Involvement of colicin in the limited protection of the colicin producing cells against bacteriophage

Yu-Hui Lin, a,1 Chen-Chung Liao, a,1 Po-Huang Liang, b Hanna S. Yuan, c and Kin-Fu Chak a,∗

a Institute of Biochemistry, University System of Taiwan–National Yang Ming University, Shih-Pai, Taipei 11221, Taiwan, ROC
b Institute of Biological Chemistry, Academia Sinica, Taipei 11529, Taiwan, ROC
c Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan, ROC

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Abstract

The restriction/modification system is considered to be the most common machinery of microorganisms for protection against bacteriophage infection. However, we found that mitomycin C induced Escherichia coli containing ColE7-K317 can confer limited protection against bacteriophage M13K07 and λ infection. Our study showed that degree of protection is correlated with the expression level of the ColE7 operon, indicating that colicin E7 alone or the colicin E7-immunity protein complex is directly involved in this protection mechanism. It was also noted that the degree of protection is greater against the single-strand DNA bacteriophage M13K07 than the double-strand bacteriophage λ. Coincidently, the $K_a$ value of ColE7-Im either interacting with single-strand DNA ($2.94 \times 10^5$ M$^{-1}$) or double-strand DNA ($1.75 \times 10^5$ M$^{-1}$) reveals that the binding affinity of ColE7-Im with ssDNA is 1.68-fold stronger than that of the protein complex interacting with dsDNA. Interaction between colicin and the DNA may play a central role in this limited protection of the colicin-producing cell against bacteriophages. Based on these observations, we suggest that the colicin exporting pathway may interact to some extent with the bacteriophage infection pathway leading to a limited selective advantage for and limited protection of colicin-producing cells against different bacteriophages.

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Colicins are a group of plasmid-encoded bacteriocins, which are active against Escherichia coli and its closely related enterobacteria [1–3]. Interestingly, colicin plasmids (Col plasmids) are found in 30% of naturally occurring populations of E. coli [4]. Recently, Feldgarden et al. [5] indicated that possession of a colicin plasmid can confer limited protection against T bacteriophage infection. The authors suggest that the phenomenon of limited bacteriophage resistance might play an important role in maintaining the high-level population of plasmid-containing cells in natural environment. The studies indicate that the specific function providing limited protection against bacteriophage infection is carried on the plasmid [6,7]. However, the mechanism by which the Col plasmid confers this protection against bacteriophages is not known. Some limited protection mechanisms against bacteriophage infection have been well documented. The restriction/modification system [8,9] is the best known of bacteriophage defense mechanisms and is encoded by many plasmids found in, for example, the lactic Streptococci [10,11] and in E. coli [12]. Other mechanisms include resistance induction [13], superinfection exclusion [14], and abortive infection [15].

The E group colicins are released from producing cells and gain access into target cells by parasitizing receptors, such as the vitamin B12 receptor (BtuB) [16] whose primary purpose is the uptake and transport of essential solutes and metabolites. BtuB also acts as the receptor for adsorption of the bacteriophages BF23,
which is closely related to phage T5 [17]. After receptor binding, a group of membrane proteins, the Tol and Ton systems, are required for translocation of the colicin across the membrane of E. coli [18]. The Tol (for tolerant) system is required for the group A colicins (A, E1–E9, K, L, N, bacteriocin 28b, and cloacin DF13) [19,20], and the Ton system is required for the group B colicins (B, D and Ia, M, and V) [21]. M13 bacteriophage is one of the members of filamentous bacteriophages, which only infect gram-negative bacteria and specifically adsorb to the tip of pili [22]. This interaction is initiated by the specific binding of the bacteriophage, via the phage gene III protein (g3p), to the tip of the F conjugative pilus. After F-pilus retraction, the first N-terminal domain of g3p binds to the C-terminal domain of bacterial TolA. Consequently, the TolA can bring the outer and inner membranes of the bacteria closer together facilitating the translocation of the phage DNA genome into the cytoplasm of a host cell [23]. The finding clearly indicates that both bacteriophage and colicin gain entry to the host cells by parasitizing the host receptors and translocation machinery such as the Tol system.

In this work, we found that expression of ColE7 operon in E. coli can confer limited protection against bacteriophage M13K07 and λ infection. Moreover, our studies showed that the degree of protection is directly related to the expression level of the ColE7 operon. Thus, this result clearly indicates that colicin or colicin in complex with the Im protein plays a central role in this limited protection process. A study of the binding affinity of ColE7-Im for single-strand and double-strand DNA using a BLAcore reveals a possible mechanism for the limited protection of cells harboring ColE7-plasmid against bacteriophage.

Materials and methods

Bacterial strains, media, and construction of plasmids. E. coli JM109 F' was used as the host strain for subcloning, expression, and bacteriophage infection. The expression plasmid pQE70 (Qiagen) was used for the over-expression of the ColE7-Im protein complex. M13K07 and λ bacteriophages were purchased from Institute of Food Industry Research and Development, Shin-Chu, Taiwan. Cultures were routinely grown in Luria–Bertani (LB) broth or on plates of LB agar and were supplemented, where required, with ampicillin (100 μg ml⁻¹), LB broth with 0.2% maltose and 10 mM MgSO₄ was used for the propagation of λ bacteriophage.

Construction of a vector for expression of the ColE7-Im complex. The plasmid pHK001, containing a completed ColE7 operon, was used as a template for polymerase chain reaction (PCR) amplification. A 2043 base pair (bp) DNA fragment containing the entire cea-ceiE7 gene of the ColE7 operon [24] was amplified by PCR using a pair of oligonucleotide primers: Col7-701 (5'-GAATTTGCATGCGCGGTGG3') and Col7-702 (5'-CAATACGATGCTGCCCTGTT3'). SpI and BglII sites were generated at either end of the PCR-amplified fragment. PCRs were carried out in 100 μl volumes using 1 U Taq polymerase with 30 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min. The PCR-amplified fragment was then cleaved with SpI and BglII and ligated into the expression vector pQE70 incubated at the same restriction sites. The resulting recombinant plasmid, pQE70 cea-ceiE7, was transformed into E. coli JM109.

Infection experiments. Bacteria were grown in LB broth at 37°C to an absorbance at 600 nm of 0.8 (~10⁶ CFU ml⁻¹). The expression of protein was induced with 0.1 mM IPTG for 0.5, 1, 5, 2.5, and 3.5 h or induced with mitomycin C (0.5 μg ml⁻¹) for 30 min. Thereafter, phage particles were added to the bacterial cells at multiplicity of infection (MOI) of 0.01 and allowed to infect for 20 min at 37°C. The samples were plated in soft agar on LB plates. The plates were incubated overnight at 37°C. The number of plaques forming units (PFU) for each of the bacteria was determined by counting the PFU on each plate. The experiments were performed in triplicate.

Purification of ColE7-Im protein complex. Overnight cultures (10 ml) of JM109 (pQE70 cea-ceiE7) were diluted into 1 L LB and incubated with shaking at 37°C for 4–5 h. IPTG was added to a final concentration of 1 mM to induce the expression of the ColE7-Im complex for 3 h. Cells were harvested and disrupted by sonication as described in Liao et al. [25]. Ni-NTA resin affinity column (Qiagen, Germany) was equilibrated with sonication buffer (50 mM sodium phosphate buffer, pH 7.8, and 300 mM NaCl) before being loaded with the crude cell extracts (150 ml). After loading, the column was washed once with washing buffer (50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl, and 75 mM imidazole) to remove the unbound protein. The bound protein was then eluted with 200 mM imidazole gradient solution from 75 to 500 mM.

Western blotting hybridization. Proteins to be tested were resolved by SDS–PAGE. The gel was then transferred to a PVDF membrane by using a semidyed blotting apparatus (American Bionetics). After blocking, the membrane was hybridized with polyclonal antibodies raised against colicin E7 followed by anti-IgG antibodies conjugated with horseradish peroxidase. Protein bands interacting with antibodies were visualized by treating the membrane with NEN Western blot chemiluminescence reagent plus. The detailed procedure for hybridization was the same as described in the NEN Western blot manual.

DNA substrates. One 5’-biotin-labelled 31 mer single-strand oligonucleotide (5’-biotin-GCATCAACGACGCATGGACTGATA CGAAAG-3’) and one unlabelled 31 mer oligonucleotide (complementary strand) were synthesized and purified by HPLC. To facilitate annealing of the oligonucleotides to form duplex, equimolar amounts of oligonucleotides were mixed together in water. The mixtures were incubated at 100°C for 5 min and allowed to cool slowly to room temperature.

Analysis of DNA–protein interaction by surface plasmon resonance. The interaction between DNA and protein was measured by SPR using the BLAcore X system. The SA sensor chips were first equilibrated with running buffer (10 mM Tris–HCl, pH 7.4, and 100 mM NaCl) for 6 min and then activated with three consecutive 2 min injections of 40 μl activating buffer (1 M NaCl and 30 mM NaOH) prior to the immobilization. The oligonucleotide was diluted in running buffer and 30 μl was injected with a flow rate of 2 μl min⁻¹. The immobilization procedure was carried out at 25°C and a constant flow rate of 30 μl min⁻¹. Blank running buffer was subtracted in the evaluation of the experimental sensorgrams. All buffers were filtered and degassed before use. Then, a 15 μl solution of ColE7-Im at each of the four different concentrations 50–400 nM in running buffer was injected over the DNA-modified sensor surface for 3 min, followed by washing with running buffer for 5 min at a flow rate of 30 μl min⁻¹. The DNA-modified chip surface was regenerated by injecting 10 μl of 1 M NaCl–50 mM NaOH for 2 min. The sensor chip surface without a DNA coating was used as the control and was injected with the analyte (ColE7-Im solution) simultaneously for each binding experiment. Each analysis was performed in triplicate. BLAevaluation software was used to fit the sensorogram data to determine the association constant (κa), which was derived from ks/kd.
Results

Limited protection of colicin-producing cells against bacteriophage M13K07

Using number of plaque-forming units as a standard to categorize the limited protection of E. coli cells against bacteriophage, we found that less than 8% of the E. coli JM109 (pColE7-K317) cells were protected after bacteriophage M13K07 infection (Fig. 1). However, when the cells were induced by a sub-lethal dose of 50 μg ml⁻¹ mitomycin C (MMC) for 30 min before infection, the plaque-forming unit of the cells containing pColE7-K317 was reduced to 54.7% compared to cells without the plasmid (Fig. 1). In other words, more than 45% of the cells containing pColE7-K317 were protected against bacteriophage M13K07 after MMC induction. The promoter of the ColE7 operon on pColE7-K317 is under SOS response control, so MMC induction triggered the expression of the ColE7 operon.

Since SOS is a global response signal induced by a DNA damage agent such as MMC, we do not know whether the limited protection of the cells harboring the pColE7-K317 is related to the products of the ColE7 operon or another SOS response regulon. In order to evaluate the possibility of colicin participating in the limited protection against bacteriophage, we cloned the colicin structural gene (ceaE7) and the immunity gene (ceiE7) into the pQE70 expression vector. In this way, expression of the ceaE7 gene is directly under the control of the lac promoter. As a result, IPTG induction of the lac promoter will only trigger the expression of the ceaE7 gene of the newly constructed recombinant plasmid (pQE70cea-ceiE7). Thus, this expression vector can be used to test the role of colicin in the limited protection against bacteriophage.

Effect of colicin expression level on the limited protection of colicin-producing cells against bacteriophage M13K07

The study of IPTG induction (0.1 mM) of E. coli JM109 (pQE70cea-ceiE7) clearly showed that there was limited protection of the cells against bacteriophage M13K07 and this was directly related to the induction level of the cea-ceiE7 genes. After 0.5 h of induction, the plaque-forming units of the cells containing pQE70cea-ceiE7 were at 51.5% and after 1.5 h of induction, the plaque-forming units had been reduced to 17.7% (Fig. 2A). The plaque-forming units were not significantly different at 2.5 and 3.5 h after induction when compared with the plaque-forming unit of the 1.5 h induction time. It is worth noting that the plaque-forming units on E. coli JM109 showed no sign of protection whatsoever against bacteriophage M13K07 at different IPTG induction times (Fig. 2A). These results support the idea that there is a degree of limited protection against bacteriophage infection that might directly relate to the expression level of the cea-ceiE7 gene. The effect of colicin and its direct involvement in the limited protection against bacteriophage was further verified by Western blotting analysis of the cell extracts with antiserum raised against colicin E7. As shown in the Western blot experiment (Fig. 2B), the increase in the amount of colicin was proportional to the induction time of the cells harboring the pQE70cea-ceiE7. Thus far, all evidence suggests that colicin plays an important role in the limited protection against bacteriophage of the cells harboring the ColE7 plasmid. However, we do not know whether the colicin E7 alone or the colicin in complex with Im7 contributes to this limited protection of the colicin-producing cells.

Fig. 1. Limited protection of E. coli containing ColE7-K317 plasmid against bacteriophage M13K07. Cells to be tested were first induced with sub-lethal dosage of mitomycin C (0.5 μg ml⁻¹) for 30 min before infected with bacteriophage M13K07 at the MOI of 0.01. The experiments were repeated three times, and standard deviation of the number of plaque-forming units (PFU) of each sample was derived from the means of the PFU. Total PFU of the wild-type cells was expressed as 100%. PFU of the cells containing ColE7-K317 plasmid expressed as percentage with respect to the total number of PFU of the cells containing no plasmid.
Comparison of limited protection of colicin-producing cells against bacteriophage M13K07 and bacteriophage λ

Since M13K07 is a filamentous bacteriophage with single-strand, closed circular deoxyribonucleic acid molecule, it is more interesting to know whether this limited protection can be extended to bacteriophage containing double-strand DNA as their genome. In order to answer this question, we set up an experiment that compared the degree of limited protection of the cells against bacteriophage M13K07 and to that against bacteriophage λ. In this experiment, infection of cells was performed at 1.5 h after IPTG induction of the cells containing the pQE70 cea-ceiE7 plasmids. Under this condition, around 88% of the cells were protected from bacteriophage M13K07 infection (Fig. 3), which was similar to that shown in Fig. 2A. However, the limited protection of the cells against bacteriophage λ was not very effective, as the protection rate was only 23% (Fig. 3). The results clearly indicated that the limited protection of colicin-producing cells is preferentially against the bacteriophage M13K07. Nevertheless, no matter what the degree of limited protection between the bacteriophage M13K07 or bacteriophage λ is, the direct involvement of colicin in this limited protection against bacteriophage infection is evident.

Kinetic studies of ColE7-Im interacting with single-strand DNA and double-strand DNA

It would be interesting to know how colicin might interfere with bacteriophage infection leading to limited protection of the colicin-producing cells. It may be speculated that the exported colicin somehow encounters the invading bacteriophage, thereby blocking the translocation pathway of the bacteriophage DNA into the sensitive cells. Alternatively, it may be possible that the resident ColE7-Im complex of the sensitive cell can interact with the incoming bacteriophage DNA and cause the hydrolysis of the foreign DNA. To study this, we measured the binding of the ColE7-Im complex with dsDNA and ssDNA.

A kinetic study of ColE7-Im complex interacting with DNA was performed using BLAcore, a commercially available biosensor. The biotin labelled dsDNA or ssDNA was anchored to the flow cells of a SA sensor chip and then the chip was flowed through with various amounts of ColE7-Im complex, and the kinetics and affinities of ColE7-Im binding with different DNA substrates were resolved (Fig. 4). Interestingly, the DNA–protein interaction experiment showed that the $K_A$ (M$^{-1}$) value of ColE7-Im interacting with --ssDNA
and dsDNA was $2.94 \times 10^7$ and $1.75 \times 10^7$, respectively (Table 1). Thus, the binding affinity of ColE7-Im with ssDNA is 1.68-fold stronger than with dsDNA. On the basis of the limited protection of a colicin-producing cell against ssDNA or dsDNA bacteriophage (Fig. 3), our protein–DNA interaction results suggest that the stronger binding affinity of the colicin–ssDNA complex results in a higher degree of limited protection for the colicin producing cell against infection by the ssDNA bacteriophage M13K07.

An in vitro DNase activity assay indicated that colicin in complex with Im cannot hydrolyze either dsDNA or ssDNA (data not shown). Furthermore, we found that **E. coli** cells containing the ColE7 plasmids electroporated with DNA either prepared from bacteriophage M13K07 or from bacteriophage λ did not provide any limited protection against infection (data not shown). These experimental results rule out the possibility that degradation of the incoming DNA by the ColE7-Im complex inside the colicin producing cells is a mechanism for the limited protection of the cells against bacteriophage infection.

### Discussion

Colicin is a well-known bacteriocin that recognizes some outer membrane receptors of enterobacteriace leading to the death of susceptible cells. It was found that cells containing CoIE plasmid can exert limited protection against T bacteriophage infection [5]. However, the mechanism for the limited protection is still unclear. In this report we found that **E. coli** cells containing ColE7-Im plasmid also exert a preferential limited protection against bacteriophage M13K07 (a single stranded bacteriophage) and bacteriophage λ (a double stranded DNA bacteriophage). We have confirmed that the limited protection is directly correlated with the expression level of the ColE7 operon. These data indicate for the first time that the endogenously expressed colicin or colicin complexed with its cognate immunity protein (Im) plays an important role in this limited protection of the colicin producing cells.

In addition to the “restriction and modification” system in lactic *Streptococci* and in *E. coli*, some other bacteriophage defense systems have been reported. Wu et al. [26] reported that the endogenously expressed nuclease (*vvn*) of the *Vibrio vulnificus* can be used as a defense system against bacteriophage infection by hydrolyzing the foreign DNA introduced by bacteriophage infection. Overexpression of the integrase gene from human immunodeficiency virus-1 (HIV) in *E. coli* can protect the cells against bacteriophage M13 infection [27]. Possibly, the effect of limited protection of the cells against bacteriophage infection may be due to the interaction between the C terminus of the integrase and the DNA introduced by bacteriophage M13 infection.

In *E. coli* cells, colicin must be co-expressed with its cognate immunity protein (Im) to form a ColE7-Im complex in order to avoid self-damage caused by the nuclease activity of the colicin they produced. Our in vitro nuclease activity assay proved that the ColE7-Im complex

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**Table 1**

Kinetics and affinities of ColE7-Im binding with different DNA ligand

<table>
<thead>
<tr>
<th>DNA ligand</th>
<th>$k_a$ (M$^{-1}$ S$^{-1}$ × $10^3$)$^a$</th>
<th>$k_d$ (S$^{-1}$ × $10^{-3}$)$^b$</th>
<th>$K_A$ (M$^{-1}$ × $10^5$)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssDNA</td>
<td>1.00 (±0.4)</td>
<td>3.40 (±0.3)</td>
<td>2.94 (±0.12)</td>
</tr>
<tr>
<td>dsDNA</td>
<td>12.0 (±1.7)</td>
<td>68.5 (±4.3)</td>
<td>1.75 (±0.26)</td>
</tr>
</tbody>
</table>

Standard deviation is shown in parenthesis.

$^a$ Association rate constant.

$^b$ Dissociation rate constant.

$^c$ Association constant.

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**Fig. 4.** Sensorgrams for ColE7-Im binding to different DNA ligands. Binding of various concentrations of ColE7-Im to sensor surfaces coated with ssDNA (A) or dsDNA (B). The kinetic parameters of the binding reactions were determined and are shown in Table 1.
Coincidently, M13K07 or bacteriophage electroporated DNA prepared from either bacteriophage cells cannot exert any limited protection against the Our unpublished data showed that the colicin-producing colicin-producing cells against bacteriophage infection? Does this interaction between ColE7-Im complex and the can bind to the ColE7-Im complex (Fig. 4 and Table 1). Demonstrated that both single and double stranded DNA of dsDNA (1.75 M−1) is 1.68-fold stronger than that of dsDNA (1.75 × 105 M−1) (Table 1). These experimental results suggest that interaction between ColE7-Im complex and DNA may play a critical role in the limited protection of the colicin-producing cell against bacteriophage infection. Recently, Bouveret et al. [22] reported that translocation of colicin E7 across the membrane and infection of bacteriophage M13 DNA into the susceptible cell both require the assistance of the Tol system, a group of membrane proteins in E. coli. They found that the endogenously expressed translocation domain of colicin fused with OmpA signal peptide in an E. coli cell can transform the cell to colicin resistance and create a so-called tol phenotype. Similarly, expression of g3p protein of the bacteriophage M13 in an E. coli can protect the cell against bacteriophage M13 infection as well as transform the cell to colicin resistance. Under this condition, expression of g3p protein also creates the tol phenotype for the cell [28,29]. It was known that both the translocation domain of colicin fused with OmpA signal peptide and the g3p protein can interact with the TolA protein [30], indicating that TolA probably forms a part of the pathway for bacteriophage M13 infection, thus any protein competing with the TolA binding site must cause an interference with bacteriophage M13 infection.

Thus far, the lysis protein and phospholipase A have been reported to be essential for colicin export [31,32]. However, the detailed pathway of colicin exportation from E. coli cells is still not well understood. In this report, we found that expression of the ColE7 operon in E. coli can cause the cell to acquire limited protection against bacteriophage infection. If competing with a major component of the infectious pathway is the mechanism for the cell acquiring limited protection against bacteriophage as proposed by Bouveret et al. [22], we would like to propose that export of the ColE7-Im complex from the colicin-producing cell may have its own specific pathway, which may overlap to a certain extent with the pathway of bacteriophage infection. If competition for a common component required for both the export of colicin and infection with bacteriophage occurs, we may envisage that a degree of limited protection against bacteriophage infection would be directly proportional to the expression level of the ColE7 operon. We have already found that the degree of protection is indeed directly related to the expression level of the ColE7 operon (Fig. 2). However, we also found that the maximum protection was roughly around 80%. This limited protection probably suggests that the pathways between colicin exportation and bacteriophage infection are not identical. This hypothesis is further supported by the fact that the infectious pathway of bacteriophage M13 is different to that of bacteriophage λ [33,34]. As a result, the limited protection of the colicin-producing cell against bacteriophage M13 is better than the limited protection against bacteriophage λ. We do not know whether the cause of the limited protection is due to the ColE7-Im complex competing for an infectious component or directly interacting with the bacteriophage DNA when these two pathways meet at some point somewhere in the membrane. Nevertheless, we like to suggest that a study of the limited protection of the colicin-producing cell may gain insight into the problem of the colicin export system.

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