

Compaction of the *Escherichia coli* nucleoid caused by Cyt1Aa

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Compaction of the *Escherichia coli* nucleoid in the cell's centre was associated with the loss of colony-forming ability; these effects were caused by induction of Cyt1Aa, the cytotoxic 27 kDa protein from *Bacillus thuringiensis* subsp. *israelensis*. Cyt1Aa-affected compaction of the nucleoids was delayed but eventually more intense than compaction caused by chloramphenicol. The possibility that small, compact nucleoids in Cyt1Aa-expressing cells resulted in DNA replication run-out and segregation following cell division was ruled out by measuring relative nucleoid length. Treatments with membrane-perforating substances other than Cyt1Aa did not cause such compaction of the nucleoids, but rather the nucleoids overexpanded to occupy nearly all of the cell volume. These findings support the suggestion that, in addition to its perforating ability, Cyt1Aa causes specific disruption of nucleoid associations with the cytoplasmic membrane. *In situ* immunofluorescence labelling with Alexa did not demonstrate a great amount of Cyt1Aa associated with the membrane. Clear separation between Alexa-labelled Cyt1Aa and 4',6-diamidino-2-phenylindole (DAPI)-stained DNA indicates that the nucleoid does not bind Cyt1Aa. Around 2 h after induction, nucleoids in Cyt1Aa-expressing cells started to decompact and expanded to fill the whole cell volume, most likely due to partial cell lysis without massive peptidoglycan destruction.

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INTRODUCTION

During sporulation, various subspecies of the Gram-positive soil bacterium *Bacillus thuringiensis* produce large amounts of insecticidal crystal proteins (ICP), the so-called δ -endotoxins (Schnepf *et al.*, 1998), each toxic against larvae of a different group of insects. The ICP of *B. thuringiensis* subsp. *israelensis* is specific against the larvae of mosquitoes and black flies (Goldberg & Margalit, 1977; Margalith & Ben-Dov, 2000), vectors of many human infectious diseases (Service, 1986). The crystal is composed of four major polypeptides, Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa (of 125, 135, 68 and 28 kDa, respectively), which are encoded by genes carried on the ~130 kDa plasmid nicknamed pBtoxis (Ben-Dov *et al.*, 1999). Cyt1Aa, which is not homologous to any of the known Cry toxins (Crickmore *et al.*, 1998), is the most prominent of the four polypeptides, but it is less specific than Cry4Aa, Cry4Ba and Cry11Aa, haemolytic and cytotoxic *in vitro* (Thomas & Ellar, 1983b; Drobniewski & Ellar, 1988; Hofte & Whiteley, 1989). The broad cytolytic activity of Cyt1Aa has been attributed to its hydrophobicity and ability to bind zwitterionic phospholipids (Thomas & Ellar, 1983a, b). Cyt1Aa is inserted into the membrane to create cation-selective single channels of

1–2 nm in diameter, leading to colloid-osmotic lysis (Knowles & Ellar, 1987; Knowles *et al.*, 1989), and induces leakage of low-molecular-mass substances from lipid vesicles (Drobniewski & Ellar, 1988, 1989).

Seven cytolytic, mosquitocidal-specific toxins are currently known (Earp & Ellar, 1987; Drobniewski & Ellar, 1989; Yu *et al.*, 1991; Koni & Ellar, 1993; Cheong & Gill, 1997; Guerchicoff *et al.*, 1997; Thiery *et al.*, 1997). Using heavy-atom isomorphous replacement, the crystal structure of Cyt2Aa from *B. thuringiensis* subsp. *kyushuensis* has recently been found to have a single pore-forming domain, composed of two outer layers of α -helix hairpins wrapped around mixed β -sheets (Li *et al.*, 1996). Due to high similarity (70%) in its amino acid sequence to Cyt2Aa, Cyt1Aa is supposed to show a similar folding pattern to Cyt2Aa (Li *et al.*, 1996; Gazit *et al.*, 1997).

The level of mosquito larvicidal activity of Cyt1Aa by itself is low, but it raises synergistically the activity of Cry4Aa, Cry4Ba or Cry11Aa, to a greater extent than the synergism obtained by combination of the three Cry polypeptides themselves (Crickmore *et al.*, 1995; Khasdan *et al.*, 2001). The role of Cyt1Aa in delaying the development of resistance to the Cry proteins is critical (Georghiou & Wirth, 1997; Wirth & Georghiou, 1997; Wirth *et al.*, 1997).

Abbreviations: CAM, chloramphenicol; DAPI, 4',6-diamidino-2-phenylindole; DPH, 1,3-diphenyl-1,3,5-hexatriene; GFP, green fluorescent protein.

Recombinant *Escherichia coli* (Douek *et al.*, 1992) and acrySTALLIFEROUS *B. thuringiensis* subsp. *kurstaki* (Wu & Federici, 1993) lose colony-forming ability upon expressing Cyt1Aa. The accessory protein P20 protects them from this lethal action (Wu & Federici, 1993; Manasherob *et al.*, 2001) and stabilizes Cyt1Aa post-translationally (Adams *et al.*, 1989; Visick & Whiteley, 1991).

The hypothesis that recombinant *E. coli* cells expressing the *cyt1Aa* gene die by inhibition of DNA synthesis (Douek *et al.*, 1992) is consistent with the high affinity of Cyt1Aa for phosphatidylethanolamine (Thomas & Ellar, 1983b), the major DNA-bound *E. coli* phospholipid (Ballesta *et al.*, 1972). This hypothesis predicts that the target is the chromosome replication complex (Douek *et al.*, 1992). Indeed, truncated Cyt1Aa (lacking 29 N-terminal amino acids) binds endogenous and heterologous DNA at similar rates *in vitro* (Yokoyama *et al.*, 1998).

In an attempt to clarify the lethal action of Cyt1Aa on *E. coli* cells expressing its gene, nucleoids were visualized and their states determined by combined fluorescence and phase-contrast microscopy of cells stained with 4',6-diamidino-2-phenylindole (DAPI). The kinetics of nucleoid compaction induced by Cyt1Aa was compared to that affected by chloramphenicol (CAM). In addition, associated changes in the permeability and viscosity of *E. coli* cell membrane were studied. *In situ* immunofluorescence labelling was used to establish independently where Cyt1Aa resides within cells.

METHODS

Bacterial strains and plasmids. The following plasmids were hosted in *E. coli* XL-Blue MRF' (Stratagene) which is *relA1*. (a) pUHE-24S, a modified version of pUHE-24 from which one *NcoI* site was removed, leaving a unique *NcoI* site in the translation start codon. pUHE-24 is a descendant of pDS (Deuschle *et al.*, 1986) containing a T5 RBS (Bujard *et al.*, 1987), two tandem copies of *lacO* and the early T7 (σ^{70} -activated) promoter P_{A1} (utilizing the usual *E. coli* RNA polymerase). (b) pRM4-C and pRM4-RC, which carry *cyt1Aa* and *p20+cyt1Aa*, respectively (Manasherob *et al.*, 2001). (c) pMC208-GFP (obtained from C. L. Woldringh, University of Amsterdam) was used as a template for green fluorescent protein (GFP) amplification. (d) pK-37, a pUC9 derivative with a 9.7 kb *HindIII* fragment (Douek *et al.*, 1992) of pBtoxis from *B. thuringiensis* subsp. *israelensis* (Ben-Dov *et al.*, 1999), was used as a template for *cyt1Aa* amplification. Plasmids pRM4-C, pRM4-RC and pREP4 (containing *lacI^q*; Qiagen) were hosted in the isogenic strains RL331T (*RelA⁺*) and RL332T (*relA*) (Ryals *et al.*, 1982).

Viable cell counts. Viability of cells was determined by colony-forming ability on Luria-Bertani (LB) plates (with 100 μ g ampicillin ml^{-1} and 10 μ g tetracycline ml^{-1}) following appropriate dilutions. The number of colonies was counted after 24 h incubation at 37 °C. Each point is a mean of a duplicate in three different dilutions. For detection of cells that were not sensitive to the Cyt1Aa lethal effect, IPTG-containing LB plates were used.

Amino acid starvation. To starve the cells of amino acids, a culture of RL331T (*RelA⁺*) or RL332T (*relA*) harbouring pRM4-C and pREP4 grown in M-9 minimal salts medium supplemented with 0.4% glucose and 1% casein hydrolysate was filtered and

resuspended (at twice the concentration) in the same medium without casein hydrolysate. Transforming these strains with pRM4-C alone failed, presumably because they lack *lacI^q* and hence express *cyt1Aa* constitutively and die on LB plates.

Membrane permeability for DAPI. Aliquots (3 ml) from steady-state cultures were transferred to a cuvette at 37 °C and stirred in a spectrofluorometer (model LS50B; Perkin Elmer). Kinetics of fluorescence intensity of DAPI (1 μ g ml^{-1}) were recorded following addition of this dye to the culture samples, using excitation and emission wavelengths of 360 and 470 nm, and 2.5 and 5.0 nm slits, respectively.

Fluorescence anisotropy. Apparent membrane viscosity and dynamics of cells were inferred from measurements of fluorescence anisotropy of 1,3-diphenyl-1,3,5-hexatriene (DPH), following the general procedure described previously (Zaritsky *et al.*, 1985; Parola *et al.*, 1990; Binenbaum *et al.*, 1999). Steady-state values were measured at 37 °C by spectrofluorometry (LS50B; Perkin Elmer) using excitation and emission wavelengths of 360 and 430 nm, and 2.5 and 7.0 nm slits, respectively, in the 'Read' mode, with a 3 s integration time. Five to six readings were taken for each sample, providing an estimated instrumental error of $\sim 1\%$.

Recombinant DNA methods. DNA modifications were performed as recommended by the enzymes' suppliers. Competent cells were prepared and plasmids were isolated by standard procedures (Sambrook & Russell, 2001). Transformants were selected on LB plates containing 100 μ g ampicillin ml^{-1} and 10 μ g tetracycline ml^{-1} . DNA run on horizontal agarose slab gels (0.7%) was visualized by staining with ethidium bromide. DNA fragments were purified from the gels by phenol extraction (Seth, 1984).

Gene expression. Cultures were grown at 37 °C in LB broth supplemented with 100 μ g ampicillin ml^{-1} and 10 μ g tetracycline ml^{-1} ; they were induced by addition of IPTG (0.5 mM) when they reached an OD₆₆₀ value of 0.2–0.3 ($\sim 2 \times 10^8$ cells ml^{-1}). Cells were harvested by centrifugation at various times after induction and resuspended in distilled water; aliquots were boiled (10 min) in sample treatment buffer [62.5 mM Tris/HCl, 2% SDS, 10% (v/v) glycerol, 0.01% Bromophenol blue and 0.1 M DTT]. Protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) was added at 4 mM (final concentration) during sample preparations to prevent proteolysis. Samples were analysed by discontinuous SDS-PAGE using 4.5% acrylamide (pH 6.8) and 12% acrylamide (pH 8.8) for the stacking and separating gels, respectively (Laemmli, 1970). The gels were stained with 0.1% Coomassie blue R-250. Protein concentrations were determined according to Bradford (1976) with BSA as standard.

Western blot. Proteins were electrotransferred from SDS-polyacrylamide gels onto nitrocellulose membranes and exposed to a specific antiserum directed against Cyt1Aa (kindly provided by S. S. Gill, University of California, USA). Protein A-alkaline phosphatase conjugate was used as a primary antibody detector. Visualization of the antigen was done with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma), the chromogenic substrate for alkaline phosphatase.

***In situ* immunofluorescence labelling.** Cells were grown in LB medium to an OD₆₆₀ value of ~ 0.6 and fixed by adding 2.8% formaldehyde and 0.04% glutaraldehyde (final concentrations). For permeabilization, the cells were harvested by centrifugation (7000 g, 5 min), washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2), and incubated in 0.1% Triton X-100-containing PBS for 45 min at room temperature. The cells were washed three times in PBS and incubated in PBS containing 100 μ g lysozyme ml^{-1} and 5 mM EDTA for 45 min at room temperature. Finally, the cells were washed three times in PBS. Non-specific

binding sites were blocked by incubation in 0.5% (w/v) blocking reagents (Boehringer) in PBS for 30 min at 37 °C. Incubation with primary polyclonal antibodies against Cyt1Aa (diluted 1:500 in blocking buffer) was carried out for 60 min at 37 °C. Excess antibodies were removed, and the cells were washed three times with PBS containing 0.05% (v/v) Tween 20. Incubation in the dark with Alexa 546 conjugated to goat anti-rabbit IgG (Molecular Probes), diluted in blocking buffer, was carried out for 30 min at 37 °C. The cells were washed three times in PBS containing 0.05% Tween 20. The nucleoids were stained with DAPI at a final concentration of 1 µg ml⁻¹ in H₂O. The cells were washed once in H₂O and resuspended in PBS.

Microscopy. Samples of steady-state exponentially growing cultures (Fishov *et al.*, 1995) were fixed (0.25% formaldehyde) and immobilized on agarose slides as described by Van Helvoort & Woldringh (1994). Prior to immobilization, the samples were stained with DAPI for visualizing nucleoids and their states by combined fluorescence and phase-contrast microscopy (ZEISS Axioplan 2 fluorescence microscope, equipped with Plan-Neofluar 100×/1.3 oil immersion lens and SPOT2 cooled CCD camera; Diagnostic Instruments). Cells were photographed by combined phase-contrast mode with a DAPI fluorescence filter (U-MWU; excitation at 330–385 nm), an Alexa filter (U-MNG; excitation at 530–550 nm) or differential interference contrast (DIC; Nomarski) optics, using the program IPLAB 3.1a (Signal Analytics).

Microscopic measurements and image analysis. Interactive measurements were performed as structured point collection on a Macintosh G3 computer by using the public domain program OBJECT-IMAGE1.62 (Vischer *et al.*, 1994), which is based on NIH IMAGE by Wayne Rasband. Photographs were stacked, and the lengths of each cell and their nucleoid(s) were measured by indicating the end points of their major axes. Relative nucleoid length, defined as the ratio between nucleoid length (sum of lengths of all nucleoids in a cell) to cell length, allows estimation of nucleoid compaction independently of the number of nucleoids per cell (Van Helvoort *et al.*, 1996). Each point is based on measurements of at least 150 cells.

Kinetics of nucleoid compaction caused by Cyt1Aa and CAM. A steady-state exponentially growing culture of cells (Fishov *et al.*, 1995) harbouring pRM4-C (carrying *cyt1Aa*) was split into two flasks; CAM (100 µg ml⁻¹) was added to one (either with or without 10 µg nalidixic acid ml⁻¹) and IPTG (0.5 mM) was added to the other. Compaction of DAPI-stained nucleoids was measured at different times using combined phase-contrast fluorescence micrographs, from which mean relative nucleoid length was calculated.

Construction of a Cyt1Aa–GFP fusion. To this end, fusion by gene splicing by overlap extension (SOE) was used (Horton *et al.*, 1989). A 737 bp amplicon with a truncated *cyt1Aa* gene was obtained by using pK-37 and the primers D-cyt [a 31-mer; 5'-GAAAGGCCAGAATTCTAATTAAGGAG-3'; containing an *EcoRI* site (shown in bold; nucleotides 10–15)] and R-cyt (a 47-mer; 5'-GTTCTTCTCCTTTGCTAGCCATCAAAGATTGGATGTTAACA-TTGTAG-3'). A 782 bp amplicon with a complete *gfp* gene was obtained by using pMC208-GFP and the primers D-gfp (a 47-mer; 5'-CTACAATGTTAACATCCAATCTTTGATGGCTAGCAAAGGAG-AAGAAC-3') and R-gfp [a 27-mer; 5'-CACGACGTTCTAGAA-CGACGGCCAGTG-3'; containing a *XbaI* site (shown in bold; nucleotides 9–14)]. The 3' end of R-cyt is complementary to the 5' region of D-gfp. Since Cyt1Aa is processed similarly at both termini by proteases to yield the activated toxin (Al-yahyaee & Ellar, 1995), *gfp* was fused to *cyt1Aa* so that Cyt1Aa was truncated at its C terminus to prevent cleavage of the Cyt1Aa–GFP fusion protein in the cell. Since Lys-225 of Cyt1Aa is the potential cleavage site for several proteases (Al-yahyaee & Ellar, 1995), *cyt1Aa* was amplified to

include Leu-224 as the last residue. The two amplicons were mixed, denatured and allowed to re-anneal. The resulting fusion product was amplified further by using primers D-cyt and R-gfp. The final 1470 bp PCR product was purified using the Wizard PCR preps DNA purification kit (Promega) and digested with *EcoRI* and *XbaI*, yielding a 1456 bp fragment that was further purified from a 1.5% agarose gel and cloned into pUHE24S to pRM4C-GFP. Amplification was carried out with high-fidelity Vent DNA polymerase (New England BioLabs) in a DNA thermal cycler (Hybaid) for a 28-reaction cycle, each with the following parameters: 1 min at 94 °C, 1 min at 50 °C and 40 s at 72 °C.

RESULTS AND DISCUSSION

Lethal effect of Cyt1Aa

Cell lysis by the Cyt1Aa protein from *B. thuringiensis* subsp. *israelensis* is not mediated by receptor binding; it is caused by perforation of the cytoplasmic membrane (Gazit *et al.*, 1997; Gill *et al.*, 1992). The pores (of 1–2 nm in diameter) are selective cation channels in the phosphatidyl-ethanolamine planar bilayer (Knowles *et al.*, 1992) that cause an influx of water that leads to colloid osmotic lysis (Knowles *et al.*, 1989). In addition to its cytolytic activity against a broad range of invertebrate and vertebrate cells (Drobniewski & Ellar, 1989; Hofte & Whiteley, 1989; Thomas & Ellar, 1983b), Cyt1Aa is lethal to *E. coli* (Douek *et al.*, 1992; Manasherob *et al.*, 2001), an effect that is associated with a block in DNA synthesis. An exponentially growing culture of *E. coli* stopped increasing in mass upon induction with IPTG (Fig. 1A), while the colony-forming ability of the cells on LB agar plates decreased by four orders of magnitude during 30 min (Fig. 1B). Cyt1Aa was detected by Western blot 2 min after induction and reached its maximum level after 10 min (Fig. 2). Plating on LB agar with IPTG (Fig. 1B) exposed the survivors, most likely spontaneous mutants that gained resistance to the lethal effect of Cyt1Aa or lost the insert before expression. The presence of pRM4-C and *cyt1Aa* in survivors was confirmed by isolating the plasmid and restriction mapping the insert, but no Cyt1Aa was detected by Western blot (data not shown).

Membrane involvement and properties

Cyt1Aa is known to non-specifically perforate plasma membranes upon binding to phospholipids (Thomas & Ellar, 1983a). Indeed, DAPI, which is not taken up by intact *E. coli* cells (e.g. Sun & Margolin, 2001), efficiently entered Cyt1Aa-producing *E. coli* cells 15 min following induction (Fig. 3A), much later than the appearance of Cyt1Aa (Fig. 2). This sequence of events is consistent with the suggestion that Cyt1Aa perforates the cell membrane as an oligomer of half a dozen monomers (Gazit *et al.*, 1997).

Two hypotheses were reported in the literature describing the mechanism of membrane disruption by Cyt1Aa – perforation (Gazit *et al.*, 1997) or detergent-like (Butko *et al.*, 1996, 1997). Membrane fluidity (measured by anisotropy of DPH fluorescence; Binenbaum *et al.*, 1999) did not change

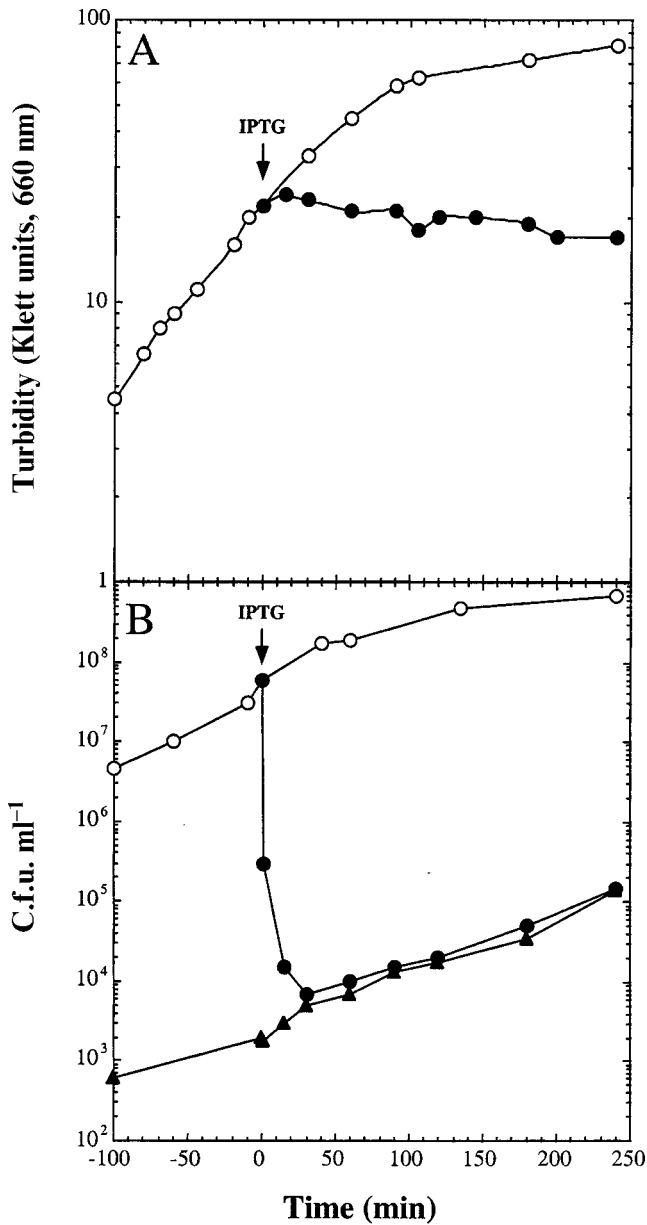


Fig. 1. Mass growth (A) and viable cell counts (B) of exponentially growing (○) *E. coli* cells (harbouring pRM4-C) and after induction (●) with 0.5 mM IPTG (producing Cyt1Aa). ▲, Viability of Cyt1Aa-resistant cells.

following induction of *cyt1Aa* (Fig. 3B), supporting the suggestion that Cyt1Aa forms distinct transmembrane pores (Gazit *et al.*, 1997; Li *et al.*, 1996). This result, however, does not preclude a detergent-like effect because minor changes at a small number of specific sites may not be detected by this gross membrane fluidity determination.

Nucleoid compaction and structure

The chromosome replication complex has been implicated as the target for Cyt1Aa in *E. coli* (Douek *et al.*, 1992)

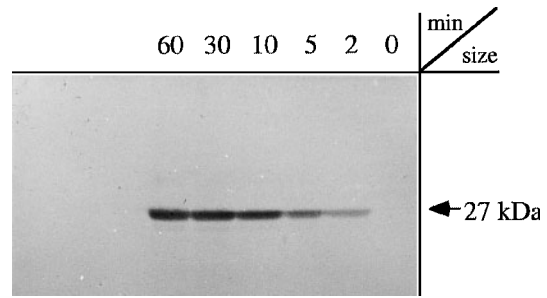


Fig. 2. Immunoblot of Cyt1Aa in extracts of pRM4-C-containing cells at various times after IPTG induction.

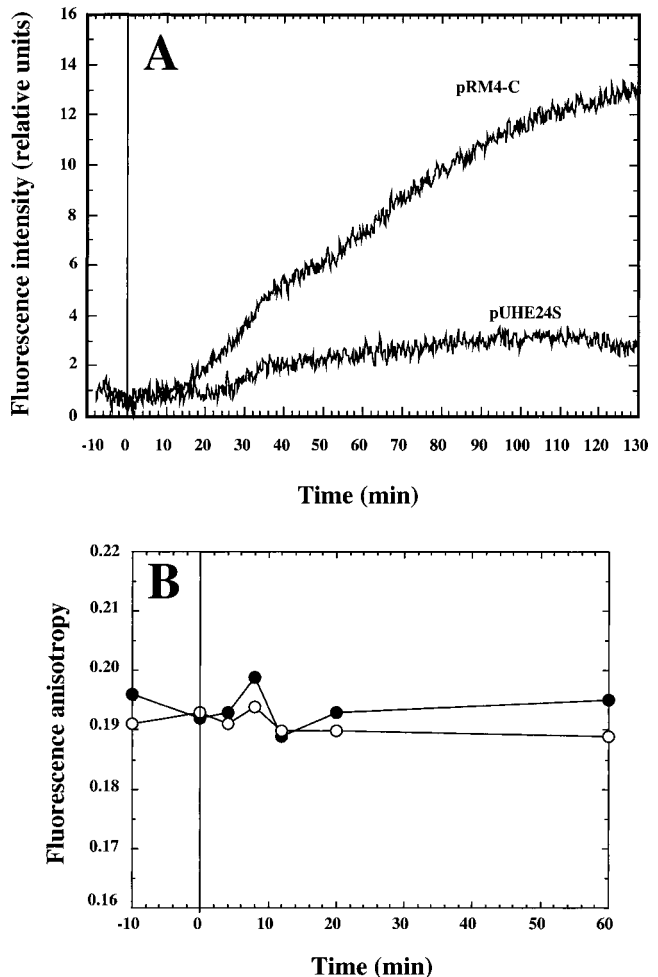


Fig. 3. Intensities of DAPI uptake (A) and DPH fluorescence anisotropy (B) displayed by exponentially growing *E. coli* cells harbouring pRM4-C or pUHE24S. (B) Cells harbouring pRM4-C before (○) and after (●) induction.

because the membrane fraction attached to DNA is enriched in phosphatidylethanolamine (Ballesta *et al.*, 1972), which is among those phospholipids known to preferentially interact with Cyt1Aa (Thomas & Ellar, 1983b). The

expanded nucleoids visualized by DAPI in exponentially growing cells, uninduced (Fig. 4A) or lacking *cyt1Aa* (Fig. 4C), were found to dramatically compact in the cell centre following intracellular synthesis of Cyt1Aa (Fig. 4B). A similar compaction caused by CAM in uninduced cells (Fig. 4D) served as a positive control. The nucleoids remained dispersed in cells expressing *p20* as well (data not shown), consistent with P20 protecting *E. coli* cells

from the lethal action of Cyt1Aa (Manasherob *et al.*, 2001). To rule out the possibility that the smaller nucleoids in Cyt1Aa-expressing cells (Fig. 4B) were the result of cell division as a consequence of DNA replication run-out and chromosome segregation, division was inhibited by cephalixin ($8 \mu\text{g ml}^{-1}$). The multiple segregating nucleoids in the filaments (Fig. 4E) seem to highly compact and fuse upon expressing Cyt1Aa (Fig. 4F). Measuring nucleoid

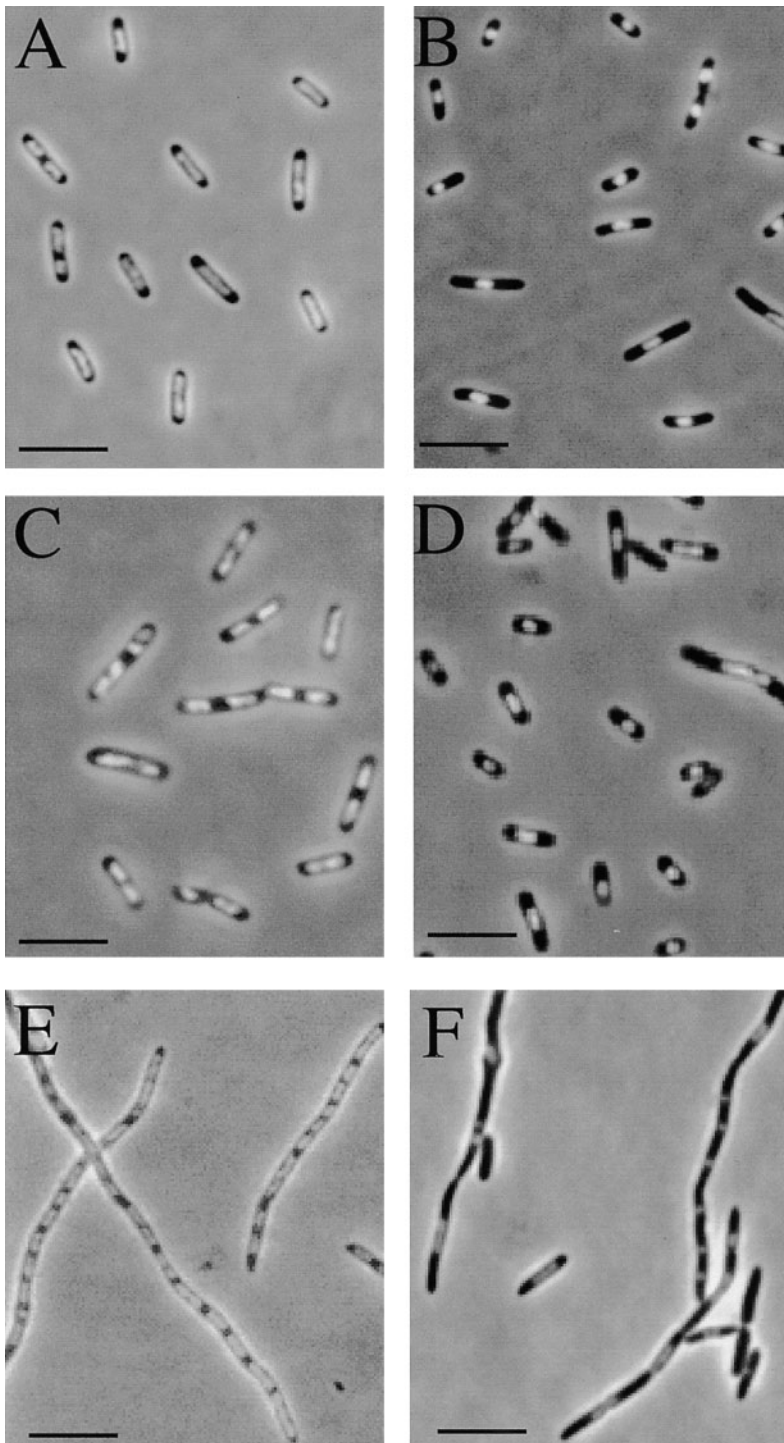


Fig. 4. Nucleoid morphologies in DAPI-stained *E. coli* cells. Cells harbouring pRM4-C, uninduced (A), after 60 min IPTG induction (B) or treated with CAM (D). (C) Cells harbouring pUHE24S 60 min after induction. Filaments (with pRM4-C) were obtained by treatment with cephalixin ($8 \mu\text{g ml}^{-1}$), IPTG-induced (F) or uninduced (E). Bars, 5 μm .

length relative to non-filamentous cell length substantiated this finding (Fig. 5). Mean nucleoid length halved (from 2.87 ± 0.08 to 1.30 ± 0.03 μm), while the mean cell length decreased by only 14% (from 4.40 ± 0.10 to 3.80 ± 0.095 μm), leading to a remarkably smaller relative nucleoid length (0.34) under the influence of Cyt1Aa than without (0.65) (Fig. 5B). This quantification demonstrates that compaction of the nucleoids did occur. The nucleoids did not compact due to cessation of DNA replication per se: no compaction occurs when DNA synthesis stops

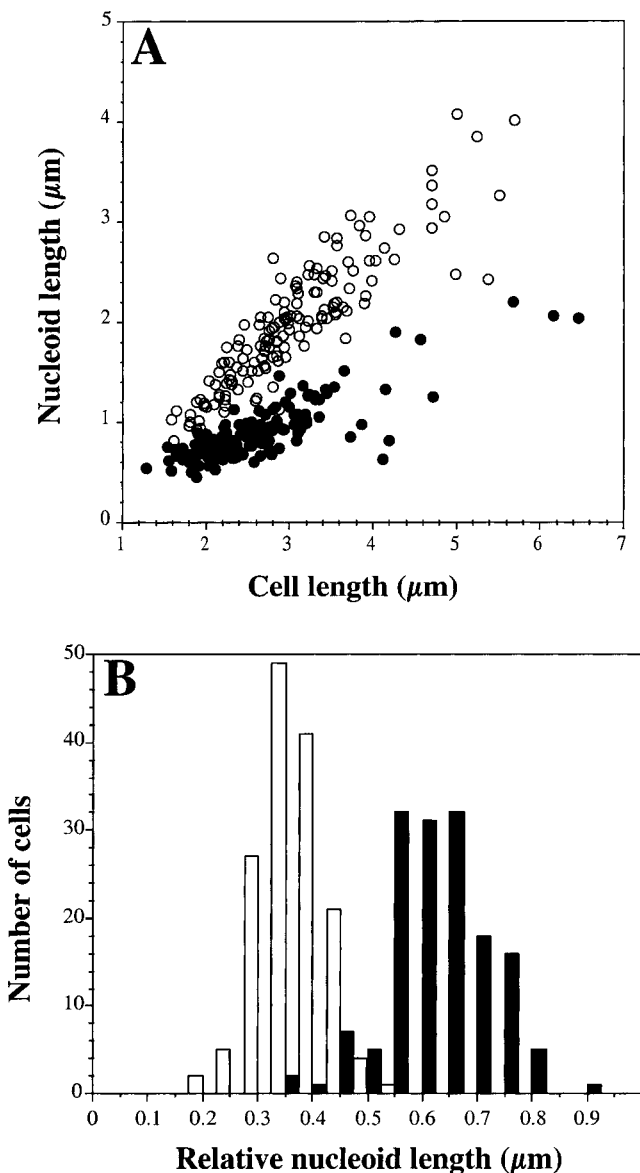


Fig. 5. (A) Nucleoid length as a function of cell length in *E. coli* cells expressing Cyt1Aa (●) or not expressing Cyt1Aa (○). (B) Frequencies of relative nucleoid lengths in 150 *E. coli* cells, uninduced (solid bars) and 60 min after IPTG induction (open bars).

rapidly in *dna-ts* mutants (Mulder & Woldringh, 1989) or with nalidixic acid (data not shown).

Nucleoid structure and shape seem to be determined by a balance between compaction and expansion forces (Woldringh, 2002). Compaction may be exerted by DNA supercoiling and phase separation from the cytoplasm, as well as by DNA-binding proteins. Coupled transcription, translation and insertion ('transertion') of the nascent membrane and exported proteins are considered to expand the nucleoid (Norris, 1995; Woldringh *et al.*, 1995). A compact nucleoid in the cell's centre and a reduced number of membrane attachment sites induced by rifampicin or CAM (Dworsky & Schaechter, 1973; Morgan *et al.*, 1967; Van Helvoort *et al.*, 1998; Zusman *et al.*, 1973) have been explained by disrupting this coupling and causing an imbalance favouring the compaction forces (Binenbaum *et al.*, 1999). Hence, nucleoid compaction by Cyt1Aa can occur by disrupting the DNA-membrane interactions. Can it also occur by membrane perforation per se, as in *cyt1Aa*-induced cells (Fig. 4B)?

To test this hypothesis, other agents that disrupt membranes were exploited. Triton X-100 (0.1%; Fig. 6A) and protamin (100 $\mu\text{g ml}^{-1}$; Fig. 6B) did not cause compaction; on the contrary, the DNA was dispersed over the whole cell volume. This effect may be explained by an efflux of low-molecular-mass compounds or a loss of macromolecular crowding. Membrane perforation results in the leakage of small molecules such as ATP from bacterial cells (Johansen *et al.*, 1997) and a reduced ability to synthesize ATP. Obstructing ATP-dependent gyrase should thus expand the nucleoid by creating an imbalance of forces. Such nucleoid expansion has indeed been observed with KCN treatment (Van Helvoort, 1996), which blocks terminal oxidation in the respiratory chain of *E. coli*. The other interpretation that this decompaction is due to the loss of macromolecular crowding in the cytoplasm is consistent with similar results published by others (Murphy & Zimmerman, 2001), where a limited loss of DNA compaction accompanied cytoplasm leakage from *E. coli* cells.

If the pores produced by Cyt1Aa in the plasma membrane of *E. coli* cells are of the same size as those produced in *Culex fumiferana* cells treated with Cyt1Aa (cut-off of about 360 Da) (Knowles & Ellar, 1987), ATP (507 Da) is very likely trapped inside *E. coli* cells, thus allowing run-out of mRNA. This run-out releases DNA from the membrane resulting in nucleoid compaction, as observed under treatment with CAM (Binenbaum *et al.*, 1999). This suggestion is consistent with nucleoid expansion in cells treated with protamin (Fig. 6B), which dramatically reduces intracellular ATP levels (Johansen *et al.*, 1997).

Upon treatment with perforating agents or Cyt1Aa, cells took up DAPI (of 350 Da) (Haugland, 1996) due to perforation of the membrane without the otherwise necessary pre-fixation with formaldehyde (Sun & Margolin, 2001). However, to prevent cell lysis by protamin (Johansen *et al.*,

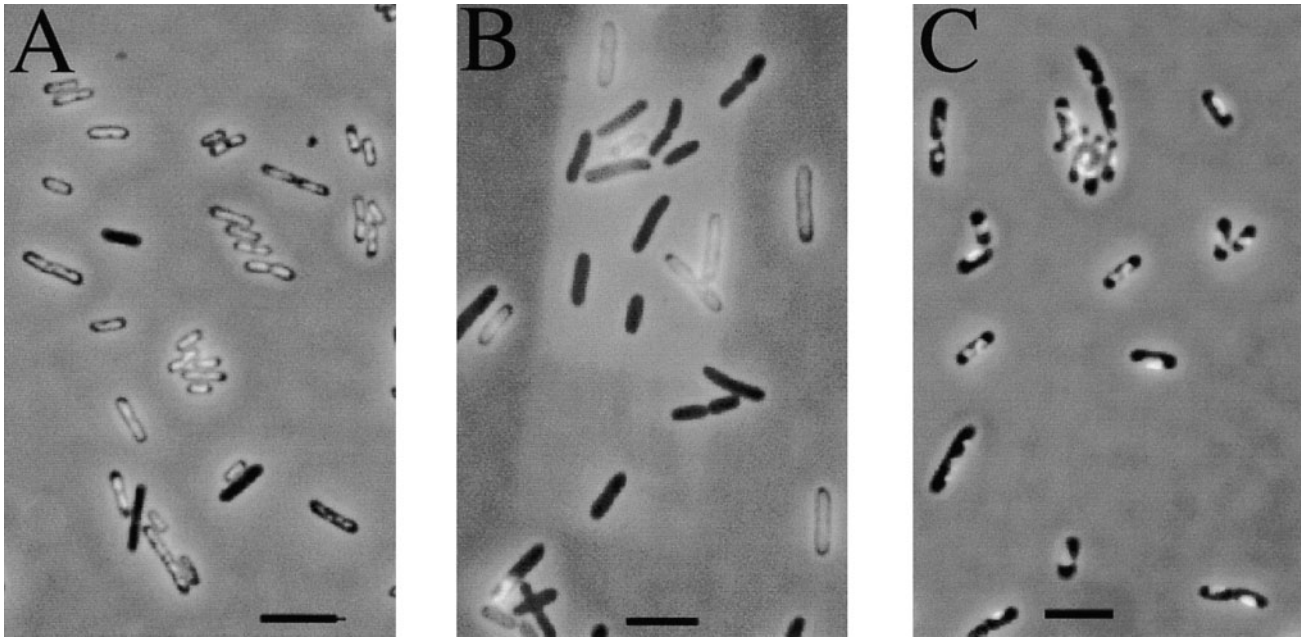


Fig. 6. DAPI-stained cells photographed under simultaneous phase-contrast/DAPI fluorescence exposure, treated with membrane-active agents: (A) Triton X-100; (B) protamin; (C) toluene. Bars, 5 μm .

1997), this agent was used at such a low concentration that not all cells were stained by DAPI (Fig. 6B). Fast nucleoid staining by DAPI in unfixed Cyt1Aa-expressing (Fig. 4B) or protamin-treated cells (Fig. 6B) confirms penetration into the cell through pores.

Treatment of the *E. coli* cells with toluene ($10 \mu\text{l ml}^{-1}$) (Fig. 6C) resulted in variable, random nucleoid positions in the cell periphery. Similar nucleoid shapes and distribution with no explanation have been observed by electron microscopy (Woldringh, 1973). These results may be due to an abrupt and uneven detachment of the nucleoid from the membrane caused by toluene, in contrast to the uniform detachment seen upon expression of Cyt1Aa. Fast detachment from large portions of the membrane leaves the nucleoid connected to other portions of the membrane that are still intact.

Relative nucleoid length

The impression that nucleoid compaction caused by Cyt1Aa is more intense than that caused by CAM (Fig. 4B and D, respectively) was substantiated by comparing the kinetics of nucleoid compaction under both conditions. In CAM-treated cells, compaction was observed after 5 min and completed after 10 min, while in cells expressing Cyt1Aa compaction started after 20 min (Fig. 7). The final compaction observed with Cyt1Aa after 40 min was significantly more effective (relative nucleoid length halved, from 0.67 to 0.3; Fig. 7B) than that observed with CAM (decrease by a third, to 0.45). This difference may reflect the different states of DNA: while there is replication run-out with CAM during about 40 min (Pato, 1975), Cyt1Aa was shown to

stop DNA synthesis immediately (Douek *et al.*, 1992). However, inhibition of replication by nalidixic acid did not change the rate or the extent of nucleoid compaction caused by CAM (Fig. 7B, inset). Furthermore, Cyt1Aa-affected nucleoid detachment from the membrane seemed to be asymmetrical (Fig. 7A): at 20 min, the nucleoid was dislodged from the cylindrical portion of cell envelope only, while the length decreased 5 min later (Fig. 7B), seen as withdrawal from cell poles (Fig. 7A). This sequential, slower compaction may allow ordered packaging, thus resulting in the observed tighter nucleoids.

The three morphometric differences in nucleoid compaction affected by Cyt1Aa and CAM (timing, degree and shape) suggest that the two agents operate to change nucleoid morphology via different modes. This is consistent with a failure to probe DNA detachment in *cyt1Aa*-induced cells by DPH anisotropy (Fig. 3B), as found in CAM-treated cultures (Fig. 2 in Binenbaum *et al.*, 1999).

Following *cyt1Aa* induction, there is an accumulation of Cyt1Aa in the cell for 10 min, to a concentration (Fig. 2) required to disrupt the membrane barrier ability after 15 min (Fig. 3A) and to compact the nucleoid after 20 min (Fig. 7). This sequence of events is consistent with a crucial role for Cyt1Aa in nucleoid compaction, but not by perforation: various perforating chemicals preferentially disperse the nucleoid (Fig. 6A and B).

Localizing Cyt1Aa

Overcompaction of the nucleoid may also be affected by DNA-binding proteins such as the histone-like HU protein

that binds throughout the nucleoid interior (Shellman & Pettijohn, 1991). Cyt1Aa has been found to bind DNA *in vivo* and *in vitro* (Yokoyama *et al.*, 1998). To locate the site

of its action, Cyt1Aa was fused with GFP (Cormack *et al.*, 1996). Cells that expressed the fused gene were not killed (data not shown), indicating that proper folding of the C

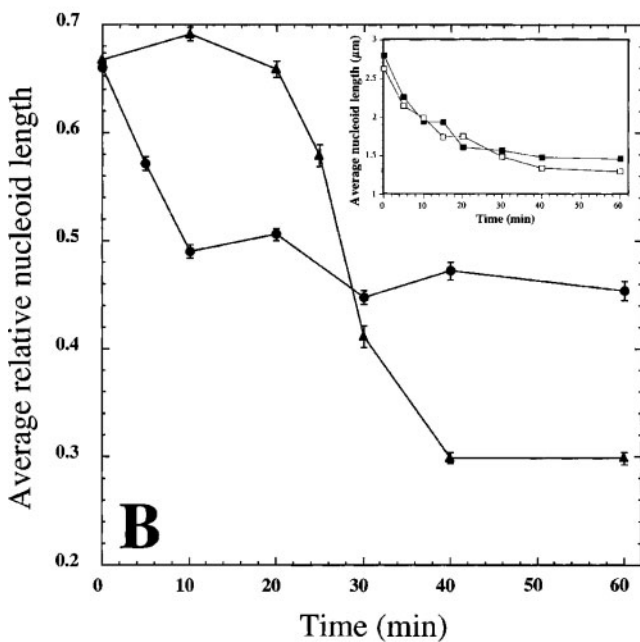
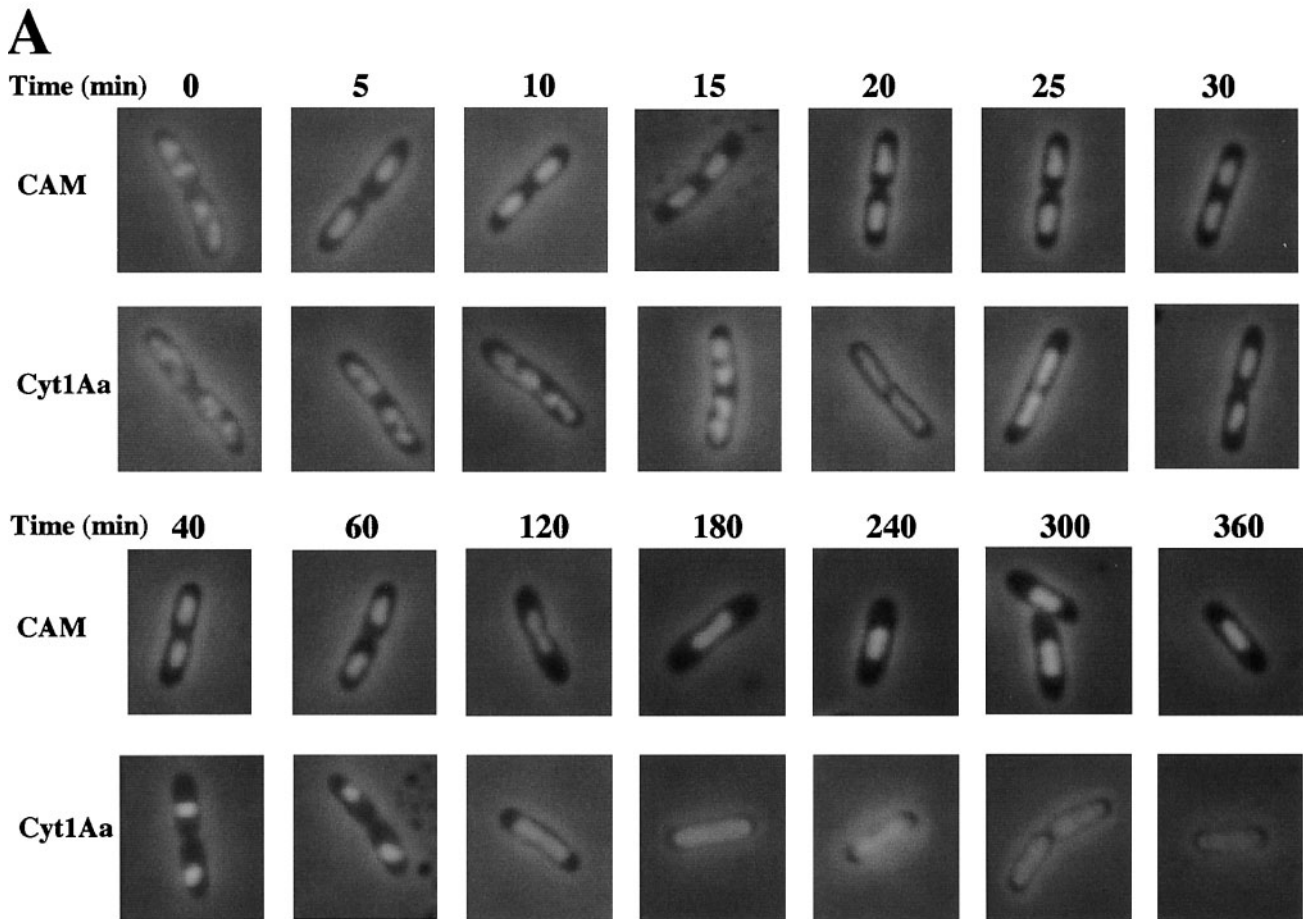


Fig. 7. Changes in nucleoid morphology (A) and relative nucleoid length (B) in cells (with pRM4-C) after addition of CAM (●) or IPTG (▲). (B) Mean and standard errors of 150 cells are displayed for each point. Inset, mean nucleoid length of *E. coli* (strain B/r) after addition of CAM alone (■) and with nalidixic acid (□).

terminus of Cyt1Aa is crucial for its activity, and supporting the view that its lethal effect is specific rather than caused by overexpression per se (Manasherob *et al.*, 2001). We have, therefore, resorted to using *in situ* immunofluorescence labelling which has been successfully applied to locate numerous bacterial proteins (e.g. Buddelmeijer *et al.*, 1998).

In cells expressing Cyt1Aa for 60 min, Alexa fluorescence was localized at the poles (Fig. 8A), where no DNA material (DAPI fluorescence) was present (Fig. 8B), while co-expression of P20 resulted in uniform granulation all over the cell (Fig. 8C), irrespective of DNA localization (Fig. 8D). This clear separation between Cyt1Aa and DNA (Fig. 8A and B) indicates that the toxin is excluded from the area of condensed nucleoid. This exclusion is not necessarily the result of a specific Cyt1Aa affinity for *E. coli* cell poles: total proteins (visualized by FITC) were similarly polarized under nucleoid compaction caused by CAM treatment (I. Fishov, unpublished observations). Nucleoid compaction itself may thus non-specifically 'push' proteins outward to the cell poles, Cyt1Aa included. This is supported by the lack of a partition between the Alexa-labelled Cyt1Aa in cells expressing it together with P20, where nucleoids are not compacted (Fig. 8D). The partition in

the absence of P20 implies no Cyt1Aa binding all over the chromosome, but does not exclude binding at the nucleoid periphery.

No great association of Cyt1Aa with the cell membrane or the typical halo was observed, as predicted if the protein were incorporated and evenly distributed at the cell membrane. This finding does not necessarily contradict the observation that Cyt1Aa perforates the membrane (Fig. 3A), because effective perforation may not need massive membrane insertion of Cyt1Aa.

Partial lysis of *cyt1Aa*-induced cells

The tightly compacted nucleoids in *cyt1Aa*-induced cells started to disperse and expand to fill the whole cell volume at around 2 h after induction, while compacted CAM-treated nucleoids remained stable in the mid-cell (Fig. 7A). In contrast to the bacteriostatic effect of CAM, Cyt1Aa is bacteriocidal (Douek *et al.*, 1992; Manasherob *et al.*, 2001; Fig. 1B). This is reflected in reduced culture turbidity (Fig. 1A) and the cells' phase-contrast (data not shown), indicating that partial cell lysis occurred without massive peptidoglycan 'destruction' (Voss, 1964) following membrane perforation by Cyt1Aa. Such partial lysis leads to loss of the macromolecular crowding effect on DNA and allows it to expand and fill the whole cell volume (Zimmerman, 1993; Woldringh, 2002) in a similar way to that obtained by treatment of the cells with Triton X-100 and protamin (Fig. 6).

Amino acid starvation

Compaction of nucleoids caused by amino acid starvation of the *relA1* mutant that does not occur in its isogenic stringent strain was explained (Binenbaum *et al.*, 1999) by run-out of mRNA releasing the transcription elements rather than freezing them, as in the wild-type. Amino acid starvation would thus result in leakage of amino acids through the membrane pores formed by Cyt1Aa. The nucleoid of the stringent strain under *cyt1Aa* induction, on the other hand, would remain expanded due to 'freezing' of the transcription elements. This hypothesis was tested by inducing *cyt1Aa* in an isogenic stringent and a relaxed strain (RL331T and RL332T, respectively). Nucleoids were compacted in both strains (data not shown), precluding this possibility.

Concluding remarks and speculations

Overexpression of Cyt1Aa in *E. coli* cells arrests growth and DNA replication, and causes membrane perforation, loss of viability and nucleoid compaction. Loss of colony-forming ability is a likely consequence of membrane perforation, but various conceivable mechanisms as primary causes for nucleoid compaction were tested and rejected: i.e. membrane perforation per se, leakage of low-molecular-mass compounds, arrest of protein or RNA synthesis and increasing macromolecular crowding. A direct effect of Cyt1Aa on macromolecular synthesis is not excluded and requires special experiments in model systems.

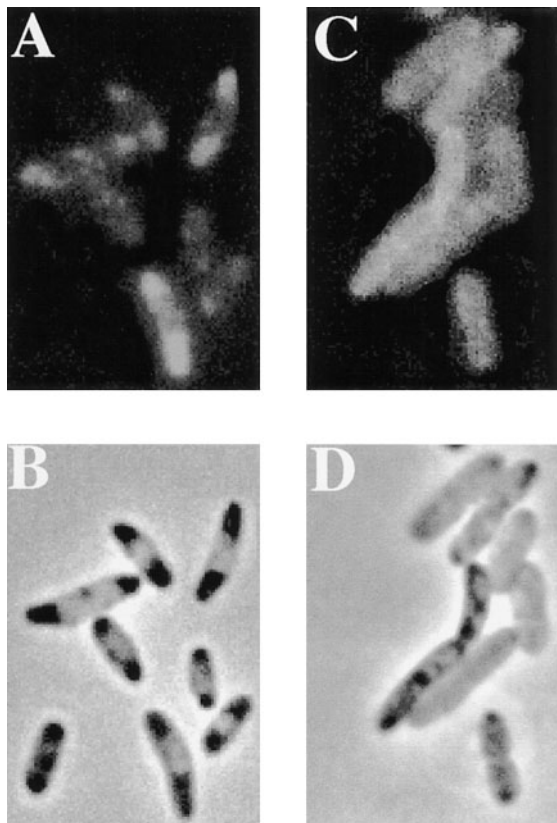


Fig. 8. Alexa-labelled Cyt1Aa fluorescence (A, C) and DAPI-stained cells (B, D) expressing Cyt1Aa alone (A, B; clone harbouring pRM4-C) or with P20 (C, D; with pRM4-RC), 60 min after induction.

Simultaneous, high-affinity interactions of Cyt1Aa with zwitterionic phospholipids as well as with DNA may enhance detachment of DNA from the membrane and, hence, affect nucleoid compaction. The observed extra compaction caused by Cyt1Aa over that caused by CAM treatment may be associated with the sequential, slower condensation of the DNA that allows ordered packaging, resulting in tighter nucleoids.

A non-insecticidal 28 kDa protein of *B. thuringiensis* subsp. *shandongensis* exhibiting cytotoxicity by swelling of human leukaemic T cells and chromatin condensation (Lee *et al.*, 2001) lends support to our observation that Cyt1Aa compacts the nucleoid of *E. coli* as well, irrespective of its mechanism(s) of action in both cases.

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