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Colicin N forms voltage- and pH-dependent channels in planar lipid bilayer membranes

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Abstract. The protein antibiotic colicin N forms ion-permeable channels through planar lipid bilayers. Channels are induced when positive voltages higher than +60 mV are applied. Incorporated channels activate and inactivate in a voltage-dependent fashion. It is shown that colicin N undergoes a transition between an "acidic" and a "basic" channel form which are distinguishable by different voltage dependences. The single-channel conductance is non-ohmic and strongly dependent on pH, indicating that titratable groups control the passage of ions through the channel. The ion selectivity of colicin N channels is influenced by the pH and the lipid composition of the bilayer membrane. In neutral membranes the channel undergoes a transition from slightly cation-selective to slightly anion-selective when the pH is changed from 7 to 5. In lipid membranes bearing a negative surface charge the channel shows a more pronounced cation selectivity which decreases but does not reverse upon lowering the pH from 7 to 5. The high degree of similarity between the channel characteristics of colicin A and N suggests that the channels share common features in their molecular structure.

Key words: Colicin N – Planar lipid bilayers – Ion channel – Voltage dependence – pH-dependence

Introduction

Colicins are plasmid-encoded protein antibiotics which kill bacteria closely related to the producing strain (generally *Escherichia coli*). Like many toxins, colicins are composed of structural domains specialized in one of the different steps of the activity, targeting, translocation and killing. Colicins of the E1-class include colicin A, B, E1, Ia, Ib and K which permeabilize the cytoplasmic membrane, thereby destroying the membrane potential (Konisky 1982; Lazdunski et al. 1988; Pattus et al. 1989). These colicins form well-defined voltage-gated ion channels in artificial membranes (Schein et al. 1978; Weaver et al. 1981; Cleveland et al. 1983; Pressler et al. 1986; Nogueira and Varanda 1988). Channel-forming colicins have become a valuable tool for the study of the basic principles of membrane channels since they offer some advantages in experimental handling. They can be obtained in large amounts and most of their amino acid sequences are known (Yamada et al. 1982; Morlon et al. 1983; Varley and Boulnois 1984; Mankovich et al. 1986; Schramm et al. 1987). Since colicins are water-soluble they are easy to employ in planar lipid bilayer experiments (Hanke 1986).

Recently the C-terminal fragment of colicin A was crystallized (Tucker et al. 1986) and its structure was determined by X-ray crystallography and refinement at 2.5 Å resolution (Parker et al. 1989). Based on this work a molecular model was suggested according to which the ion-conducting channel is delineated by alpha-helices and which may account for the voltage-dependent incorporation and channel gating (Parker et al. 1989; Parker, personal communication).

Colicin N presents some interesting unusual features among channel-forming colicins. It has a relative molecular mass of 42 000 and thus it is smaller than the other colicins which have relative molecular masses $\geq 57\,000$ (Pugsley 1984c). It uses a major outer membrane protein, the OmpF-porin, as a receptor (Tommassen et al. 1984). Moreover, it causes cell lysis, a property which it shares with colicin M, but not with other colicins (Pugsley 1984a, b).

Recent studies strongly suggest that colicin N belongs to the channel-forming class of colicins. Firstly, it was shown that the primary effect of colicin N treatment is a depolarisation of the cytoplasmic membrane resulting in the loss of the ability to accumulate proline (Pugsley 1987). Secondly, nucleotide sequencing of the structural gene for colicin N revealed a strong homology between the C-terminal domains of colicin A and N (Pugsley 1987).

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In this study we provide direct evidence that colicin N forms well-defined ion channels in planar lipid bilayer membranes and we compare the channel-forming properties of colicin N with those of other well known colicins of the E1-class.

Material and methods

Purification of colicin N

Colicin N was purified from the *E. coli* K-12 strain BZB 1019 (hsdR) containing a plasmid called pCHAP4, (Pugsley 1987).

Cell growth, colicin induction, salt extraction of colicin N and ammonium sulfate precipitation were carried out as described for colicin A (Cavard and Lazdunski 1979). The protein was dialyzed against 10 m*M* sodium phosphate (pH 6.8), 10 m*M* procaine (buffer A) and loaded on a CM-Sephadex C-50-120 column equilibrated in the same buffer at 4 °C. The protein was eluted by a 0 *M*–1 *M* NaCl gradient. The colicin N-containing peak was equilibrated against buffer A containing 1 *M* NH_4Cl, loaded on a Phenyl-Sepharose column (Cl-4B, Pharmacia) and eluted by a 1 *M*–0 *M* NH_4Cl-gradient. After this last chromatographic step colicin N gave a single band in polyacrylamide gel electrophoresis after Coomassie Blue or silver staining.

Planar lipid bilayer experiments

Unless otherwise stated, the following buffer was used throughout: 1 *M* NaCl, 10 m*M* *Tris*-acetate, 5 m*M* $CaCl_2$. In some single-channel experiments NaCl was replaced by an equal concentration of CsCl, KCl, LiCl or NH_4Cl respectively. At pH-values > 8.5 glycine was used as buffer. For determination of reversal potentials the buffer on the cis-side (see below) was 100 m*M* NaCl, 10 m*M* *Tris*-acetate, 5 m*M* $CaCl_2$. All buffer solutions were prepared in double quartz-distilled water using reagent grade materials.

Planar lipid bilayer membranes were formed either according to the method described by Schindler and Feher (1976) or according to the method described by Montal and Mueller (1972). For the first method phosphatidylcholine from soybeans (Sigma Chemical Company, St. Louis, USA, type IIS) was purified according to Kagawa and Racker (1971). The Montal-Mueller technique was employed to form neutral planar lipid bilayers. For this purpose a 1 : 1 mixture of POPC [1] and DOPE [2] (Avanti Polar Lipids, Birmingham, USA) was used. These lipids were dissolved in chloroform and kept in small aliquots at −40 °C. For an experiment the lipid was dried under a stream of nitrogen and by exposure to low pressure under a vacuum pump for one hour. The lipids were then dissolved in hexane (2 mg/ml) and spread from this solution on the surface of the aqueous phase. In all cases bilayers were formed across a 150-μm hole in a teflon septum which was pretreated with a 1 : 40 solution (v/v) of hexadecane in pentane before use to increase the mechanical stability of the membrane.

We term the sides of the membrane as *cis*- and *trans*-compartments. The membrane voltage was clamped to the desired value using two Ag/AgCl-electrodes which were connected to the buffer in the *cis*- and *trans*-compartments via agar bridges. The membrane current was amplified using an I-V converter with an operational amplifier (Burr Brown 3528) and feedback resistors ranging from 10^7 to 10^9 Ω. The trans-compartment was connected to the I-V converter and held at virtual ground potential. The sign of the membrane potential refers to that on the cis-side of the membrane. Current was defined as positive when cations flowed into the trans-compartment. The protein was always added to the cis-side while the aqueous solution was stirred vigorously with a magnetic bar.

Reversal potentials were determined by applying a slow triangular wave voltage ($2 \cdot 10^{-3}$ Hz) using a function generator (Wavetek, model 143).

Results

Figure 1 shows the increase of the membrane current I after colicin N was added to the cis-side of a bilayer at a constant applied voltage. This reflects an increase in the macroscopic conductance λ of the bilayer which was low at the beginning. Usually there was a lag-time between the addition of the protein and the start of the current

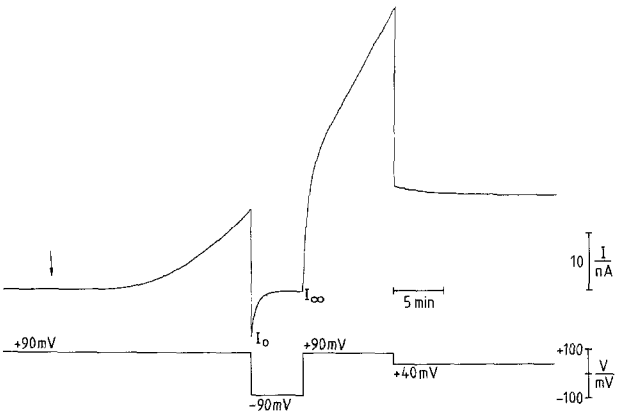


Fig. 1. The macroscopic membrane current I after addition of colicin N (arrow; final concentration $3.7 \cdot 10^{-8}$ g/ml) at different membrane voltages. After a lag time of a few minutes I increased at a constant applied voltage of +90 mV, reflecting an enhancement of the macroscopic membrane conductance λ, which was low before the addition of colicin N (≈5 pS). When the voltage was switched to −90 mV the current reached an initial peak value I_0 and then declined to reach a steady state value I_∞ which was close to the baseline, indicating an almost complete current inactivation. The relation $\lambda(+90\text{ mV})/\lambda_0(-90\text{ mV})$ between the macroscopic conductances immediately before and after the voltage step to −90 mV is 1.65 : 1. When the voltage was switched back to +90 mV the current increased steeply at first, this being followed by a slower but linear increase after about 2 min. After a step to +40 mV the inactivation was far weaker than at −90 mV. Note that there was no net increase of current at this voltage. The pH in this experiment was 7.0

[1] 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidyl choline
[2] 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine

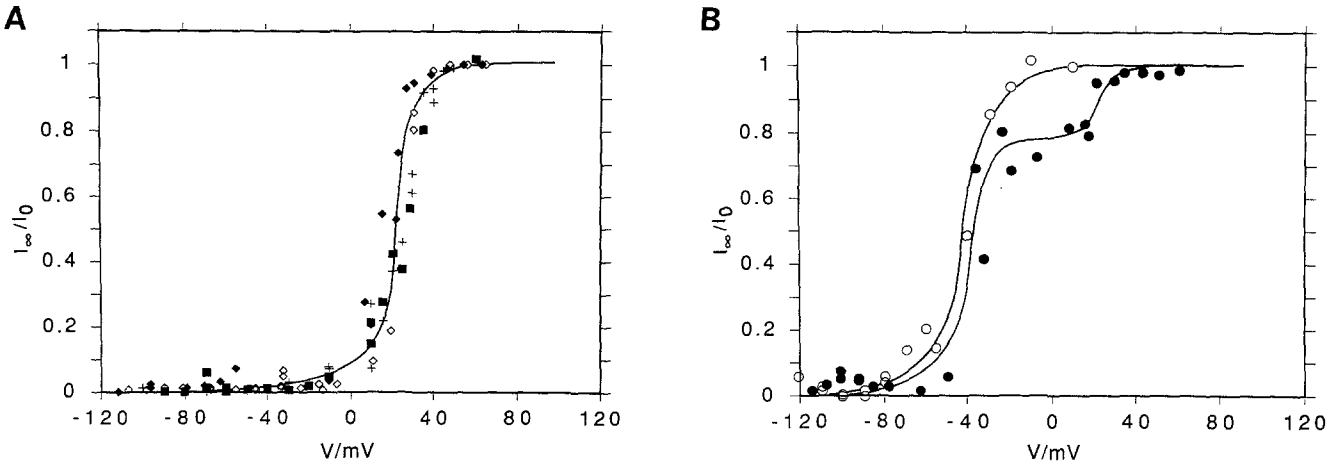


Fig. 2 A, B. The relation between I_∞ and I_0 as a function of voltage. I_∞ and I_0 were obtained by switching the membrane voltage to the desired value after the membrane had been clamped to a high positive voltage at which the membrane current was fully activated. **A** The curves are shown for pH 5 (♦), 6 (◇), 7 (■) and 8 (+). The mean value of V_0 is 21.5±3 mV at these pH values. When the data points are plotted in a linearized form according to the Boltzmann relation (Schein et al. 1978) a mean gating charge of $n=2.6$ can be derived from the slope of the straight lines (not shown). **B** The relation I_∞/I_0 at pH 4 (○) and pH 4.5 (●). The V_0-value of the pH 4.0 curve is about −47 mV. At pH 4.5 the data points cannot be fitted with a single sigmoidal curve. An intermediate plateau between −30 mV and +30 mV indicates that two sigmoidal curves of the "acidic" and the "basic" type are superimposed. The linearized plot of the data points at pH 4 yields a gating charge of $n=2.3$ (not shown)

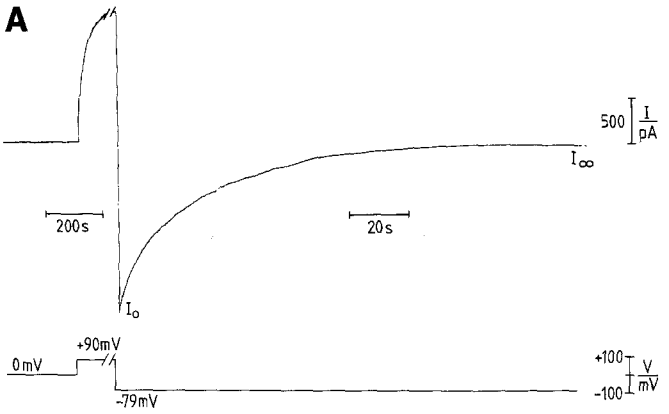
increase. The length of this lag-time depended on both the final protein concentration in the bath solution and the intensity of stirring. Often we observed that the current increased in a fairly linear fashion if the process was allowed to proceed at a constant voltage. Generally a current increase as shown in Fig. 1 could only be obtained when high positive voltages were applied (V > +60 mV). At lower voltages no constant current increase was observed.

When the membrane voltage was switched to −90 mV the membrane current decreased in a time-dependent fashion and reached a final steady state value which was close to the current baseline (Fig. 1). Under these conditions no net increase of the membrane current was observed. Two quantities can be derived directly from a current inactivation curve as shown in Fig. 1, an initial peak current value I_0 at the onset of the inactivation process and a final steady state current value I_∞. It should be pointed out here that the initial peak current at the negative voltage, I_0 (−90 mV), is lower than the current value which was reached at +90 mV immediately before the voltage step. The ratio between the corresponding macroscopic conductances λ_0 (−90 mV) and λ (+90 mV) is 1 : 1.65. Such an asymmetry of the macroscopic conductance of colicin N-containing membranes favouring the membrane current at positive membrane voltages was generally observed at pH-values ≥ 6.

After a voltage step back to +90 mV there was an instantaneous and rapid increase in current which after slowing down continued in a linear fashion. When the membrane voltage was switched to a lower positive value (e.g. +40 mV in Fig. 1), again a time-dependent inactivation may be seen, the degree of which was lower than that observed at −90 mV. Note that again a steady state current value was reached and no net increase of the membrane current appeared.

The relation I_∞/I_0 is a measure of the voltage-dependent inactivation of a current through a membrane containing colicin N. The degree of inactivation depended on both the membrane voltage and the pH of the bulk solution. This is clarified in Fig. 2 A and B. When I_∞/I_0 is plotted versus the membrane voltage, sigmoidal curves are obtained. They clearly show that the membrane current tends to inactivate almost completely towards negative voltage values as depicted in Fig. 1. At basic and slightly acidic pH values (pH ≥ 5), the voltage dependence is strongest between 0 mV and +40 mV, i.e. the curves are steepest in this voltage range (Fig. 2 A). In the following we define the position of these sigmoidal curves along the voltage axis by the so-called "switching voltage" V_0, i.e. the voltage at which 50% inactivation of the membrane current is obtained. In the pH-range between 5 and 8 the switching voltage of the I_∞/I_0-curves did not vary much, the mean value of V_0 at these pH values was +21.5± 3 mV (Fig. 2 A). However, at pH values lower than 5 a different behaviour was found (Fig. 2 B). At pH 4.0 the curve was shifted markedly towards the negative voltage range, the V_0-value being now about −47 mV. This indicates that two types of voltage-dependent inactivation processes of colicin N can be distinguished: a "basic" one which is observed at pH values ≥ 5 and an "acidic" one which becomes apparent at pH values ≤ 5. There is an intermediate pH-range between pH 4 and 5 in which the data points could not be described by a single sigmoidal curve alone. Figure 2 B exemplifies this for pH 4.5. The I_∞/I_0-curves in the pH-range between 4 and 5 typically showed an intermediate plateau and hence it appears that they are composed of a superposition of the "acidic" and the "basic" curve-types.

The data from Fig. 2 A and B can be replotted in an alternative, linearized form according to the Boltzmann relation (see e.g. Schein et al. 1978). In this way the data points for pH 4, 5, 6, 7 and 8 could be fitted well by straight lines (not shown), the slopes of which can be regarded as a direct measure of the voltage dependence of the inactivation process and which are generally referred



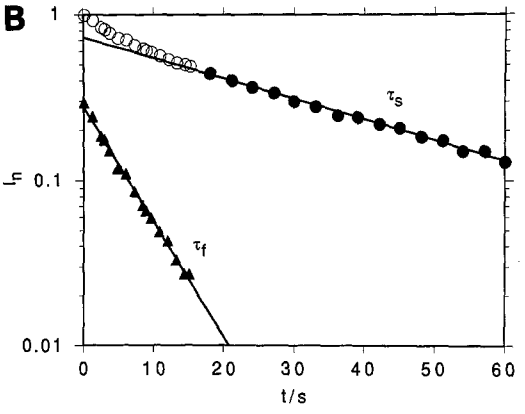
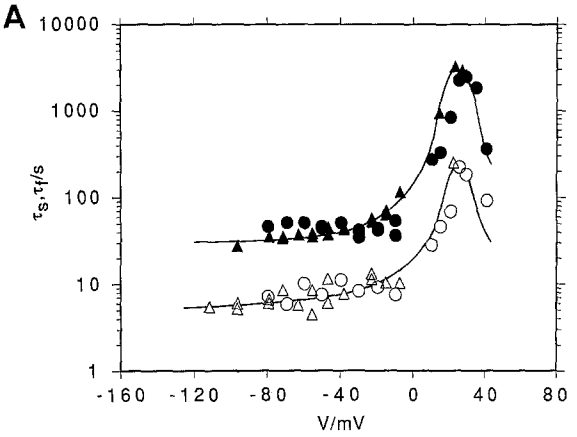


Fig. 3 A, B. The time course of the inactivating membrane current. **A** shows the original current trace recorded at pH 5.0. Note that the time scale of the trace changed when the voltage was switched to −79 mV. The ratio $\lambda(+90\text{ mV})/\lambda_o(-79\text{ mV})$ between the membrane conductances immediately before and after the voltage step is 1 : 1.5. **B** shows the time course of this current on a semi-logarithmic scale (circles). The current was normalized according to $I_n = [I(t)-I_0]/[I_\infty - I_0]$. The upper line is a least squares fit to the filled symbols, which gives a time constant $\tau_s = 34.8$ s. The lower line is a least squares fit to the residual current (triangles) which can be obtained as the difference between the normalized current and the experimental relaxation of time constant τ_s. This fit gives a second time constant $\tau_f = 6.3$ s



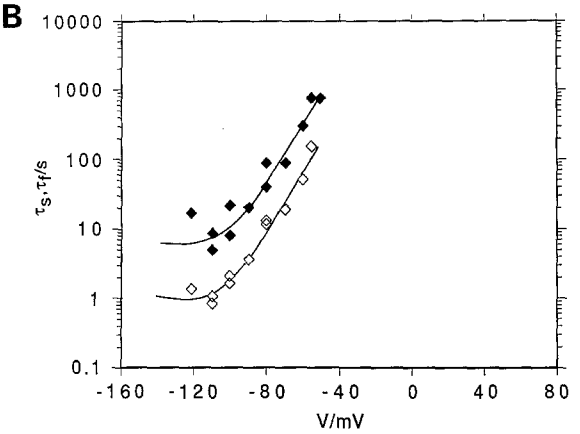


Fig. 4 A, B. The slow (τ_s) and the fast (τ_f) time constants of the macroscopic current inactivation as functions of membrane voltage. **A** shows τ_s (filled symbols) and τ_f (open symbols) at pH 5 (triangles) and pH 7 (circles). At negative voltages τ_f and τ_s were almost voltage-independent and reached a lower limit of about 40 s (τ_s) and 7 s (τ_f) respectively. At positive voltages both time constants display a pronounced voltage dependence and pass through a maximum at the switching voltage. **B** τ_s (filled symbols) and τ_f (open symbols) at pH 4.0. As compared to basic and neutral pH values (**A**) both curves are shifted along the voltage axis towards the negative voltage range. Because of the much longer time constants as compared to colicin A, entailing ageing of the lipid bilayer membrane and induction of new channels, it was not possible to collect more sufficient reliable data at voltages more positive than the switching voltage at this pH

to as "gating charge". Thus for the data points in Fig. 2 A an apparent mean gating charge of $n = 2.6$ could be deduced while at pH 4 (data points in Fig. 2 B) a value of $n = 2.3$ was obtained for the acidic inactivation process.

Kinetics of inactivation

Figure 3 A shows an example of the macroscopic membrane current through a colicin N-containing membrane after a switch to a negative membrane voltage. When the normalized current $I_n = [I(t) - I_\infty]/[I_0 - I_\infty]$ is plotted as a function of time on a semi-logarithmic scale it becomes obvious that the curve can be fitted well by two exponential functions with two time constants τ_f and τ_s (Fig. 3 B). This indicates that the inactivation process of the colicin N-induced membrane current comprises a "fast" and a "slow" component. We followed this approach to describe the time course of the inactivation current by two time constants since two exponential components appeared to fit the experimental data satisfactorily and we found a good reproducibility of such biphasic kinetics from one experiment to another.

Both time constants were controlled by the membrane voltage and the pH of the bulk solution, as demonstrated in Fig. 4 A and B. Here τ_s and τ_f are shown for pH 7, 5 and 4 respectively. As found with colicin A (Collarini et al. 1987), at pH values ≥ 5.0, τ_s and τ_f were almost constant at negative voltages (Fig. 4 A). At positive voltages, however, both time constants displayed a pronounced voltage dependence. The same behaviour was found at pH 6 and 8 (not shown). At pH 4.0, however, the curves of τ_s and τ_f were shifted along the voltage axis towards the negative voltage range (Fig. 4 B). At this pH a voltage dependence of both time constants was ob-

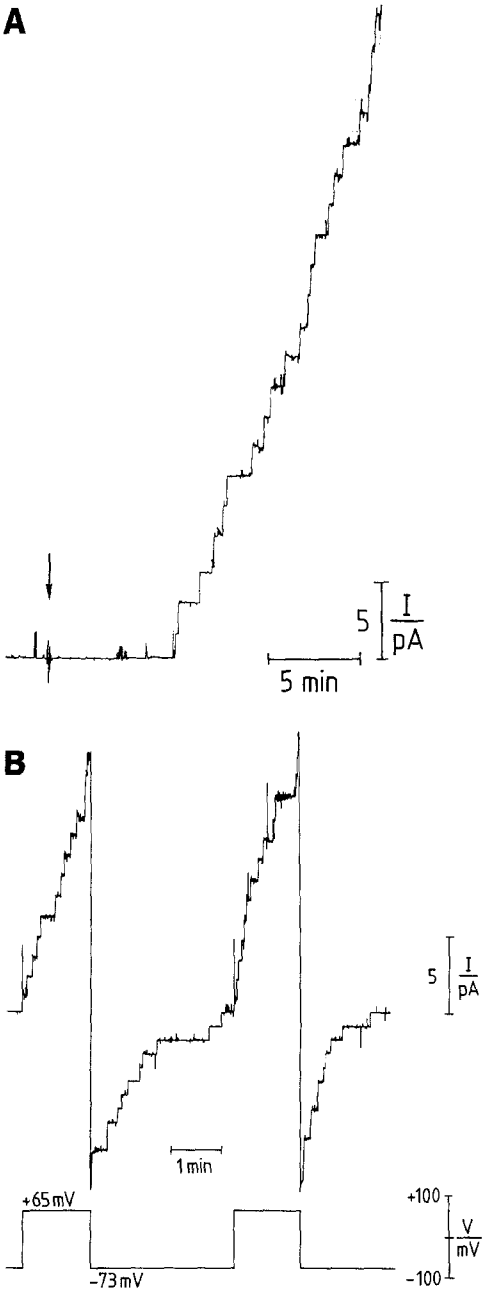


Fig. 5 A, B. The gating behaviour of single colicin N channels at a high current resolution. **A** The opening of single channels at a constant applied voltage of $V = +100$ mV. The arrow marks the application of colicin N to a final concentration of $6.4 \cdot 10^{-10}$ g/ml. After application the chamber was stirred with a magnetic bar which produced some current noise. Note that hardly any closing events could be observed. **B** The opening and closing events of single colicin N channels at positive (+65 mV) and negative (−73 mV) voltages. Note that the channels closed completely at −73 mV and that the current reached the baseline. The pH in this experiment was 7.0

served in the voltage range between −120 mV and −40 mV. Assuming that the voltage dependence of the time constants obeys the model described previously by Collarini et al. (1987), τ_f and τ_s should pass through a maximum at the switching voltage. This is apparent at pH 7.0 as shown in Fig. 4A. Generally, however, it was not possible to collect sufficient reliable data at voltages more positive than the switching voltage because of the extremely long time constants obtained with colicin N as compared to colicin A, involving problems of induction of new channels and ageing of the lipid bilayer membrane (Fig. 4B).

At pH values between 4 and 5 the time courses of membrane currents appeared to be more complex. They could not be fitted satisfactorily with two time constants alone when plotted on a semilogarithmic scale and we do not consider them here.

Both time constants were independent of the holding voltage applied before the step to a lower inactivating voltage.

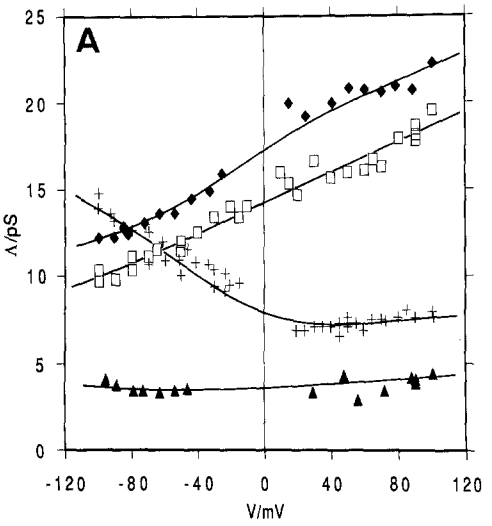
Single-channel characteristics

When low concentrations of colicin N were applied to the cis-chamber ($3-8 \cdot 10^{-10}$ g/ml) the occurrence of steplike current increases could be observed at a high current resolution (Fig. 5A). This is clear evidence that colicin N formed discrete ion-conducting pathways through planar lipid bilayers. It is also obvious from Fig. 5A that opening events prevailed at a high positive voltage and closing steps were rather rare events. Thus under these conditions colicin N channels preferred the open state once they had opened. At negative voltages, however, colicin N channels reacted in the opposite way. This is depicted in Fig. 5B in which a positive and a negative membrane voltage were applied alternately. At the positive voltage the steplike current increase continued whereas the channels closed completely when the negative voltage was applied.

The single-channel conductance Λ of colicin N channels depended markedly on both the membrane voltage and the pH of the bulk solution. This is shown in Fig. 6A. At basic and neutral pH values (pH 9 and 7) Λ increased with the applied membrane voltage, i.e. Λ was higher at positive than at negative voltages. At pH 5.0 this voltage dependence reversed. Now Λ was higher at negative than at positive voltages. Thus colicin N channels did not behave like ideal ohmic resistors at these pH values but rather showed a rectification behaviour the direction of which was pH-dependent. When the pH was decreased further to pH 4, Λ became fairly constant with voltage and the distinct rectification characteristic was lost.

It is also obvious from Fig. 6A that the single-channel conductance Λ decreased with the pH of the bulk solution. This is demonstrated by the single-channel conductances at 0 mV, Λ_0, which can be derived from single-channel I-V curves like those shown in Fig. 6A as intersections with the y-axis. Figure 6B shows Λ_0 as a function of pH. At acidic pH-values Λ_0 approached a value of about 4 pS as a lower limit and increased with rising pH values. In the basic pH-range no clear saturation value of Λ_0 could be obtained up to pH 10.

Besides being a function of the membrane voltage and the pH the single-channel conductance Λ was dependent on the electrolyte used in the experiments. This is shown in Table 1 in which Λ_0 is listed for different chloride salts. Λ_0 was maximum with NH_4^+ as cation, while the lowest value was obtained with Cs^+.



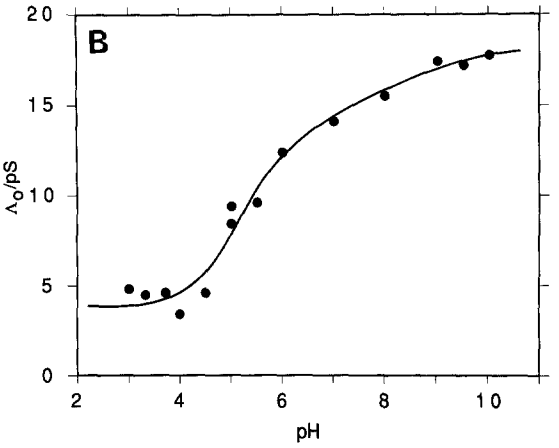


Fig. 6. A The single-channel conductance Λ at four different pH values as a function of voltage. The symbols are: ◆ = pH 9; □ = pH 7; + = pH 5; ▲ = pH 4. At pH 9 and 7, Λ increased towards positive membrane voltages while at pH 5 the reversed characteristic was observed. At pH 4, Λ is almost constant and independent of voltage. **B** The single-channel conductance of colicin N at 0 mV, Λ_0, as a function of pH. At pH-values <4 Λ_0 approaches a lower limit of 4 pS. In the basic pH-range no clear saturation value is obtained up to pH 10. Λ_0-values were obtained from single-channel I-V curves like those shown in **A** as intersections with the *y*-axis

Table 1. The single-channel conductances of colicin N at 0 mV, Λ_0, in 1-M solutions of different chloride salts. Λ_0-values were obtained from single-channel I-V curves like those shown in Fig. 6 A. At the pH-value used (pH 7) the data points could be fitted well by linear regression in the voltage range between −110 mV and +110 mV. Λ_0 was then calculated as the intersection of the theoretical curve with the *y*-axis. For each salt a total of 350–600 single-channel events were evaluated

Cation	Λ_0/pS
NH_4^+	22.7±0.9
Na^+	14.2±0.6
K^+	13.6±0.7
Li^+	12.4±0.2
Cs^+	8.7±0.4

Table 2. The selectivity ratios between Na^+ and Cl^- in membranes of different composition and at different pH. The ratios were calculated according to the Goldman-Hodgkin-Katz equation (see text). In neutral membranes composed of the synthetic phospholipids POPC and DOPE a shift from cation-selectivity to anion-selectivity was observed when the pH was lowered from 7 to 5. In membranes formed of a natural mixture of soybean phospholipids, a higher cation selectivity was obtained at pH 7 as compared to neutral membranes. Unlike in POPC-DOPE membranes the channel retained its cation selectivity when the pH was lowered to 5

Lipid	Permeability ratio $Na^+ : Cl^-$	
	pH 7	pH 5
POPC/DOPE	2.3 : 1	1 : 2.5
soybean phospholipid	7.0 : 1	1.5 : 1

Interaction with neutral lipid bilayers and ion selectivity

It was our intention to characterize the ion selectivity of colicin N channels in bilayers made from a natural mixture of phospholipids as well as in bilayers which were composed of synthetic phospholipids and which bore no electric net surface charge. To form neutral planar lipid bilayer membranes we employed the method described by Montal and Mueller (1972; see Material and methods). When colicin N was applied to neutral membranes the observed macroscopic current increases were roughly at least 10 times lower than in experiments with soybean phospholipids at identical colicin N concentrations while the single-channel conductance was not changed much (not shown). This implies that the incorporation rate of colicin N into neutral lipid bilayers was markedly lower than that observed with membranes composed of soybean phospholipids.

The ion selectivity of colicin N was determined using a 10-fold concentration gradient of NaCl across the membrane which corresponds to about an 8.4-fold gradient in ion activity. Depending on the ion selectivity of the colicin N channel a reversal potential E_{rev} developed across the membrane which is given by the Goldman-Hodgkin-Katz equation:

$$E_{rev} = \frac{RT}{F} \ln \frac{b\,\alpha^c_{Na} + \alpha^t_{Cl}}{b\,\alpha^t_{Na} + \alpha^c_{Cl}} \qquad (1)$$

where b is the ratio P_{Na}/P_{Cl} between the permeabilities of the cation and the anion. α^c and α^t denote the activities of the anion and the cation on the *cis*- and *trans*-side respectively. E_{rev} was determined as that voltage at which the net current became zero when a slow triangular wave voltage was applied. E_{rev} was found to depend on both the membrane lipid used and the pH of the bulk solution (Table 2). In neutral lipid bilayers which consisted of POPC and DOPE at a ratio of 1 : 1, colicin N channels showed a higher permeability for Na^+-ions than for Cl^--ions at pH 7. At pH 5 the selectivity reversed, the channels being slightly more selective for Cl^--ions than for Na^+-ions.

In bilayers made of soybean phospholipids the channel's preference for Na^+ to Cl^- at neutral pH was even more pronounced as compared to neutral membranes judged by the reversal potentials. At pH 5 the selectivity ratio between Na^+ and Cl^- decreased but, unlike in neutral membranes, did not reverse.

Discussion

The results presented in this study show clearly that colicin N creates well-defined pathways for ions through lipid bilayers and thus belongs to the channel-forming class of colicins as was suggested on the basis of sequence homology of its C-terminal domain with that of colicins of the E1-class and earlier in vivo studies (Pugsley 1984c; Pugsley 1987).

The induction of colicin N channels proved to be voltage-dependent since an increase in membrane conductance occurred only at positive voltages higher than a threshold voltage of +60 mV. On the other hand, no current increase was observed when negative and low positive membrane voltages were applied at pH values ≥ 5. Very similar effects have been described for a series of channel-forming colicins such as colicin A, B, E1, Ia, Ib and K (Schein et al. 1978; Weaver et al. 1981; Bullock et al. 1983; Pattus et al. 1983; Nogueira and Varanda 1988). It should be emphasized at this point that such a voltage-dependent induction of channel activity is clearly different from the voltage-dependent gating process of incorporated channels (see below). This becomes apparent from the fact that the latter process is characterized by switching voltages V_0 which are distinctly different from the threshold voltage for channel induction and which characterize the reversible gating of the colicin channel between the open and closed states.

Recently a model for the insertion of the C-terminal fragment of colicin A into lipid bilayers was proposed which is based on its 3-dimensional structure obtained from X-ray crystallography (Parker et al. 1989; Parker, personal communication). According to this model the interaction of colicin comprises two steps: binding to the membrane surface and insertion of the protein into the hydrophobic core of the bilayer. The first step is probably of electrostatic nature, requiring the interaction of a ring of positively charged residues on one face of the protein with a negatively charged membrane surface. The second step involves the insertion of the hydrophobic hairpin, initially buried inside the structure, into the lipid bilayer. Both steps may be voltage-independent and lead to a closed state of the channel with most of the amphipathic helices lying at the surface of the membrane. A positive membrane voltage could be necessary to insert amphipathic alpha-helical portions of the C-terminal domain in the membrane which may be followed by oligomerization of the peptide as a final step to form channels (Parker et al. 1989).

It is tempting to assume that colicin N inserts into lipid bilayers in a similar manner since its C-terminal domain reveals a high degree of sequence homology with that of colicin A and it may thus possess a similar structure. Our observations are in line with the hypothesis that a negatively charged membrane surface may accelerate channel incorporation since we observed a considerably lower insertion rate of colicin N in membranes which bore no net surface charge.

As illustrated by the sigmoidal I_∞/I_0-curves in Fig. 2 the membrane conductance attained its maximum value at membrane voltages much more positive than the switching voltage while it could be switched off almost completely towards much more negative membrane voltages. Viewing the behaviour of colicin N on the single-channel level (Fig. 5 B) it becomes obvious that this macroscopic conductance-voltage relation reflects an inherent voltage-dependent gating of the channel: single colicin N channels adopt the open state at positive membrane voltages while they can be caused to close entirely at negative membrane voltages thus enhancing or reducing the membrane conductance.

On the basis of this voltage-dependent gating behaviour we could clearly distinguish two types of colicin N channels. A "basic" channel predominates at pH values ≥ 5, while an "acidic" one becomes apparent at pH values ≤ 4. The channel types are distinguished by their different switching voltages (V_0). In an intermediate pH-range ($4 < pH < 5$) a superposition of the two curves is observed, indicating that both types of colicin N channel coexist in comparable amounts under these conditions.

Qualitatively the same voltage- and pH-dependent gating behaviour has been described for the native colicin A channel (Collarini et al. 1987). Like colicin N it can also adopt an acidic (Colicin A_A) or basic form (Colicin A_B) which are distinguished by different switching voltages V_0. These findings confirm our hypothesis that the channel structures of colicin A and N may be related due to the high identity of their amino acid sequence (Pattus et al. 1985; Pugsley 1987; Parker et al. 1989). In contrast, the thermolytic fragment of colicin A displays only one type of voltage-dependent gating with one switching voltage over the whole pH-range (Collarini et al. 1987). On the basis of this result, together with others on colicin A deletion mutants (Frenette et al. 1989), it was proposed that the channel-forming domain and the receptor domain of colicin A interact at neutral pH and that this interaction is disrupted at acidic pH. The fact that there are two distinguishable channel types indicates that a similar intramolecular interaction exists for colicin N, the formation and disruption of which leads to two different conformations of the protein molecule. A titration of one or more amino acid residues may be involved in this mechanism (Frenette et al. 1989), the pK of which may be even closer to 4 in the case of colicin N as compared to colicin A. In contrast, colicin Ia, the amino acid sequence of which bears little resemblance with that of colicin A and N, does not display a one-step transition between two clearly distinguishable channel types. In this case the I_∞/I_0-curves rather shift continuously along the voltage axis when the pH is varied (Nogueira and Varanda 1987).

The time course of the current inactivation could be fitted satisfactorily by two exponential components with different time constants τ_f and τ_s. Both time constants

were dependent on the membrane voltage and the pH of the aqueous solution. Like the gating characteristics (see above) the voltage- and pH-dependence of τ_f and τ_s reveals that the colicin N channel can adopt two different channel types. They differ in that the slow and the fast time constant of the basic channel type show a pronounced voltage dependence in the positive voltage range whereas τ_f and τ_s of the acidic channel type display a steep voltage dependence at negative voltages (Fig. 4).

As compared to the inactivation kinetics of colicin A, the time constants of colicin N are substantially longer. In the case of the basic channel type of colicin A a maximum time constant of about 100 s was observed (Collarini et al. 1987), whereas for colicin N values of τ_s were obtained which were as high as 3000 s and more. Thus the rate of inactivation of colicin N is 1–2 orders of magnitude slower than that of colicin A.

The fact that the time course of inactivation can be described by two exponential components indicates that the channel may pass through more than two different channel states during the closing reaction (Chiu 1977; Colquhoun and Hawkes 1977). This biphasic kinetic behaviour is apparent in the acidic as well as in the basic channel type, implying that both share a common gating mechanism by which they open and close. However, at present we lack sufficient single-channel data to suggest an elaborate kinetic model of the colicin N channel.

The single-channel conductance Λ of colicin N depends on the pH as well as on the membrane voltage. In the pH-range between 3 and 10, Λ_0 increases with pH by a factor of about 4.5, and up to pH 10 no clear saturation was reached. Such a broadened pH-dependence indicates that more than one titratable residue within the channel lumen or at the channel mouth control the entry and passage of ions into and through the channel and thus the single-channel conductance. The single-channel properties of colicin A and N are similar inasmuch as both channels show voltage-dependent single-channel conductances at pH values ≥ 5. Both colicins have in common that at basic and slightly acidic pH values the single-channel conductance increases towards positive voltages while it decreases towards negative voltages. This phenomenon is pronounced at basic pH values (Collarini et al. 1987). In the case of colicin N this asymmetry reverses at pH 5.0, Λ being now larger at negative voltages. Recently a similar non-ohmic single-channel conductance has been reported for colicin B (Pressler et al. 1986). Conversely, single colicin Ia channels have a rather linear current-voltage relationship (Nogueira and Varanda 1988).

The non-ohmic single-channel conductance Λ of the colicin N channel accounts well for the asymmetry in the macroscopic membrane conductance λ which was observed in voltage step experiments (see Fig. 1 and Fig. 3 at pH 7 and 5 respectively). If $\lambda(V_x)$, $\lambda(V_y)$ and $\Lambda(V_x)$, $\Lambda(V_y)$ denote the macroscopic and single-channel conductances at two membrane voltages V_x and V_y, the simple relation

$$\frac{\lambda(V_x)}{\lambda(V_y)} = \frac{\Lambda(V_x)}{\Lambda(V_y)} \qquad (2)$$

holds if the numbers of open channels contributing to the macroscopic conductance are equal. As an example, in Fig. 1 a ratio of 1.65 : 1 between the macroscopic conductances at +90 mV and −90 mV was observed. This agrees well with a corresponding ratio of 1.72 : 1 between the single-channel conductances at +90 and −90 mV which can be derived from Fig. 6A. Likewise the voltage dependence of the single-channel conductances at pH 5 can account for the reversed asymmetry of the macroscopic conductances at this pH (i.e. higher conductances at negative membrane voltages, see Fig. 3A). In this case a ratio of 1 : 1.5 (macroscopic conductances) corresponds well with a ratio of 1 : 1.6 between the single-channel conductances derived from Fig. 6A. These examples illustrate a good agreement between single-channel and multi-channel data of colicin N.

Among the monovalent cations investigated, Λ_0 was maximum with NH_4^+-ions, whereas a minimum value of Λ_0 was observed with Cs^+-ions. Similar sequences have been reported in the case of colicin A and B (Pressler et al. 1986; F. Pattus, unpublished results). This is again an indication that colicins, which share a high degree of sequence identity, may show a very similar channel architecture.

The phospholipid environment as well as the pH control the ion selectivity of the colicin N channel. In membranes bearing no net surface charge colicin N is slightly more permeable for monovalent cations than for anions (Na^+ and Cl^-) when the pH of the medium is neutral. At pH 5 this ratio reverses, the channel being more permeable to anions. This indicates that titratable groups, the pK of which lie between these two pH values, are involved in the regulation of the cation/anion selectivity. Protonation of these residues may lead to a change of the charge at the channel mouth or in the channel lumen, thus favouring the permeability of anions.

The presence of charged phospholipids in the bilayer may affect the entry of ions into the colicin N channel. This becomes apparent from the reversal potentials obtained in membranes made from soybean phospholipids, which contain about 20% negatively charged lipid (Raymond et al. 1985). In these membranes the preference of the channel for cations appears to be higher as compared to neutral membranes. This is conceivable since a negatively charged surface leads to an enhancement of the local cation concentration (and a corresponding lowering of the anion concentration) at the membrane and thus to an increased entry of cations into the channel lumen. Hence the mouth of the colicin N channel should be located in this layer of enhanced cation concentration and may not project too far into the free bulk solution.

A similar dependence of ion selectivity on pH and surface charge has been observed for the colicin E1-channel (Raymond et al. 1985). In this case the authors could show that titratable residues within the channel lumen as well as at the channel ends contribute to the ion selectivity.

Conclusion

The properties of colicin N channels show a great resemblance to that of the biochemically related colicins A and B, implying that the high correspondence in their amino acid sequence brings about a common channel design.

If we regard the considerable difference between the molecular sizes of colicin N and A it appears surprising at first glance that both share a great similarity in their channel characteristics. A plausible explanation of this discrepancy is that the channel-forming properties of both colicins are restricted to particular domains of the molecule. It has been shown in fact that the potency of colicin A to form transmembrane channels resides exclusively in its C-terminal part (Baty et al. 1985; Collarini et al. 1987).

The high sequence homology between the C-terminal parts of colicin N and A accounts for the strong similarities between the properties of both channels. They have in common a voltage- and pH-controlled gating mechanism as well as a pH-dependent and non-ohmic single-channel conductance. Furthermore, and most characteristically, both channels are able to adopt either a basic or an acidic channel type.

There exist some differences between the channel characteristics of colicin A and N such as the slower inactivating kinetics of colicin N and slightly differing switching voltages of the basic and acidic channel types. However, none of the channel characteristics studied so far can account satisfactorily for the two main differences observed in the mode of action of colicin N in vivo as compared to other colicins, i.e. the inhibition by Mg^{2+}-ions and the cytolytic activity at moderate and high protein concentrations (Pugsley 1987). Mg^{2+}-ions do not affect the colicin N channel properties in vitro (data not shown). This implies that Mg^{2+}-ions may affect either receptor binding or translocation through the cell envelope. As discussed by Pugsley (1987), the cytolytic activity of colicin N is a late event and may probably be due to the high density of the colicin N receptor (OmpF-porin) at the cell surface and consequently the entry of many more colicin N molecules than the other colicins which bind to minor receptor proteins.

Considering the sequence homology of colicin A and N, differences in their channel characteristics may be due to differences within narrow parts of their primary structure. Hence it appears as a challenging future objective for us to detect these essential parts within the amino acid sequences.

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References

Baty D, Knibiehler M, Verheij H, Pattus F, Shire D, Bernadac A, Lazdunski C (1985) Site-directed mutagenesis of the COOH-terminal region of colicin A: Effect on secretion and voltage-dependent channel activity. Proc Natl Acad Sci USA 84:1152–1156

Bullock JO, Cohen FS, Dankert JR, Cramer WA (1983) Comparison of the macroscopic and single-channel conductance properties of colicin E1 and its COOH-terminal tryptic peptide. J Biol Chem 258:9908–9912

Cavard D, Lazdunski C (1979) Purification and molecular properties of a new colicin. Eur J Biochem 96:519–524

Chiu SY (1977) Inactivation of sodium-channels: second order kinetics in myelinated nerve. J Physiol 273:573–596

Cleveland MvB, Slatin S, Finkelstein A, Levinthal C (1983) Structure-function relationship for a voltage-dependent ion channel: Properties of COOH-terminal fragments of colicin E1. Proc Natl Acad Sci USA 80:3706–3710

Collarini M, Amblard G, Lazdunski C, Pattus F (1987) Gating processes of channels induced by colicin A, its C-terminal fragment and colicin E1 in planar lipid bilayers. Eur Biophys J 14:147–153

Colquhoun D, Hawkes AG (1977) Relaxation and fluctuations of membrane currents that flow through drug-operated channels. Proc R Soc London B 199:231–262

Frenette M, Knibiehler M, Baty D, Geli V, Pattus F, Verger R, Lazdunski C (1989) Interactions of colicin A domains with phospholipid monolayers and liposomes: relevance to the mechanism of action. Biochemistry 28:2509–2514

Hanke W (1986) Incorporation of ion channels by fusion. In: Miller C (ed) Ion channel reconstitution. Plenum Press, New York London, pp 141–153

Kagawa Y, Racker E (1971) Partial resolution of the enzymes catalyzing oxidative phosphorylation: XXV. Reconstitution of particles catalyzing $^{32}P_i$-adenosin triphosphate exchange. J Biol Chem 246:5477–5487

Konisky J (1982) Colicins and other bactericins with established modes of action. Ann Rev Microbiol 36:125–144

Lazdunski CJ, Baty D, Geli V, Cavard D, Morlon J, Lloubes R, Howard SP, Knibiehler M, Chartier M, Varenne S, Frenette M, Dasseux J-L, Pattus F (1988) The membrane channel-forming colicin A: synthesis, secretion, structure, action and immunity. Biochim Biophys Acta 947:445–464

Mankovich JA, Hsu C, Konisky J (1986) DNA and amino acid sequence analysis of structural and immunity genes of colicins Ia and Ib. J Bacteriol 168:228–236

Montal M, Mueller P (1972) Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. Proc Natl Acad Sci USA 69:3561–3566

Morlon J, Lloubes R, Varennne S, Chartier M, Lazdunski C (1983) Complete nucleotide sequence of the structural gene for colicin A, a gene translated at non-uniform rate. J Mol Biol 170: 271–285

Nogueira RA, Varanda WA (1988) Gating properties of channels formed by colicin Ia in planar lipid bilayer membranes. J Membr Biol 105:143–153

Parker MW, Pattus F, Tucker AD, Tsernoglou D (1989) Structure of the membrane-pore-forming fragment of colicin A. Nature 337: 93–96

Pattus F, Cavard D, Verger R, Lazdunski C, Rosenbusch J, Schindler H (1983) Formation of voltage dependent pores in planar bilayers by colicin A. In: Spach G (ed) Physical chemistry of transmembrane ion motions. Elsevier, Amsterdam, pp 407–413

Pattus F, Heitz F, Martinez C, Provencher SW, Lazdunski C (1985) Secondary structure of the pore forming colicin A and its C-terminal fragment. Eur J Biochem 152:681–689

Pattus F, Massotte D, Wilmsen HU, Lakey J, Tsernoglou D, Tucker A, Parker M (1990) Colicins: Procaryotic killer-pores. Experientia (in press)

Pressler R, Braun V, Wittmann-Liebold B, Benz R (1986) Structural and functional properties of colicin B. J Biol Chem 261: 2654–2659

Pugsley AP (1984a) The ins and outs of colicins. Part 1: production and translocation across membranes. Microbiol Sci 1:168–175

Pugsley AP (1984b) The ins and outs of colicins. Part 2: lethal action, immunity and ecological implications. Microbiol Sci 1: 203–205

Pugsley AP (1984c) Genetic analysis of col N plasmid determinants for colicin production, release and immunity. J Bacteriol 158: 523–529

Pugsley AP (1987) Nucleotide sequencing of the structural gene for colicin N reveals homology between the catalytic C-terminal domains of colicins A and N. Mol Microbiol 1:317–325

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

Schein SJ, Kagan BL, Finkelstein A (1978) Colicin K acts by forming voltage dependent channels in phospholipid bilayer membranes. Nature 276:159–163

Schindler H, Feher G (1976) Branched bimolecular lipid membranes. Biophys J 16:1109–1113

Schramm E, Mende J, Braun V, Kamp RM (1987) Nucleotide sequence of the colicin B activity gene cba: consensus pentapeptide among TonB-dependent colicins and receptors. J Bacteriol 169: 3350–3357

Tommassen J, Pugsley AP, Korteland J, Verbakel J, Lugtenberg B (1984) Gene encoding a hybrid OmpF-PhoE pore protein in the outer membrane of *Escherichia coli* K12. Mol Gen Genet 197: 503–508

Tucker AD, Pattus F, Tsernoglou D (1986) Crystallization of the C-terminal domain of colicin A carrying the voltage-dependent pore activity of the protein. J Mol Biol 190:133–134

Varley JM, Boulnois GJ (1984) Analysis of a cloned colicin Ib gene: complete nucleotide sequence and implications for regulation of expression. Nucl Acids Res 12:6727–6739

Weaver CA, Kagan BL, Finkelstein A, Konisky J (1981) Mode of action of colicin Ib. Formation of ion-permeable membrane channels. Biochim Biophys Acta 645:137–142

Yamada M, Ebina Y, Miyata T, Nakazawa T, Nakazawa A (1982) Nucleotide sequence of the structural gene for colicin E1 and predicted structure of the protein. Proc Natl Acad Sci USA 79:2827–2831