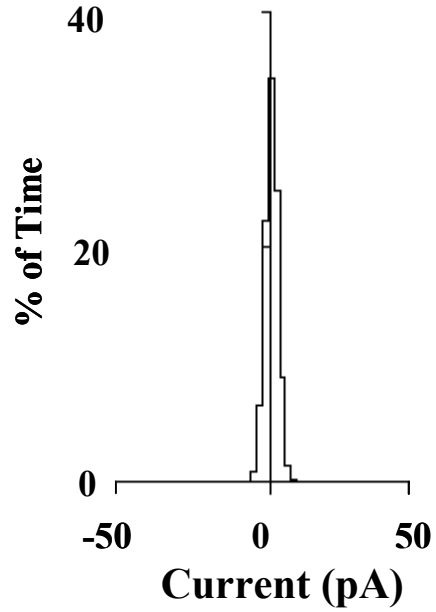

F
Fr
eq
ue
nc
y
of
E9
D
N
as
e
ga
2
2
1
1
5
0
0
80
16
0
24
0
32
0
40
0
48
0
56
0
64
0
72
0
80
0
88
0
96
0
10
40
11
20
Conductance

Figure 2

b **E9 DNase**



c **E3 rRNase**



d

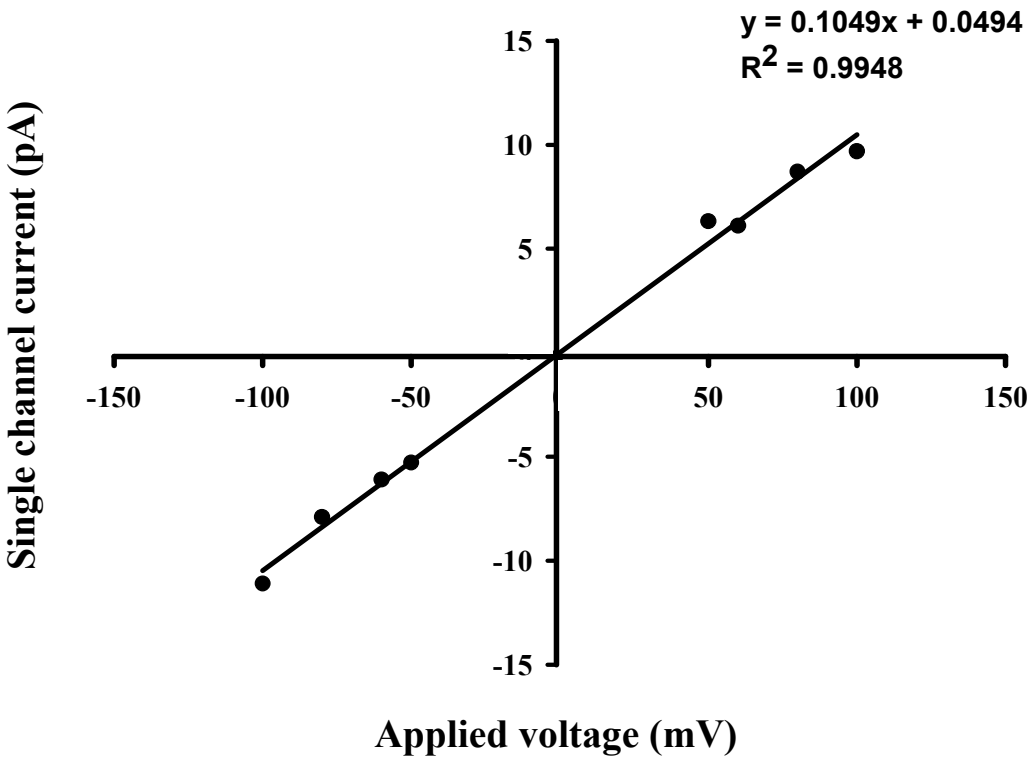

y = 0.1049x + 0.0494
R2 = 0.9948
Single channel current (pA)
15
10
5
-5
-10
-15
-150
-100
-50
50
100
150
Applied voltage (mV)

Figure 3

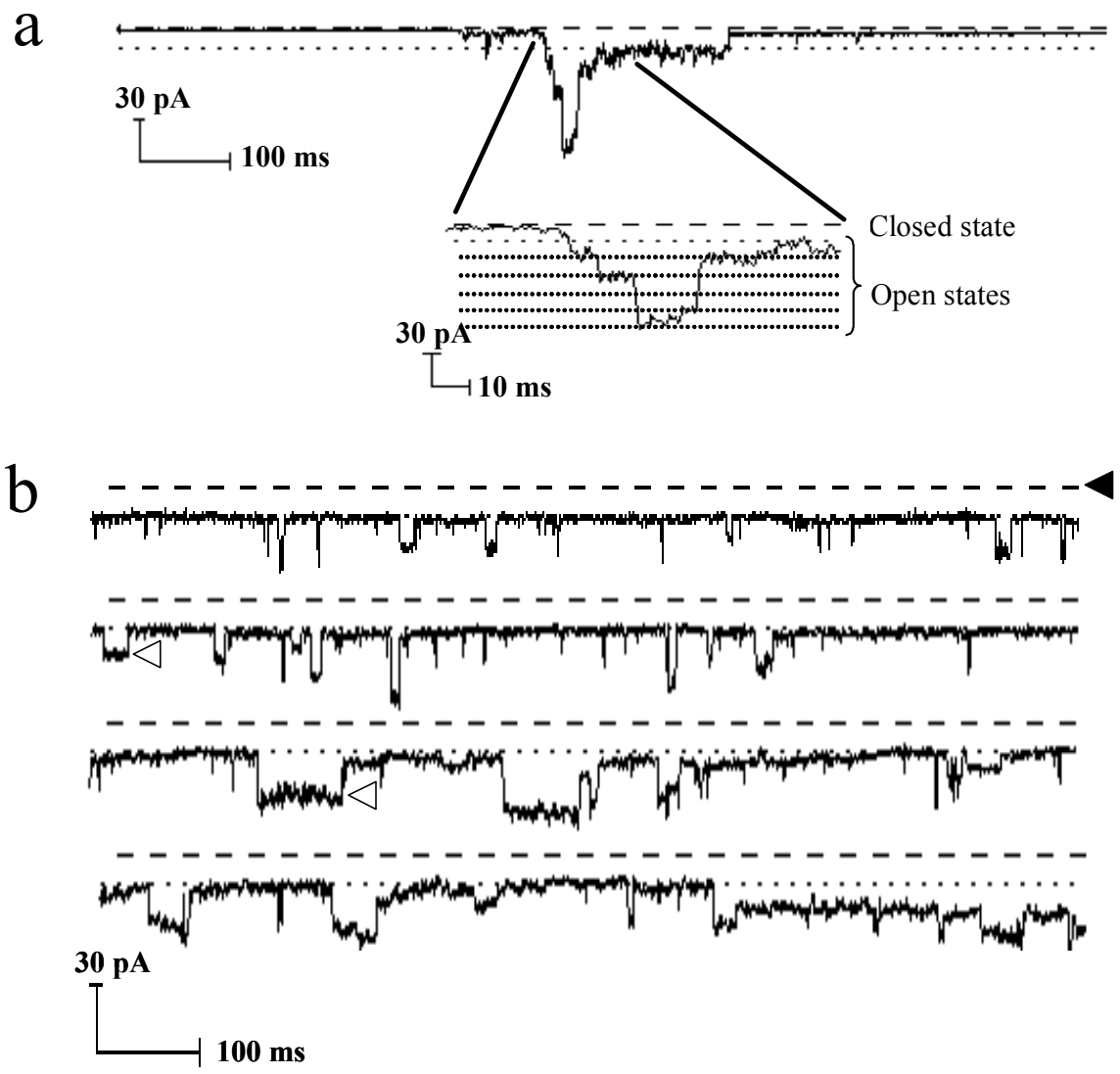
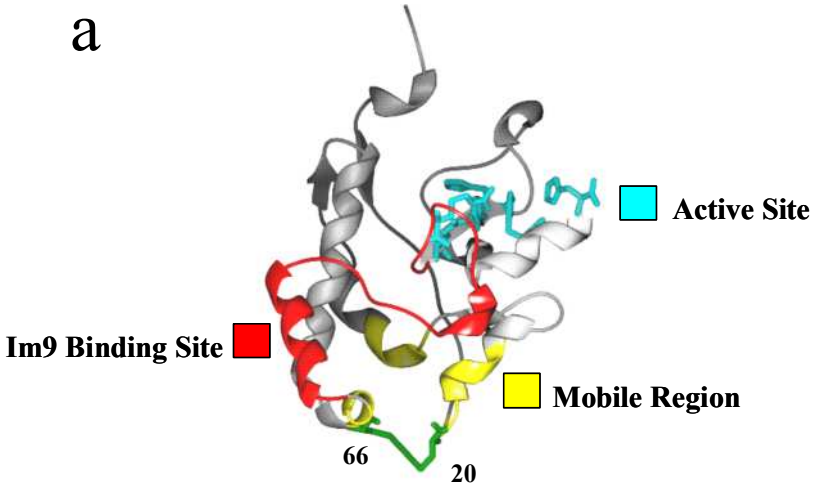
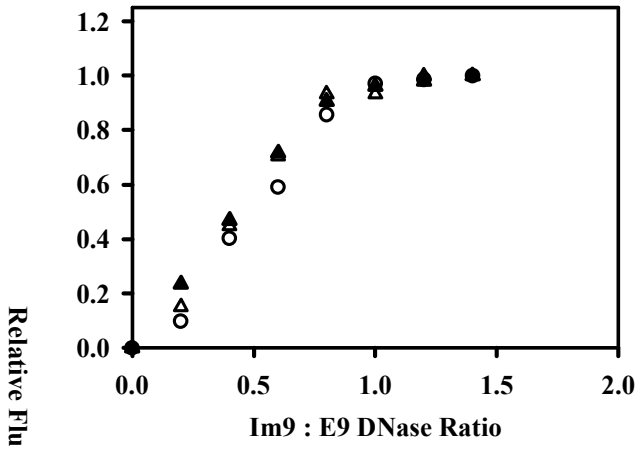


Figure 4

a


Active Site
Im9 Binding Site
Mobile Region
66
20

b


1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.5
1.0
1.5
2.0
Relative Flu
Im9 : E9 DNase Ratio

c

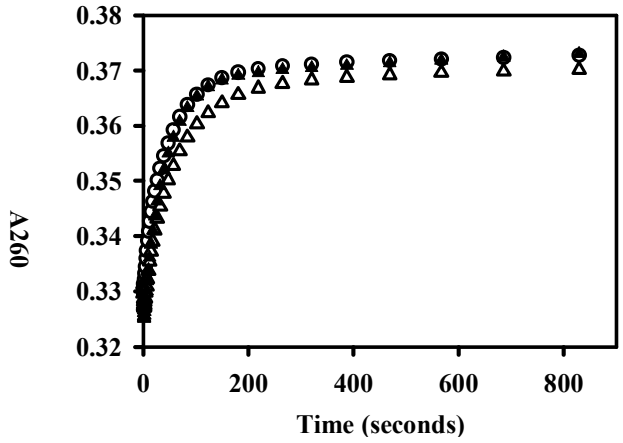

0.38
0.37
0.36
0.35
0.34
0.33
0.32
0
200
400
600
800
A260
Time (seconds)

Figure 5

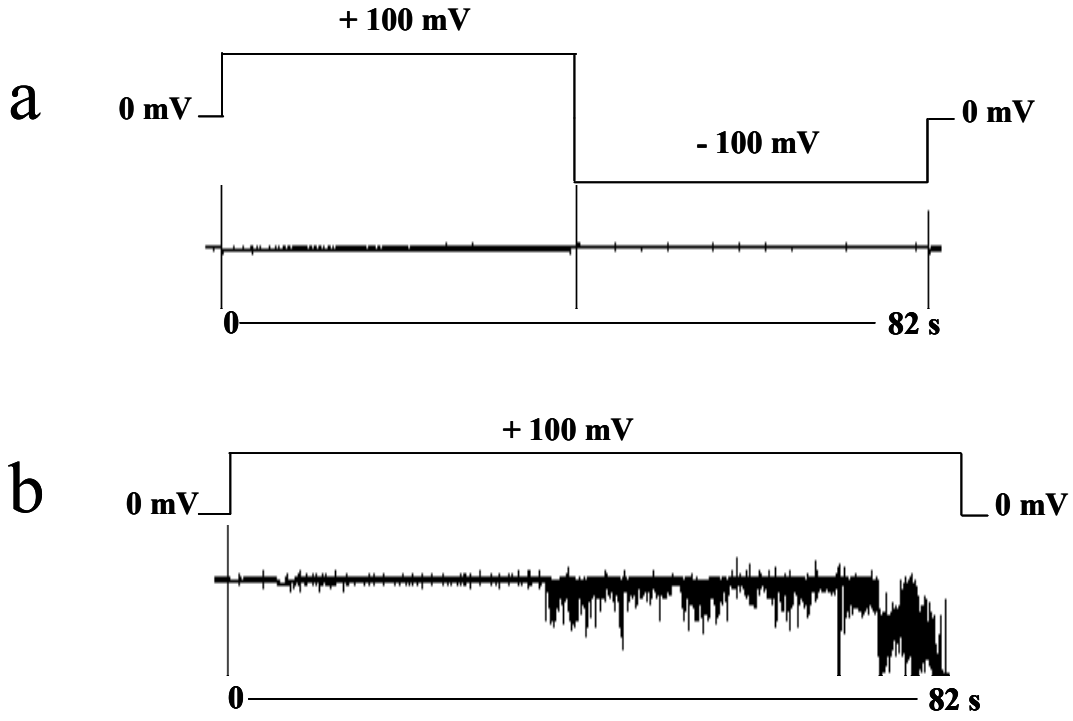


Figure 5

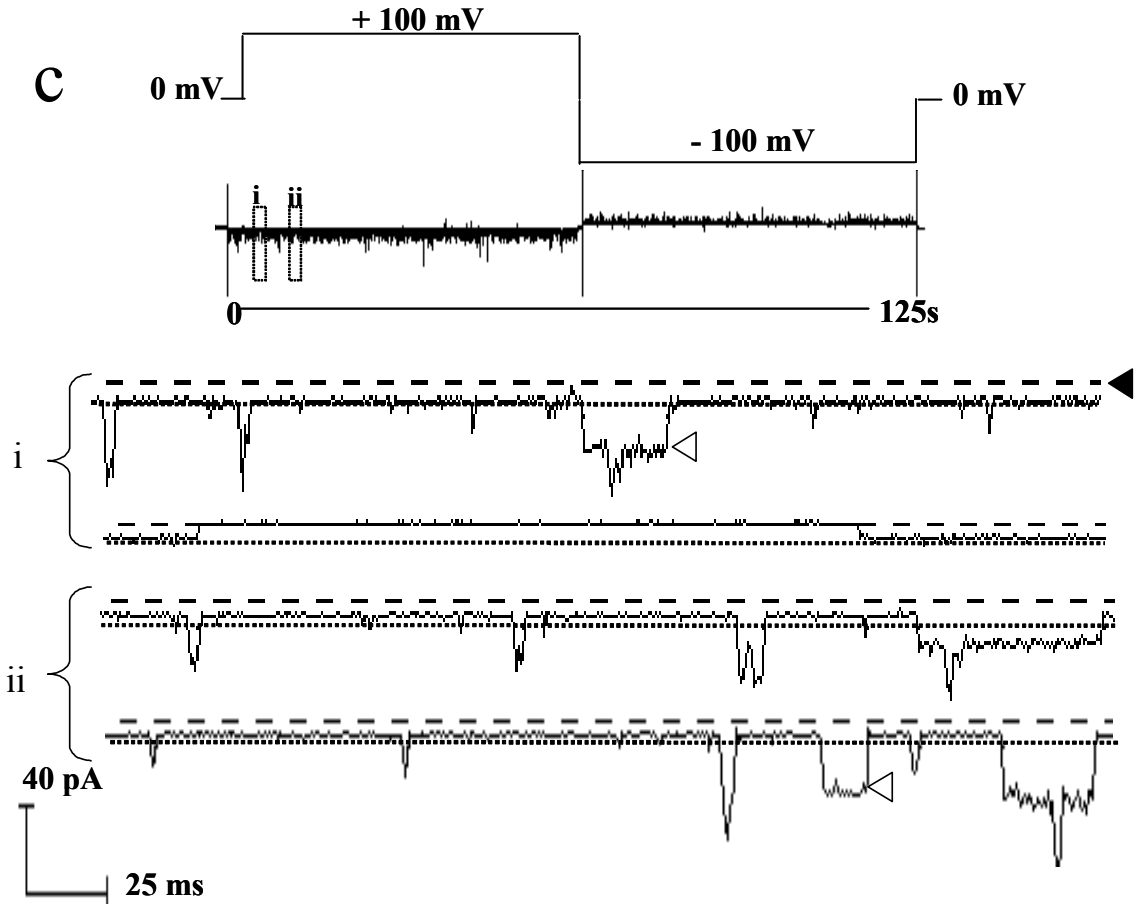


Figure 6

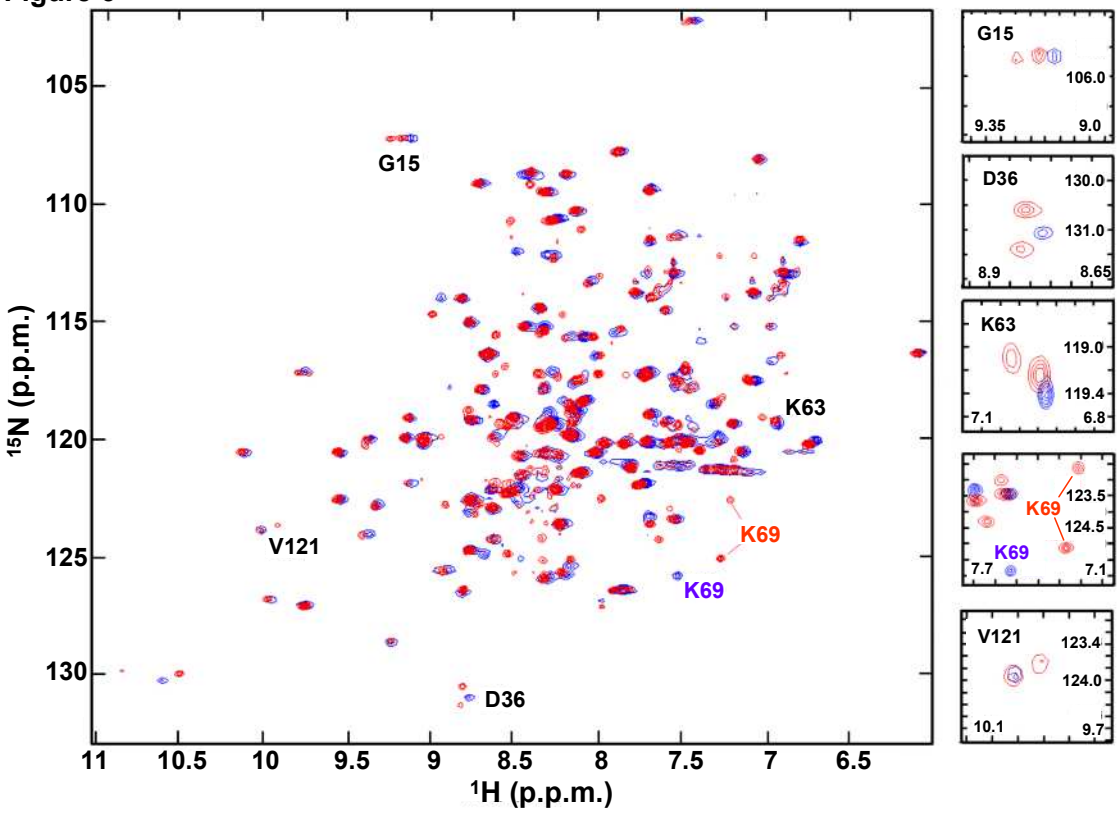

G15
D36
K63
K69
K69
V121
^{15}N (p.p.m.)
^{1}H (p.p.m.)
105
110
115
120
125
130
11
10.5
10
9.5
9
8.5
8
7.5
7
6.5
G15
106.0
9.35
9.0
D36
130.0
131.0
8.9
8.65
K63
119.0
119.4
7.1
6.8
K69
123.5
124.5
K69
7.7
7.1
V121
123.4
124.0
10.1
9.7

Figure 7

a

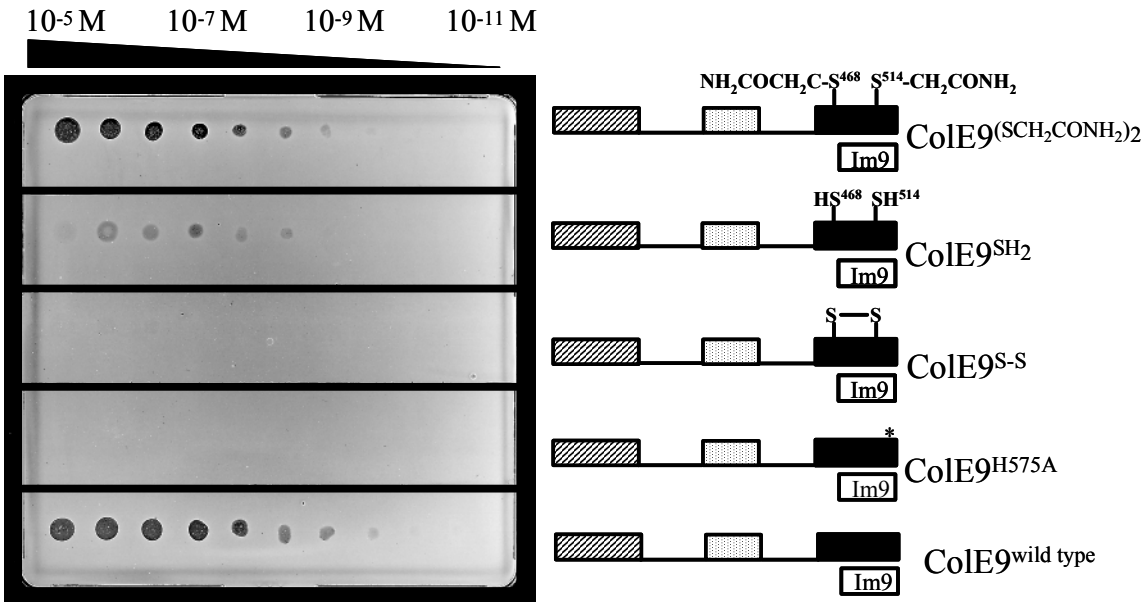
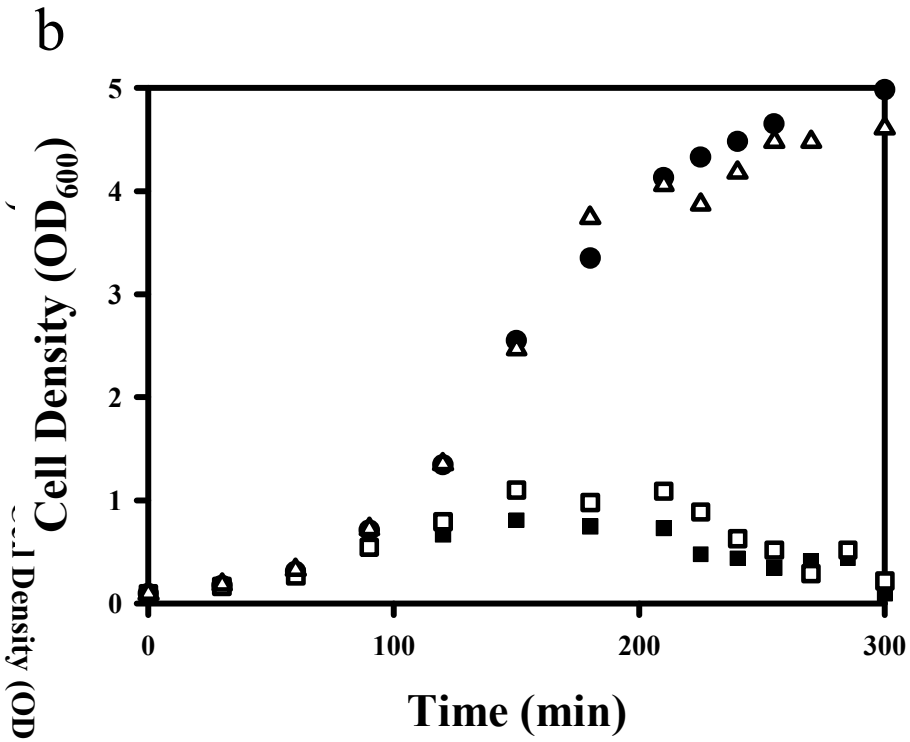

10-5 M
10-7 M
10-9 M
10-11 M
NH2COCH2C-S468 S514-CH2CONH2
Im9
ColE9(SCH2CONH2)2
HS468 SH514
Im9
ColE9SH2
S—S
Im9
ColE9S-S
*
Im9
ColE9H575A
Im9
ColE9wild type

Figure 7


b
Cell Density (OD600)
Cell Density (OD
0
1
2
3
4
5
0
100
200
300
Time (min)