# Colicin N and its thermolytic fragment induce phospholipid vesicle fusion

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Colicin N, a bacteriocin encoded on a plasmid belonging to the pore-forming class of colicins, induces phospholipid vesicle fusion at acidic pH as demonstrated by fluorescence resonance energy transfer. Its C-terminal thermolytic fragment has properties very similar to the native molecule. The fusion is protein concentration-dependent and is regulated by (a) group(s) with a p$K$ of approximately 4.6. The physiological relevance of this characteristic common to all colicins tested so far is discussed.

Colicin N; Membrane fusion; Fluorescence energy transfer; Thermolytic fragment

## 1. INTRODUCTION

Colicins are plasmid-encoded protein antibiotics which kill bacteria closely related to the producing strain (generally *Escherichia coli*) in a three step manner: (i) binding to a specific receptor protein on the outer membrane of the target cell; (ii) translocation through the periplasmic space; and finally (iii) killing activity sensu stricto (for a review see [1]). Colicins A, B, E1, K, Ia, Ib and N form voltage-dependent channels in vitro and this is very likely in vivo [2]. Colicin E2 has a DNase activity and colicin E3 a RNase one. Colicin M inhibits peptidoglycan biosynthesis [3]. In spite of their different mode of action, all these molecules are composed of three domains corresponding to the three steps mentioned above: the central domain is involved in the receptor binding, the COOH terminus contains the channel or nuclease activity, and the NH$_2$ terminus is probably involved in the translocation step [4].

Although some progress has been made in the determination of the gene products involved in the translocation of some colicins through the cell envelope [5,6], not much is known about the mechanism by which colicins reach their target inside the cell.

Pattus et al. [7] have previously shown that the pore-forming colicins A, E1 and K but also the colicins with nuclease activity, E2 and E3, are able to induce vesicle fusion at acidic pH regardless of their modes of action

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in vivo. However, the thermolytic fragment of colicin A corresponding to the pore-forming domain [8] was unable to provoke any fusion whilst in a series of colicin A constructs, only those containing the receptor-binding domain were able to fuse vesicles at acidic pH [9]. Similarly Escuyer et al. [10] found that exposure to low pH triggers an increase of hydrophobicity of the N-terminal domain of colicin E3. It was therefore of great interest to test colicin N and its thermolytic fragment since colicin N has a smaller size (PM 42000) [11] than the other colicins (PM 57000-70000) whilst its thermolytic fragment (PM 23000) is slightly larger than that of colicin A (PM 21800) and accounts thus for about half of the protein.

Although the true meaning of the fusogenic activity is unknown, this apparently general property of colicins may have a role in, or reflect a feature required for penetrating the target cell.

We report here the fusogenic ability of both colicin N and its thermolytic fragment on phospholipid vesicles made of *E. coli* lipids at acidic pH.

## 2. MATERIALS AND METHODS

Colicin N was purified from the *E. coli* K-12 strain BZB 1019 (hsdR) containing a plasmid called pCHAP4 [11]. Cell growth, colicin induction, salt extraction of colicin N and ammonium sulfate precipitation were carried out as described for colicin A [12]. Colicin N precipitates at low ionic strength, therefore the extracts were dialyzed against 10 mM sodium phosphate buffer, 300 mM NaCl, 10 mM procain, pH 6.8, loaded onto a Sephadex CM 50-120 column and eluted with a 0.3–1 M NaCl gradient at 4°C in the same buffer. The fractions containing colicin N were pooled and (NH$_4$)$_2$SO$_4$ added to a concentration of 1 M. After loading onto a phenyl-Sepharose column, colicin N eluted with an inverse 1 to 0 M (NH$_4$)$_2$SO$_4$ gradient in 10 mM sodium phosphate, 10 mM procain, pH 6.8 at 4°C. Fractions which gave a single band on SDS-PAGE electrophoresis gel [13] were pooled.
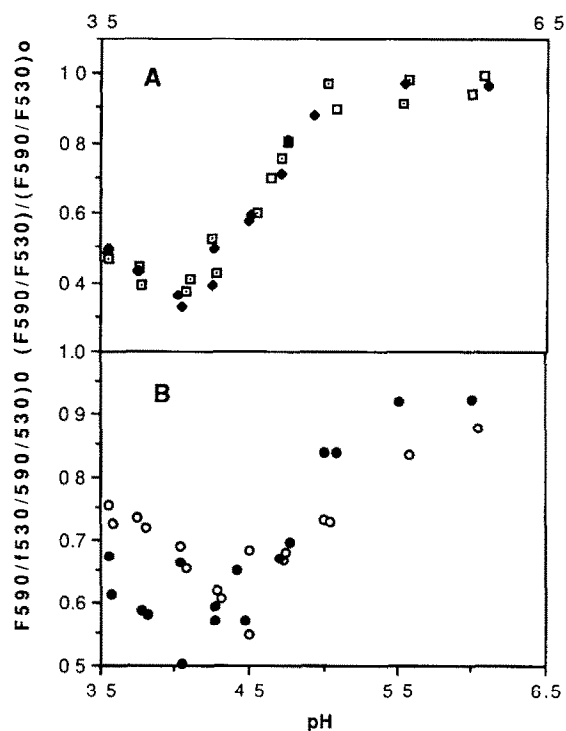
Fig.1. pH dependence of the fusion of *E. coli* phospholipid vesicles promoted by colicin N (A) or its thermolytic C-terminal fragment (B) as shown by the plot of the fluorescence ratio at 590 nm and 530 nm. Tris acetate buffer, 10 mM, NaCl, 100 mM. The protein concentration in both cases was $1.2 \times 10^{-7}$ M (2 independent preparations of colicin or thermolytic fragment were tested).

The thermolytic fragment was prepared like the colicin A COOH terminal fragment [14].

A fusion assay by resonance energy transfer was performed as previously described [7] except that the phospholipid vesicles were prepared by detergent dialysis at pH 4.5 for the experiments on colicin concentration dependence. The principle of the assay is based on the loss of fluorescence energy transfer between the fluorescent phosphatidyl ethanolamine N-NBD-PE ($Em_{max}$ = 530 nm) and N-Rho-PE ($Em_{max}$ = 590 nm) (Avanti Biochemicals) due to the surface

dilution that occurs upon fusion of labelled vesicles with unlabelled ones [15]. The samples were excited at 450 nm and the increase of fluorescence at 530 nm and the decrease at 590 nm were recorded with an Aminco SLM 8000 fluorimeter.

Turbidity measurements were performed with a Cecil CE595 double beam spectrophotometer.

## 3. RESULTS

### 3.1. pH dependence of the fusogenic activity

At neutral and basic pH, colicin N has no effect on *E. coli* phospholipid vesicles. As found with colicin A [7], an increase in the turbidity and fusion of liposomes was observed upon lowering the pH. The pH dependence of this fusion activity is shown in fig.1A. The most efficient fusion is promoted around pH 4 while no fusion is observed at pH values higher than 5. The titration curve shows that a group with an approximate p$K$ of 4.6–4.7 governs the fusion activity. This p$K$ is around 1 unit lower than that found with colicin A acting on the same phospholipid vesicles.

A similar, although less pronounced, titration curve is obtained with the thermolytic fragment and this also gives a p$K$ of around 4.6 (fig.1B). This indicates that both the entire molecule and its fragment possess similar properties while the latter has a slightly reduced activity. The small increase in the ratio of fluorescence at pH values below 4 (corresponding to a small decrease in the fusogenic activity) is correlated with an increase in turbidity observed with the vesicles alone before any protein addition. This reflects, at least partly, an influence of pH on the lipidic system itself.

### 3.2. Molecularity of the fusogenic event

Vesicle fusion and the increase in turbidity are colicin N concentration-dependent as shown in figs 2a and 3, respectively. Similar concentration dependences of turbidity increase were observed at 600 and 350 nm although some chromophore contribution to the spec-
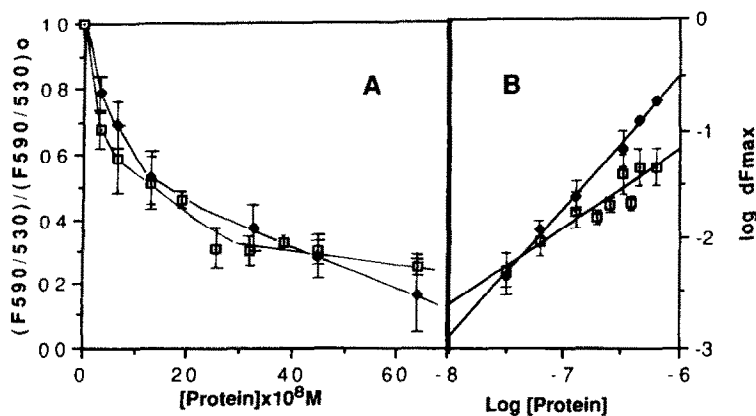


Fig.2. Concentration dependence of the fusion of *E. coli* phospholipid vesicles: (A) the fluorescence ratio at 590 nm and 530 nm 15 min after colicin N (□) or its thermolytic C-terminal fragment (◆) injection is plotted as a function of protein concentration (2 independent preparations of colicin or thermolytic fragment). (B) Molecularity of the fusion reaction. Logarithmic plot of the initial rate of fusion monitored by the rate of fluorescence increase at 530 nm as a function of protein concentration. The correlation coefficients are 0.949 and 0.998 for colicin N (□) and the C-terminal peptide (◆) data, respectively. Tris acetate, 10 mM, NaCl, 100 mM, pH 4.5.
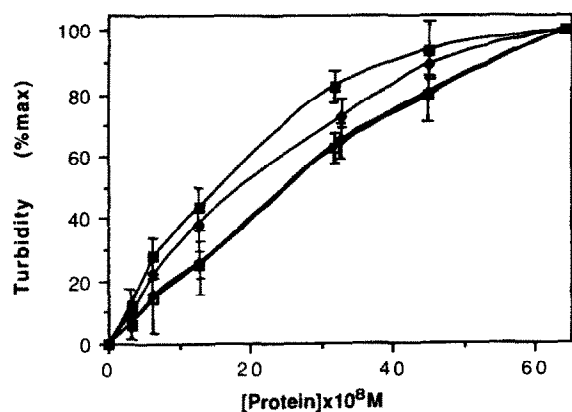
Fig.3. Concentration dependence of the turbidity at 350 nm (open symbols) and 600 nm (closed symbols) for colicin N (□, ■) and its thermolytic fragment (◊,♦), respectively expressed in % of the maximum value obtained. Same conditions as in fig.2.

tra cannot be excluded at 350 nm. Colicin N fusion activity is almost identical to that found with colicin A previously [7]. In contrast to colicin A, the thermolytic fragment exhibits a concentration dependence of the fusion and turbidity almost identical to that of the native molecule (figs 2A and 3).

For each concentration, fusion kinetics were monitored by recording the fluorescence increase at 530 nm during the first 15 min following protein injection. The molecularity of the fusion event can be deduced from the slope of the log-log plot of initial rates versus concentration (fig.2B). This slope is 0.74 and 1.22 for colicin N and its thermolytic fragment, respectively. This molecularity close to one indicates that colicin N molecules fuse vesicles as monomers.

### 3.3. Sustained fusion activity and reversibility of vesicle aggregation

Fluorescence measurements record only true fusion events while increase in turbidity reflects the increase in particle size due to both fusion and vesicle aggregation. The colicins studied so far promote both aggregation and fusion as confirmed by electron microscopy [7]. It was shown with colicin A that aggregation prevents fusion from proceeding further. This is also observed with colicin N and its thermolytic fragment as seen when pH cycles are applied to the system. The protein is injected at pH 4; after 15 min, no more fusion occurs. The pH is then raised above 8 and subsequently decreased again to 4. Fluorescence spectra indicate that fusion starts again at acidic conditions (fig.4). The turbidity decreases when raising the pH to 8, indicating deaggregation of the vesicles and increases again at pH 4 up to a higher value than before at the same pH. These pH dependent changes can be repeated 2 or 3 times with fluorescence intensity at 530 nm decreasing down to zero. This phenomenon resembles that observed with colicin A: (i) raising the pH reverses vesicle aggregation; and (ii) decreasing the pH to its original value allows new fusion events to occur. As hypothesized before, fusion seems to be inhibited by aggregation. This experiment also shows that colicin N maintains its fusogenic activity after the first fusion event, i.e. fusion is not due to an irreversible change in the protein.
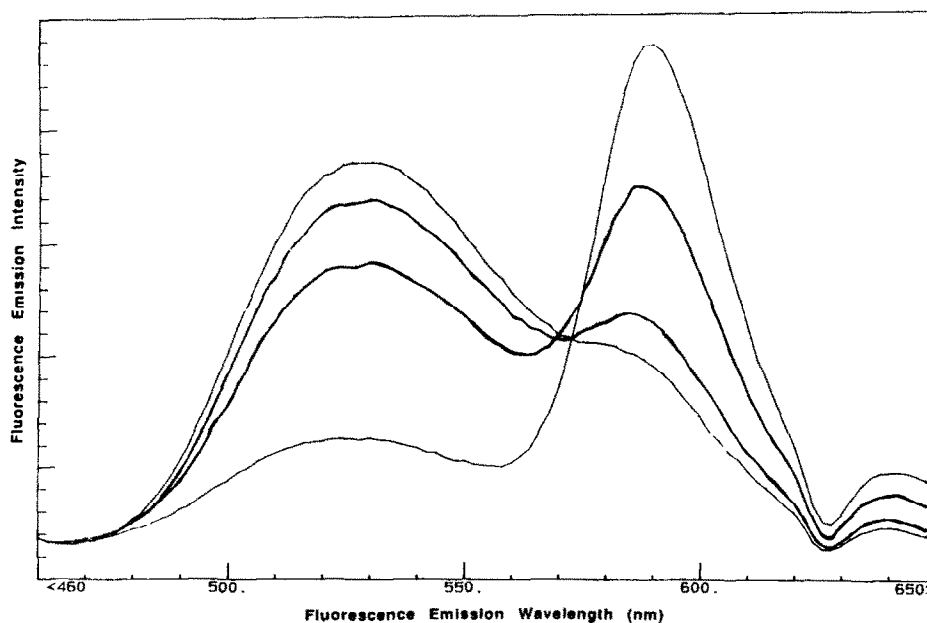


Fluorescence Emission Wavelength (nm)

Fig.4. Sustained fusion activity and reversibility of the vesicle aggregation. *E. coli* phospholipid vesicle suspension containing colicin N (1.2 × $10^{-7}$ M) was subjected to cyclic 4.5–8.5 pH steps. The spectrum with the highest fluorescence intensity at 590 nm corresponds to the spectrum of the lipids before protein injection. After this, the order of decreasing fluorescence intensity at 590 nm are the spectra corresponding to: (i) 15 min after protein injection at pH 4.5; (ii) after 1 pH cycle; and (iii) after 2 cycles. Tris acetate, 10 mM, NaCl, 100 mM. The pH was adjusted with 5 M acetic acid or 5 M NaOH. Similar results are obtained with the thermolytic fragment.

## 4. DISCUSSION

The results presented in this study show that colicin N like colicins A, $E_1$, $E_2$, and $E_3$ was able to promote phospholipid vesicle fusion at acidic pH.

It was previously shown that one fusion event was induced by one colicin molecule [7] and our results extend that conclusion to colicin N. Fusion requires by definition two sites of interaction with lipids: for pore-forming colicins, one should be localized in the COOH terminus, capable of tight interactions with lipids [16], and the other one in the remaining part of the molecule, most probably in the central domain as deduced from the fusogenic activity of colicin A constructs [9]. It was also shown in planar lipid bilayer [17] and lipid monolayer [18] experiments that colicin A, but not its COOH terminal fragment, undergoes a pH-dependent transition between an 'acidic' and a 'basic' form of the pore with an apparent p$K$ of 5.3 suggesting a pH-dependent association between the COOH terminal domain and another part of the molecule which affects the channel activity. Although no p$K$ value was determined for the fusogenic activity of colicin A, the data suggest a similar value. Such a transition between acidic and basic forms has been found recently with colicin N in planar lipid bilayers (Wilmsen, H.U., personal communication); as compared to colicin A, the p$K$ is shifted to around 4.5 and is in good agreement with our findings of a p$K$ of 4.6–4.7 in the fusogenic activity. This suggests that acidic conditions introduce protein rearrangements and modify the interactions between domains by, for example, opening the structure so that the contact regions are accessible towards the outside. In colicin E3 [10], acidic pH leads to an increased hydrophobicity and possibly a better binding to lipid bilayers with, consequently, a facilitated penetration in the target cell.

The fusogenic activity of the colicin N thermolytic fragment is an unexpected result. However, colicin N is only two-thirds the molecular mass of colicin A, while the colicin N thermolytic fragment is slightly larger than the colicin A thermolytic peptide (23 000 Da and 21 800 Da, respectively). The thermolytic fragment of colicin N may therefore contain more than the killing activity domain and at least partly the central domain responsible for the receptor binding where the second lipid site is believed to be located. The less pronounced pH dependence compared to that of the entire molecule may indicate that several groups are involved in the intramolecular interactions, some of them being in the $NH_2$ part of the molecule. This situation might be similar to that observed in colicin E1: its pore activity shows a strong pH dependence governed by a p$K$ of 4.5–4.6 that is absent in the COOH terminal tryptic fragment (equivalent to our thermolytic fragment) [19]. Mutagenesis experiments lead to the conclusion that several carboxylic groups are involved therein because no single mutation completely abolishes the pH dependence [20].

As outlined before, the fusogenic activity is common to all colicins tested so far whatever their modes of action may be. It is however noteworthy that all of them use the Tol QRAB translocation pathway [1]. It would therefore be of great interest to test a colicin translocated through the Ton B system to allow us to consider whether fusion activity is a general characteristic shared by all colicins. Nevertheless, the fusogenic activity must reflect an important property for the penetration in the target cell since colicins with different modes of action are concerned. Although there is no evidence for an acidic compartment in *E. coli* cells, enhanced binding to lipid membrane and membrane fusion may occur by some triggering event which is mimicked by low pH in vitro.

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