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# BACTERICIDAL MECHANISMS OF ESCAPIN, A PROTEIN IN THE INK OF A SEA HARE

by

KO-CHUN KO

Under the co-Direction of Charles Derby and Phang-Cheng Tai

## ABSTRACT

A 60 kDa monomeric protein isolated from the defensive purple ink secretion of the sea hare *Aplysia californica* has broad antimicrobial activity in tryptone peptone rich medium. This protein, which we call 'escapin', belongs to an L-amino acid oxidase family. The goals of my project were 1) to determine the products of escapin's oxidation of its main substrate L-lysine, 2) to characterize the antimicrobial effects of escapin's products, and 3) determine bactericidal mechanisms of action of these products.

Escapin is a powerful bactericidal agent against several bacteria species including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Vibrio harveyi*. Escapin operates through a two-step process: 1) deamination of L-amino acids (especially L-lysine) by enzymatic activity to produce escapin intermediate products of L-



lysine (EIP-K), hydrogen peroxide, and ammonia; and 2) EIP-K simultaneously reacts with hydrogen peroxide to generate escapin end products (EEP-K). EIP exists as an equilibrium mixture of the linear  $\alpha$ -keto analogue of lysine and its cyclic forms, and the relative amount of the linear form increases with pH decreases. The powerful bactericidal effect of escapin requires the simultaneous presence of hydrogen peroxide and EIP-K in weak acidic conditions, which suggests that linear form of EIP-K with hydrogen peroxide is responsible for the bactericidal effect of escapin. Using *E. coli* MC4100 as a model, the mechanism of action of escapin was examined. Brief treatment with EIP-K + H<sub>2</sub>O<sub>2</sub>, but not EIP-K or H<sub>2</sub>O<sub>2</sub> alone, causes irreversible DNA condensation with a time course similar to the bactericidal effect. A mutant strain resistant to EIP-K + H<sub>2</sub>O<sub>2</sub> was isolated, and a single point mutation was found in the oxidative stress regulator gene (*oxyR*). Through a complementary assay, it was shown that wild type *E. coli* is conferred resistance to EIP-K + H<sub>2</sub>O<sub>2</sub> by carrying mutated *oxyR* plasmid. Furthermore, in this bactericidal effect, heat or cold shock does not substitute for hydrogen peroxide induced oxidative stress. Thus, escapin's powerful bactericidal effect may be through irreversible DNA condensation mediated through hydrogen peroxide generating an oxidative stress response, but the pathway mediating EIP-K's synergistic effect is still unclear.

INDEX WORDS: Toxin, Chemical defense, Flavin, Gastropod, Inking, Opisthobranchia, L-Amino acid oxidase (LAAO), Amino acids, Enols, Imino compounds, Oxidation, DNA condensation.

BACTERICIDAL MECHANISMS OF ESCAPIN, A PROTEIN IN THE INK OF A SEA  
HARE

by

KO-CHUN KO

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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2011



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HARE

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KO-CHUN KO

Committee Chair: Charles Derby

Committee: Phang-Cheng Tai  
Chung-Dar Lu

Electronic Version Approved:

Office of Graduate Studies  
College of Arts and Sciences  
Georgia State University  
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## 1 INTRODUCTION

Some animals chemically defend themselves from predators. Defensive chemical secretions are produced by many animals, including a marine snail, the sea hare *Aplysia californica*. When vigorously attacked by predators, sea hares release an inky secretion. This ink secretion is a mixture of the products of two glands: a purplish ink from the ink gland and a whitish, mucousy ink from the opaline gland (1). This secretion protects sea hares through a diverse number of chemicals and mechanisms, some acting on predators to abate their attack and others acting on conspecific sea hares as alarm cues to evoke escape behaviors (1-8). One of the bioactive ingredients in the ink gland secretion of *A. californica* is escapin, an L-amino acid oxidase (LAAO). The product of escapin with its substrate, L-lysine, is a deterrent to spiny lobsters, blue crabs, and other predators (9). Antipredatory chemical defenses also have antimicrobial, antiviral, and other effects. Triterpene glycosides isolated from Caribbean sponges have antipredatory and anti-fouling activity (10). Furthermore, this activity, while not necessarily of ecological relevance, can and often does help in identifying antibacterial, antiviral, and antitumor agents (11-14). It will be important to determine if the mechanisms of bactericidal effects and the mechanisms of anti-predatory effects are similar. My work also has potential importance in the development of new antibacterial drugs.

**L-Amino acid oxidases.** L-Amino acid oxidases (LAAOs) have been isolated from skin mucus of rockfish *Sebastes schlegeli*, venom of snakes, and mu-

cus of the African giant land snail *Achatina fulica*. They show oxidization activity of L-amino acid and produce  $\alpha$ -keto acids, hydrogen peroxide ( $H_2O_2$ ), and ammonia ( $NH_3$ ) (11, 12, 15-19). However, they are reported to have different antibacterial activities, either bactericidal activity or bacteriostatic (18, 20, 21), and the antibacterial mechanisms have not been clarified. Immunostaining with colloidal gold showed that achacin, an LAAO isolated from giant African snails, can pass through bacterial outer membrane and cell wall and attack the cytoplasmic membrane; however, bacteria with achacin-induced damage of cytoplasmic membranes, absence of peptidoglycan layer, or absence of outer membranes were not otherwise different from control bacteria (15). Otsuka-Fuchino et al. (1992) suggested that this cell-selective membrane binding leads to a local increase in the concentration of  $H_2O_2$ . Although  $H_2O_2$  induces filamentation of bacteria as achacin does, the minimal inhibition concentration of  $H_2O_2$  generated by achacin is lower than that of  $H_2O_2$  alone. This inconsistency suggests that a novel mechanism involving molecules in addition to  $H_2O_2$  itself enhances the antibacterial effect (12). Similarly, cell death triggered by LAAOs from the venomous snake venom *Agkistrodon halys* is ~70% rescued by catalase (22). Another LAAO, apoptosis-inducing protein (AIP) from parasite-infected fish, causes apoptosis even in the presence of catalase (23). Butzke et al. (2004) reported that  $H_2O_2$  generated via LAAO activity of APIT (*Aplysia punctata* ink toxin) kills tumor cells. Aplysianin E, isolated from eggs of sea hares, *Aplysia kurodai*, displays bacteriostatic activity that is caused by complete inhibition of synthesis of nucleic acid without a change in bacterial morphology or release of ATP (24). This bacte-

ricidal activity is greater in the growth phase than stationary phase, which is similar to the situation observed from tumor cells and normal cells (11). In conclusion, the binding ability of LAAOs affects the extent cell toxicity but this binding are not required for cell death (22). Besides toxicity caused by  $H_2O_2$ , there is another mechanism that may cause cell toxicity: modification of essential enzymes that function in protecting cells from oxidative stress but do not require energy consumption. This type of cell death preferentially occurs in actively metabolizing cells.

**Mechanisms of bactericidal action.** Mechanisms of bactericidal effect include: 1) inhibition of synthesis of macromolecules such as proteins, DNA, and RNA; 2) damage of essential enzymes; 3) fragmentation of DNA; 4) damage of membranes; and 5) disruption of cell walls. Bactericidal activity, which is different from bacteriostasis, must be an irreversible effect. For example, the major bactericidal effect of streptomycin is to disturb the process of protein synthesis by binding to the 30s subunit but with several secondary consequences including an effect on RNA and DNA synthesis, the integrity of the cell membrane, and respiration (25).

The overall goal of this project was to determine the antimicrobial mechanism of the enzyme, escapin. This work was built on previous results showing that escapin, like other LAAOs, has long-term antimicrobial activity. Therefore, this work first focused on characterization of escapin's antimicrobial activity, the molecules responsible for this activity, and the conditions for activity. Bactericidal assays were performed to test escapin's effect on a range of microbes, including

diverse bacteria, yeast, and fungi. NMR and mass spectroscopy were applied to identify the products of the escapin pathway and determine the effect of pH on this mixture, since the pH of ink secretion varies over several orders of magnitude (pH 4-8). The final aim was to use *E. coli* MC4100 as a model to examine the mechanisms of action of escapin products – in particular, examining the ability of escapin products to cause irreversible DNA condensation and the pathway mediating this effect.

## **2 Characterization of Escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica***

This work has been published as part of: Hsiuchin Yang, Paul M. Johnson, Ko-Chun Ko, Michiya Kamio, Markus W. Germann, Charles D. Derby, and Phang C. Tai. (2005) Cloning, characterization and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica*. *Journal of Experimental Biology* 208: 3609-3622. NMR and mass spectroscopy of the co-factor FAD were performed and analyzed by M. Kamio and M. W. Germann. I performed all other experiments written in this chapter.

## 2.1 Summary

A 60 kDa monomeric protein isolated from the defensive purple ink secretion of the sea hare *Aplysia californica* was cloned and sequenced, and is the first sea hare antimicrobial protein to be functionally expressed in *E. coli*. Sequence analysis suggested that this protein is a flavin-containing L-amino acid oxidase (LAAO), with one predicted potential glycosylation site, although the glycosylation could not be experimentally confirmed. This protein, which we call 'escapin', has high sequence similarity to several other gastropod proteins. Escapin was verified by NMR, mass spectroscopy and HPLC to have FAD as its flavin cofactor. Escapin's antimicrobial effects, bacteriostasis and bactericidal, were determined using a combination of two assays: (1) incubation of bacteria on solid media followed by assessment of inhibition by direct observation of zones of inhibition or by turbidity measurements; and (2) incubation of bacteria in liquid media followed by counting viable colonies after growing on agar plates. Native escapin inhibited the growth of Gram-positive and Gram-negative bacteria, including marine bacteria (*Vibrio harveyi* and *Staphylococcus aureus*) and pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa*). Escapin also inhibited the growth of yeast and fungi, with different efficacies. Escapin was bacteriostatic and not bactericidal in minimal media (e.g. salt media) with glucose, yeast extract, and a mixture of 20 amino acids each at 50  $\mu\text{mol l}^{-1}$ , but was bactericidal in media enriched with Tryptone Peptone. Escapin was also strongly bactericidal in media with L-lysine at concentrations as



low as 3 mmol l<sup>-1</sup> and slightly bactericidal in 50 mmol l<sup>-1</sup> L-arginine, but not in most other amino acids even at 50 mmol l<sup>-1</sup>. Escapin had high oxidase activity (producing hydrogen peroxide) with either L-arginine or L-lysine as a substrate and little to no oxidase activity with other L-amino acids. Hydrogen peroxide alone (without escapin or amino acids) was strongly bacteriostatic but poorly bactericidal, similar in this respect to L-arginine but different from L-lysine in the presence of escapin. Together these results suggest that there are multiple mechanisms to escapin's antimicrobial effects, with bacteriostasis resulting largely or entirely from the effects of hydrogen peroxide produced by escapin's LAAO activity, but bactericidal effects resulting from lysine-dependent mechanisms not directly involving hydrogen peroxide.

## 2.2 Introduction

Several proteins isolated from different tissues and secretions of marine opisthobranch gastropod molluscs known as sea hares have been reported as antimicrobial and antitumor agents (11, 20, 21, 26-37). All are derived from either albumen glands that package sea hare egg masses, the egg masses themselves, or ink secretions from defensive glands in the sea hare's mantle cavity. Achacin, a protein purified from mucus of the African giant land snail *Achatina fulica*, and apoxin I, a protein purified from venom of the western diamondback rattlesnake *Crotalus atrox*, also possess similar bioactivity (12, 13, 15, 16, 22, 23, 38-40). Several of these proteins, including three from sea hares, are known to

be L-amino acid oxidases (11, 34, 36), but the cofactors responsible for oxidase activity have not been experimentally demonstrated. These studies differ in their findings as to whether the proteins are bacteriostatic or bactericidal (15, 20, 21, 30). In addition to the interesting biological roles these proteins may play for their host organisms, some may also have practical industrial or medical uses, yet none have been successfully expressed in high output systems such as bacteria (11, 37, 40-42).

In this study, we have isolated and sequenced a 60-kDa monomeric antimicrobial protein from the purple ink of the sea hare *Aplysia californica* (Fig. 1.1A) and named it 'escapin', because of its potential role in sea hare defense, because it is only released when a sea hare is attacked by predators and it has cytotoxic effects against a predatory sea anemone (43). When attacked by natural predators, *A. californica* releases secretions from two glands in its mantle cavity: a purple secretion from its ink gland, and a sticky white secretion from its opaline gland (1). Nearly 30% of the dry mass of *A. californica* ink is protein (44, 45) while the remaining portion is algal-derived pigments from the sea hare's seaweed diet (46). The ink-opaline secretion of *A. californica* is an effective deterrent against predatory sea anemones (8, 47), and escapin is the major protein component of ink. Thus, as an abundant and potentially bioactive protein in ink, escapin may play a role in sea hare chemical defense.

We found that escapin contains flavin adenine dinucleotide (FAD) and is predicted to have one potential glycosylation site, although the glycosylation could not be experimentally confirmed. Escapin has a wide spectrum of antimi-

crobial activities, including against bacteria, yeasts and fungi, but with different efficacies. Escapin is bacteriostatic but not bactericidal under many growth conditions (e.g. in a mixture of amino acids each at  $50 \mu\text{mol l}^{-1}$ , or  $50 \text{ mmol l}^{-1}$  L-valine), weakly bactericidal under some conditions (e.g. in yeast extract or in  $50 \text{ mmol l}^{-1}$  L-arginine or L-histidine), and strongly bactericidal under other conditions (e.g. in Tryptone Peptone or  $50 \text{ mmol l}^{-1}$  L-lysine). Hydrogen peroxide plays a prominent role in the bacteriostatic effect but only a weak role in the bactericidal effect.

## 2.3 Materials and Methods

**Animals.** Sea hares (*Aplysia californica* Cooper 1863) were collected in California by Marinus Scientific (Garden Grove, CA, USA). The sea hares were dissected on the day of arrival in our laboratory. Experiments were performed according to Georgia State University regulations and national guidelines.

**Collection of purple ink.** Ink glands were dissected from anesthetized animals and frozen at  $-80^{\circ}\text{C}$  until they were used. Purple ink was collected by gently squeezing dissected ink glands in a Petri dish with the blunt end of a scalpel handle.

**Purification of proteins from purple ink.** Proteins were isolated and purified using an ÄKTA 100 Automated FPLC (Amersham Pharmacia Biotech, Piscataway, NJ, USA). A preparative grade Hi-Load Superdex 200 16/60 column (Amersham Pharmacia Biotech) or an in-house-packed Sephacryl 300·HR 26/60 column was used for initial size separation with fractions collected in an automated fraction collector. Fractions identified to have activity by bacteriostatic assay (described in the next section) were concentrated using a Biomax 5K NMWL membrane Ultrafree Centrifugal Filter Device (Millipore, Billerica, MA, USA). Active fractions were further purified on a cation exchange Mono S column, and fractions were collected, assayed, concentrated and frozen at  $-80^{\circ}\text{C}$ . One purified protein of interest, which we call 'escapin', was bright yellow. Escapin's concentration was determined by Bradford (1976) assay using bovine serum albumen (BSA) as a standard. The molecular mass of purified escapin was determined by gel filtration using a Superose-6 10/30 column (Amersham Pharmacia Biotech) eluted with 50 mmol  $\text{l}^{-1}$  potassium phosphate buffer (pH 7.2) containing 150 mmol  $\text{l}^{-1}$  KCl at a flow rate of 0.5 ml  $\text{min}^{-1}$ . The molecular mass markers were BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa).

**N-terminal protein sequencing.** *Aplysia californica* ink was analyzed for protein content using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. A single dominant protein band was found at about 60,000 Da, and was named escapin. This band was blotted from the polyacrylamide gel to a PVDF membrane using CAPS (transfer buffer) and N-terminal sequencing of

escapin was carried out in GSU protein sequencing facility using a protein sequencer (Procise 492; Applied Biosystems, Foster City, CA, USA).

**Isolation and identification of the yellow pigment associated.** Purified escapin was heated at 70°C for 15 min followed by centrifugation at 25,000 x g for 20 min to separate the pigment from the protein. Yellow pigment in the supernatant was purified by high pressure liquid chromatography (HPLC) according to Light et al. (1980), using a Beckman system equipped with a 168 photodiode array set at 200-600 nm with a Phenomenex Luna C18 (0.46 x 250 mm) column (Phenomenex, Torrance, CA, USA). Isocratic reversed phase chromatography was performed using 5 mmol l<sup>-1</sup> ammonium acetate and 20% methanol in water as a mobile phase with a flow rate of 1ml min<sup>-1</sup>. Retention times of a FAD standard (Sigma, St. Louis, MO, USA) and the yellow pigment were compared by co-injection, ESI-TOF mass spectrometry was performed using an Applied Biosystems QSTAR XL and run in positive ion mode. ESI samples were injected into a flow of 50/50 water/ acetonitrile containing 0.2% formic acid. NMR spectra were acquired on a 500 MHz Bruker Avance NMR (Rheinstetten, Germany) equipped with a triple resonance cryoprobe. 1 μmol l<sup>-1</sup> of FAD standard was purified using the same method as for the yellow pigment. Spectra were recorded in D<sub>2</sub>O at 309K. spectra for the FAD standard were obtained under identical conditions except the experimental time. Proton assignments for the FAS standard were based on established 2D NMR methods (COSY, ROESY). The amount of FAS in

the supernatant of heated protein was calculated based on an extinction coefficient value,  $\epsilon_{450}$  for FAD of  $11.3 \text{ mmol l}^{-1} \text{ cm}^{-1}$  (48).

**Detection of glycosylation of escapin.** GelCode Glycoprotein Staining Kit (Pierce Biotechnology, Rockford, IL, USA) and DIG Glycan Detection Kit (Roche) were used to determine the carbohydrate component of escapin.  $5 \mu\text{g}$  of escapin, BSA, and *E. coli* protein (negative controls, since they lack glycosylation), and various concentrations of horseradish peroxidase (a positive control and standard, since it is 15% carbohydrate by mass) were analyzed by SDS-PAGE followed by staining for carbohydrates according to the manufacturers' protocols, as well as by Coomassie Blue labeling of proteins.

**L-Amino acid oxidase (LAAO) assay.** LAAO activity of escapin was determined by an enzyme-coupled assay (49). Purified escapin in  $50 \text{ mmol l}^{-1}$  phosphate buffer and  $150 \text{ mmol l}^{-1}$  KCl was added to a  $100 \mu\text{l}$  reaction mixture containing  $0.1 \text{ mol l}^{-1}$  Tris- HCl, pH 7.6,  $10 \mu\text{g}$  horseradish peroxidase,  $0.2 \text{ mmol l}^{-1}$  o-dianisidine, and indicated concentration of various L-amino acids. Reactions were performed at room temperature for 1–60 min; the activity was monitored by absorbance at 436 nm and the increase in absorbance was transformed into molar concentration of end product based on  $\epsilon$  of o-dianisidine =  $8.31 \times 10^3 \text{ mol l}^{-1}$ . The  $K_m$  and  $V_{\text{max}}$  values were determined by Lineweaver–Burk plots.

**Microbial species and strains.** Eleven species or strains of microbes were examined for antimicrobial activity of escapin. These are the following: Gram-negative bacteria *Escherichia coli* (MC4100), *Pseudomonas aeruginosa* (PAO1), and *Salmonella typhimurium* (AA140), *Vibrio harveyi* BB170 (a marine species), Gram-positive bacteria *Bacillus subtilis* (2 strains, 168 and WB600), *Streptococcus pyogenes* (NZ131), and *Staphylococcus aureus* (6538; a pathogenic species); yeast *Candida krusei* and *Saccharomyces cerevisiae* (BY4761); and fungus *Cladosporium sphaerospermum*.

**Antimicrobial assay.** Antimicrobial effects, which can be bacteriostatic and/or bactericidal, were determined for escapin using a combination of two assays. In the first assay, which measured inhibition, microbes were incubated on solid medium in the presence of escapin or controls, followed by assessment of escapin's effects either by direct observation of zones of inhibition or by turbidity measurements of cell density (measured as  $A_{600}$  in a spectrophotometer). In the second assay, bacteria were incubated in liquid medium in the presence of escapin or controls, followed by plating onto agar-filled Petri dishes and counting viable colonies. No effect on bacterial growth in the first assay indicated that the compound was not antimicrobial – neither bacteriostatic nor bactericidal. If there was inhibition of growth according to the first assay but no reduction in number of colonies according to the second assay, then bacteriostasis was indicated. If there was inhibition of growth according to the first assay and a reduction in

number of colonies according to the second assay, then bactericidal effects were indicated.

In the first assay, growth inhibition plate assays, various bacteria species and strains were plated as a lawn of ca.  $1\text{--}2 \times 10^8$  cells on Petri dishes with solid medium in 1.5% agar. Growth inhibition was examined by spotting 1  $\mu\text{l}$  of escapin onto the plate, incubating overnight at 37°C or room temperature, and assaying for the presence of a clear zone around at the spot. In this assay, different microbes were cultured in an appropriate medium. *E. coli* cells were cultured in either minimal medium (e.g. M9+glucose+thiamine) or enriched medium [e.g. Tryptone Peptone (Try) or LB]. Other bacteria species were cultured in LB medium, except for *Streptococcus pyogenes*, which was cultured in Todd Hewitt broth. Fungi were cultured in Sabouraud Dextrose medium. Yeast was cultured in YEPD solid medium (1% yeast extract, 2% peptone, 2% dextrose).

In the second assay, using liquid medium, bacteriostatic or bactericidal activity was determined by co-incubating bacteria in liquid medium with supplements and escapin, followed by measuring bacterial cell density either by turbidity measurement at  $A_{600}$  or by counting the number of viable colonies after incubating on Petri dishes with solid medium.



## 2.4 Results

### **Purification and characterization of native escapin from purple fluid secretion of *A. californica*.**

Raw purple from ink *A. californica* (Fig. 2.1A) run on a denaturing SDS-PAGE gel contains many proteins, including a dominant one at 60 kDa (Fig. 2.1B, left lane). The 60 kDa denatured protein was purified to homogeneity using a MonoS column (see Materials and methods; Fig. 2.1B, right lane). Analytical gel filtration shows that native (non-denatured) ink purified using the MonoS column contains a single molecule with a molecular mass of ca. 60 kDa (Fig. 2.1C). Furthermore, this purified 60 kDa protein has complete antibacterial activity (see later). Together, these results show that the 60 kDa protein occurs as a bioactive monomer in native ink. We called this 60 kDa protein 'escapin' because it is only released when a sea hare is attacked by predators and has cytotoxic effects against one predator (43). Escapin also has similarity to other proteins isolated from sea hares (20, 26, 29-31, 37).

### **Sequence analysis of escapin cDNA.**

A BLAST search found that escapin shares identity with a number of L-amino acid oxidase (LAAO) flavoproteins (Fig. 2.2). Escapin has highest identity (93%) with APIT, a protein from the purple ink secretion of the sea hare *Aplysia punctata* (GenBank accession nos. 442281, 442282, 442283) (11). Escapin shared 61% identity with cyplasin L (Accession no. AJ304802) (37), likely iso-

lated from an ink-opaline secretion of *A. punctata*. Escapin shared 61% and 60% identity with aplysianin A precursor protein isolated from albumen glands of *A. kurodai* (Accession no. D83255) (27) and *A. californica* (Accession no. AY161041) (41), respectively. Escapin also had 48% identity with achacin precursor, and antibacterial protein isolated from a land snail *Achatina fulica* (Accession no. X64584) (42). It also showed 21% identity with other L-amino acid oxidases from various species, including apoxin I from venom gland of the pit viper *Crotalus atrox* (Accession no. AF093248) (40).

The alignment results indicate that the two characteristic sequence motifs of flavoproteins- 'GG' (RxGGRxxS/T) and  $\beta\alpha\beta$  dinucleotide-binding (DMB) motifs are well conserved among these proteins (Fig. 2.2). Glycosylation is commonly observed among LAAOs and is reported to be critical for the enzyme's activity (12, 38, 40). However, only one possible N-glycosylation site (Thr 463) was predicted from escapin using the analytical program NetOGlyc (<http://www.cbs.dtu.dk/services/NetOGlyc/>). We directly examined the level of glycosylation of purified escapin using two glycoprotein staining methods. With the GelCode Glycoprotein Staining Kit, which has a sensitivity of 1.5% carbohydrate by mass, no carbohydrate was detected. Using the DIG Glycan Detection Kit, escapin contained <0.03% carbohydrate by mass and even less than dactylomelin-P (a homologue of escapin in *Aplysia dactylomela*, which has been reported to have <0.05% carbohydrate); however, this assay yielded false positives for our negative control *E. coli* proteins, so it is questionable whether any glycoprotein was present. We also attempted to identify glycoproteins in purified dactyl-

lomelin-P using a ConA-sepharose column (Amersham Pharmacia Biotech), and again we did not identify any glycoproteins. We also attempted to identify carbohydrates from purified dactylomelin-P, using the University of Georgia Complex Carbohydrate Research Center (<http://www.ccrc.uga.edu/home.html>); this assay, which is extremely sensitive, yielded negative results. Collectively, our results predict that escapin has one N-glycosylation site, but this prediction could not be experimentally confirmed. Thus, we conclude that escapin is minimally glycosylated, if at all.

### **Escapin is a member of the flavoprotein family.**

Amino acid sequence analysis suggested that escapin is a member of the flavoprotein family, and contains 'GG' (RxGGRxxS/T) and  $\beta\alpha\beta$  dinucleotide-binding (DMB) motifs. Purified homogeneous escapin was bright yellow, and it was assumed that this yellow pigment was the flavin. Since flavin adenine dinucleotide (FAD) is the typical flavin cofactor for this protein family, we used NMR, ESI-TOF mass spectroscopy, and HPLC to detect for the presence of FAD. The aromatic region of the  $^1\text{H}$  NMR spectrum of the yellow pigment isolated from escapin showed essentially identical resonances to the FAD standard, although the spectrum of the yellow pigment contained signals of impurity at 8.45 p.p.m. (Fig. 2.3A). Similar features were also obtained for the aliphatic region of the FAD standard and the yellow pigment (data not shown). Particularly noteworthy is the observation that two of the aromatic protons (7.8 p.p.m.) that have long  $T_1$  relaxation times in FAD are also observed for the pigment. A high resolution

ESI-TOF mass spectrum of purified yellow pigment from escapin showed a peak with an  $m/z$  value of 786.2 (Fig. 2.3B); this corresponds to the molecular formula  $C_{27}H_{34}N_9O_{15}P_2$ , which was designated as a  $(M+H)^+$  ion of FAD. ESI-TOF mass spectrum of the yellow pigment also showed peaks at  $m/z$  of 808.1 and 830.1, which correspond to the  $(M+Na)^+$  and  $(M-H+2Na)^+$  ions of FAD (Fig. 2.3B). In this spectrum, signals below 400  $m/z$  were due to solvents, and signals of 400–600  $m/z$  were not identified. Ions corresponding to another flavin, FMN (molecular mass=478.3), were not found. In addition, in reversed phase HPLC, the yellow pigment had the same retention time (17.6-min) as FAD, and co-injection of the yellow pigment and FAD showed only one peak. The UV-visible absorbance spectrum of the peak of the yellow pigment showed absorbance at 263, 375 and 450 nm, which is characteristic for FAD. Thus, the yellow pigment released from escapin is FAD. Based on  $\epsilon_{450}$  values, 17.2 nmoles FAD were extracted from 16.7 nmoles of purified escapin, yielding an escapin: FAD molar ratio of about 1:1. (*Analysis of Mass spectroscopy and NMR are done by Dr. Michiya Kamio*).

Results from an NCBI conserved domain search predicted that escapin is an  $L$ -amino acid oxidase (LAAO), which oxidizes  $L$ -amino acids to produce hydrogen peroxide. To test this prediction, we performed an enzyme-coupled oxidase assay. Our results confirmed the prediction, showing that amino acids serve as a substrate for escapin, resulting in generation of hydrogen peroxide (Fig. 2.4). Unlike LAAOs from African snail and snake venom (12, 39, 49-51), but similar to aplysianin A isolated from the albumen gland of *Aplysia kurodai* (36), escapin preferentially utilized positively charged amino acids. The most effective amino

acids were L-lysine and L-arginine. When escapin and 2 mmol l<sup>-1</sup> amino acids were incubated for 1 min, L-lysine and L-arginine proved excellent LAAO substrates, L-tyrosine and L-histidine were low quality substrates, and other amino acids were poor substrates (Fig. 2.4).  $K_m$  (μmol) and  $V_{max}$  (μmol s<sup>-1</sup>) values of were 31–85 μmol and 1.92–2.70 μmol s<sup>-1</sup> for lysine and 25–120 μmol and 1.56–3.30 μmol s<sup>-1</sup> for arginine (Fig. 2.4). Similar efficacies for the substrates were seen for 60-min incubation times. The reactions were completed within 30 s at room temperature for lysine or arginine concentrations of 0.02–2 mmol l<sup>-1</sup> (data not shown). LAAO assays for lysine and arginine in the presence of *E. coli* produced similar effects (data not shown), demonstrating that hydrogen peroxide is produced from escapin's oxidation of lysine or arginine under the conditions of the antimicrobial assays (described later).

### **Antimicrobial activity of escapin.**

Escapin inhibited the growth of several types of bacteria including Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Vibrio harveyi*) and Gram-positive (*Bacillus subtilis*, *Streptococcus pyogenes* and *Staphylococcus aureus*) (Table 2.1). *Vibrio harveyi*, which is a marine bacterium, was most sensitive to escapin, with a minimum inhibitory concentration (MIC) of 0.25 μg ml<sup>-1</sup>. The pathogenic species *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the next most sensitive to escapin, with MIC values of 0.31 μg ml<sup>-1</sup>. *Bacillus* of both wild-type and protease deficient strains showed the highest resistance to escapin, with MIC=2.5 μg ml<sup>-1</sup>. Escapin also exhibited anti-fungal

and anti-yeast activity, though it was less effective against them than bacteria. The MIC values of escapin against a common pest mold *Cladosporium sphaerospermum* and a pathogenic yeast strain *Candida krusei* were 62 and 5  $\mu\text{g ml}^{-1}$ , respectively, which is 10–100 times higher than against *E. coli* (Table 2.1).

Escapin can be either bacteriostatic or bactericidal against *E. coli*. The bacteriostatic effect occurs in either minimal medium (e.g. M9+ glucose+ thiamine) or in enriched medium (e.g. LB). Fig. 2.5A shows this effect, in which growth in the absence of escapin ('Control') is indicated as a line with a positive slope, and an absence of growth in the presence of escapin ('Esc') is indicated by a flat line. Hydrogen peroxide, which is produced under the conditions of this assay, appears to be necessary for bacteriostasis, since the addition of catalase, a scavenger of hydrogen peroxide, strongly reduced bacteriostasis in a concentration dependent manner (Fig. 1.5A). The observation that catalase alone (in the absence of escapin) actually enhanced bacterial growth (Fig. 2.5A: compare '2 mg ml<sup>-1</sup> Cat' vs. 'Control') might result from the catalase scavenging naturally produced hydrogen peroxide. Escapin's bactericidal effect in enriched medium is shown in Fig. 2.5. Killing occurred rapidly, within 10 min, and was maintained for up to 2 hours when cells were incubated at 37°C (Fig. 2.5B). No killing occurred at 0°C (Fig. 2.5B). Hydrogen peroxide was also produced under these assay conditions (see earlier results).

The bactericidal effect of escapin occurred in enriched media but not minimal media, even though minimal media supported growth inhibition. When *E.*

*coli* cells were grown in minimal medium (M9) alone or M9 plus yeast extract, escapin was either weakly or not at all bactericidal (Fig. 2.6A). However, when *E. coli* cells were grown in M9 plus Tryptone Peptone, escapin was strongly bactericidal (Fig. 2.6A).

The strong enhancement of escapin's bactericidal effect on *E. coli* also occurred by adding L-lysine to minimal medium. L-Lysine at 50 mmol l<sup>-1</sup> dramatically enhanced escapin's bactericidal effect (Fig. 2.6B). L-Arginine and L-histidine at 50 mmol l<sup>-1</sup> produced a small enhancement of escapin's bactericidal effect, whereas L-valine or other amino acids did not affect escapin's bactericidal effects (Fig. 2.6B). A similar large enhancement of escapin's bactericidal effect by L-lysine but not by L-arginine or other L-amino acids was also seen for *Staphylococcus aureus* incubated in LB medium. The killing effect by escapin on *S. aureus* in LB medium was not apparent, presumably because of the presence of a naturally occurring catalase (Fig. 2.6C).

To examine the concentration dependency of the ability of L-amino acids to mediate escapin's antimicrobial effects, we performed both types of assays with *E. coli*, using amino acid concentrations up to 50 mmol l<sup>-1</sup>. In the growth inhibition plate assay, escapin inhibited bacterial growth with a similar concentration dependence and threshold in L-lysine and L-arginine (Fig. 2.7A). Escapin did not inhibit growth of *E. coli* in the presence of up to 50 mmol l<sup>-1</sup> L-tyrosine, L-histidine, or any other amino acid (Fig. 2.7A). Hydrogen peroxide alone (without either escapin or amino acids) had a concentration dependence and threshold to bacterial growth inhibition that was similar to that of lysine and arginine in the

presence of escapin (Fig. 2.7A), demonstrating that hydrogen peroxide is sufficient to mediate growth inhibition. Together with the observation that hydrogen peroxide is also largely or completely necessary for growth inhibition (Fig. 2.5A), these results are consistent with the idea that escapin exerts its inhibition of bacterial growth through hydrogen peroxide, which is a product of escapin's oxidation of either L-lysine or L-arginine.

In contrast, the bactericidal effect of escapin occurred in a concentration-dependent fashion only for L-lysine (Fig. 2.7B). L-Arginine at any concentration did not mediate escapin's bactericidal effect, even though L-arginine is a good substrate for escapin's LAAO activity. L-Arginine's inability to mediate escapin's bactericidal effect is similar to L-tyrosine, which unlike L-tyrosine is a poor substrate for escapin's LAAO activity (Fig. 2.7B). In addition, hydrogen peroxide at 8–11 mmol l<sup>-1</sup> had no to little bactericidal effects on *E. coli* (Fig. 2.6B), even though L-lysine is an excellent substrate for escapin's generation of hydrogen peroxide at 8–11 mmol l<sup>-1</sup> greatly enhanced escapin's bactericidal effects (Fig. 2.7B).

The bactericidal effect of escapin did not depend directly on protein synthesis, since addition of chloramphenicol, a protein synthesis inhibitor, did not affect escapin's bactericidal effects in LB medium. In contrast, it enhanced the killing effect by at least one-log unit (Fig. 2.8).

Finally, since some of escapin's antibacterial effects depend on the concentration of L-lysine in the growth medium, and since the microbes tested in this study (Table 2.1) used different growth media, we wanted to know whether the



different inhibitory efficacies of escapin were dependent on the lysine concentration in the media. So, we analyzed the concentration of lysine and other free amino acids in the media, using an ion exchange, post-column ninhydrin detection system (Beckman Model 6300/7300 Amino Acid Analyzer, The Scientific Research Consortium, Inc.: [www.aminoacids.com](http://www.aminoacids.com)). We found that the media contained the following concentrations of lysine: tryptone peptone medium (for bacteria), 2.636 mmol l<sup>-1</sup>; YEPD (for yeast), 4.980 mmol l<sup>-1</sup>; and Sabouraud Dextrose (SD) medium (for fungus), 0.812 mmol l<sup>-1</sup>. Thus, the lysine concentration was different in the media. But even so, lysine concentration cannot explain the lower inhibitory efficacy of yeast compared to bacteria because yeast has lower efficacy even though yeast medium has a higher lysine concentration: yeast MIC of 5 µg ml<sup>-1</sup> at 4.980 mmol l<sup>-1</sup> lysine, *E. coli* MIC of 0.62 µg ml<sup>-1</sup> at a lysine concentration of 2.636 mmol l<sup>-1</sup>. We increased the concentration of L-lysine in SD medium to 4.980 mmol l<sup>-1</sup>, and found that its inhibitory efficacy against fungus increased (MIC of 62 µg ml<sup>-1</sup> in 0.812 mmol l<sup>-1</sup> lysine, and 15 µg ml<sup>-1</sup> in 4.980 mmol l<sup>-1</sup>). Thus, the inhibitory efficacy of escapin against different types of microbes is dependent on lysine concentration, with greater inhibition at higher lysine concentrations (results not shown). But nonetheless, escapin has different efficacies against different microbes that are independent of lysine concentration, with the relatively efficacies being bacteria > yeast > fungi for the species tested.

## 2.5 Discussion

These L-amino acid oxidases have been of interest to biomedical researchers for more than a decade, largely for the promise they show both as antitumor agents and as a new class of broad antimicrobial compounds. They may also be of use as antifouling agents in aquatic environments. They are also of interest to chemical ecologists for the potential role that they may play in protecting sea hares from microbial infections or from predators, and in protecting their egg masses from both predators and biofoulers. The research presented here focuses primarily on the practical antimicrobial activity of escapin. However, there are implications for how escapin, as the dominant protein in the defensive ink secretion of *Aplysia californica*, may also function as an antipredatory or antiseptic compound.

L-Amino acid oxidase (LAAO) activity of antimicrobial and antineoplastic agents from a variety of species has been reported as a major (11, 12, 22, 33, 39, 51, 52) or partial (23, 36) mechanism for their effects. Escapin also possesses LAAO activity: it shows strong and rapid activity under our assay conditions when using arginine or lysine as a substrate, with reactions being completed within 30 s at room temperature (Fig. 2.4). Similar to other LAAOs from snails and snakes, escapin is a flavoprotein. We show that the flavin in escapin is FAD (Fig. 2.3). Escapin has one potential glycosylation site, but this is probably not essential for its antimicrobial activity. Dactylomelin-P, a homologue of escapin in *Aplysia dactylomela*, was likewise found to have no or minimal glycosy-

lation (<0.05% carbohydrate by mass) (20) and was still fully functional. This is in contrast to other reports that for related LAAOs, the sugar moiety is necessary for antimicrobial effects (35, 37, 38, 40, 42).

Escapin has a broad antimicrobial spectrum compared to many known antimicrobial agents, and thus far escapin has been effective against all tested microorganisms (Table 2.1). It was most effective against the bacteria found in the marine environment (*Vibrio harveyi* and *Staphylococcus aureus*), so it can be highly active against microbes that occur in the environment in which escapin normally acts. Escapin was also effective against pathogenic species (*Staphylococcus aureus*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa*), demonstrating its potential in use against medically important species.

Escapin is an effective inhibitor of many microbes (Table 2.1), but we mostly used *E. coli* in our characterization of antimicrobial effects of escapin. Its activity is concentration dependent. Escapin exerts its bactericidal effects very quickly – within 10 min (Fig. 2.5).

Escapin is bacteriostatic in minimal media and bactericidal in enriched media (Fig. 2.5). Unlike previous reports of similar proteins isolated from sea hares (20, 21, 24, 30), we demonstrate that substrates determined the bactericidal or bacteriostatic effects of escapin. Bacteria cultured in M9 and glucose medium without high levels (50 mmol l<sup>-1</sup>) of selected amino acids or without Tryptone Peptone supplements were not killed (Figs 2.5, 2.6). Escapin's bacteriostatic effect seems to be mediated through its oxidation of the L-amino acids lysine and/or arginine and the consequent subsequent production of hydrogen

peroxide. Both lysine and arginine are substrates for escapin's oxidase activity (Fig. 2.4). Hydrogen peroxide is sufficient to cause bacteriostasis, and does so with a concentration dependency and threshold (ca. 3 mmol l<sup>-1</sup>) similar to that of amino acids as substrates for escapin (Fig. 2.7A). Thus, hydrogen peroxide appears to be both necessary (Fig. 2.5) and sufficient (Fig. 2.7) for bacteriostasis under our conditions, and likely mediates escapin's bacteriostatic effects.

We have identified L-lysine as a major cofactor in escapin's bactericidal effect. Lysine is much more effective than arginine in enhancing escapin's bactericidal activity. Lysine's effect is over three-log units greater than that of arginine at the same concentration (50 mmol l<sup>-1</sup>; Fig. 2.6B), and lysine mediates the bactericidal effect with a threshold of ca. 3 mmol l<sup>-1</sup> whereas arginine is ineffective in killing bacteria at concentrations as high as 50 mmol l<sup>-1</sup> (Fig. 2.7B). This is true even though arginine and lysine have similar LAAO activities (Fig. 2.4) and similar thresholds for bacteriostasis (Fig. 2.7A). In addition, arginine's limited enhancement of escapin's bactericidal effect is similar to that of histidine (Fig. 2.6), even though arginine has much greater LAAO activity than histidine (Fig. 2.4). Thus LAAO activity alone, and the resultant production of hydrogen peroxide, cannot explain escapin's pronounced killing effect in the presence of lysine as compared to arginine. Hydrogen peroxide production thus plays little to no direct role in escapin's bactericidal effect. We are currently examining escapin's bactericidal mechanisms.

Analysis of the free amino acids found in the opaline secretion of *Aplysia californica* show that it has a very high lysine concentration (145 mmol l<sup>-1</sup>), while

the purple ink secretion, containing escapin, has none. Only small amounts of arginine ( $<0.4 \text{ mmol l}^{-1}$ ) are found in opaline and none in ink. This raises the possibility that the lysine is mixed with escapin, which is only in ink, when ink and opaline are released and mixed by sea hares following attack by predators. In fact, ink and opaline are normally co-released and mixed in the sea hare's mantle cavity, then pumped toward the attacking predator (1). It should be noted, however, that the lack of any detectible lysine or arginine in the ink could also be a result of stores being used as substrates by escapin by the time we collect the ink and thus prior to analysis of free amino acids. We are currently conducting experiments to evaluate this and related hypotheses. Understanding the natural roles that escapin plays for sea hares will likely aid our understanding of how it may function as a practical antimicrobial or antineoplastic agent.

Given our finding of a lysine-dependent antimicrobial effect for *E. coli*, it may be that the different inhibitory efficacies of escapin against the species of microbes tested (Table 2.1) were due to the concentration of lysine in their growth media (which are necessarily different). Although the concentration of lysine did influence the efficacy of escapin against a fungus to a small degree (Table 2.1), nonetheless escapin had different efficacies against different microbes when tested in media with similar lysine concentrations, with escapin being most potent against bacteria and least against fungi for the species tested.

Although the mechanisms of antimicrobial activity of escapin are not yet known in detail, our results give some suggestions. Escapin preferentially kills bacteria that are metabolically active (i.e. in log-growth phase vs. stationary

growth phase; (17)). An interesting observation was that although escapin exerted its bactericidal effect in a concentration-dependent manner, the killing never reached 100% as the protein concentration increased or over longer time periods (Fig. 2.5). In addition, these cells were not resistant to escapin, since they were sensitive to more cycles of killing when re-inoculated with fresh medium (data not shown). These results suggest the existence of persister cells, which neither grow nor die in the presence of microbial antibiotics (53), and that more persisters were produced in stationary phase than in log-growth phase at the same protein concentration (as in other studies: (53, 54)). Thus, it appears that escapin preferentially kills cells that are metabolically active. In this way, escapin is similar to other antimicrobial agents such as penicillin, streptomycin, ampicillin and O-oxacin in being bactericidal on log-growth phase cells and not stationary cells, in contrast to bismuth, which is bactericidal on stationary cells but not log-growth phase cells (55, 56).

The existence of persister cells in our cultures of *E. coli* may explain why several sea hare homologues of escapin, including aplysianin-E (28), aplysianin-P (30) and dactylomelin-P isolated from purple fluid of *Aplysia dactylomela* (20), have been characterized as bacteriostatic and not bactericidal, whereas we found escapin to be bactericidal. To test this idea, we extracted and purified dactylomelin-P from *Aplysia dactylomela* and found that it was also bactericidal under our assay conditions (unpublished data). These differences in our results with escapin and dactylomelin-P vs. those of the earlier studies of related proteins could be due to the presence of persister cells in our culture conditions; other ex-

planations could include methodological differences such as the concentrations of lysine in culture media or the species and strains of microbes used.

In addition to escapin's preferential killing of bacteria that are metabolically active, escapin also kills cells in the presence of chloramphenicol to stop protein synthesis (Fig. 2.8), and it does so without lysing the cells. Comparison of the mechanisms of action of escapin with other antimicrobial agents can give some clues as to escapin's mode of action. Streptomycin, which affects ribosomes and cell membrane, is similar to escapin in that it kills bacteria without lysing cells but dissimilar in requiring protein synthesis for its bactericidal effect (55). Penicillin, which inhibits cell wall formation, is more dissimilar to escapin in that it kills bacteria through lysis and requires protein synthesis for its effect (55).

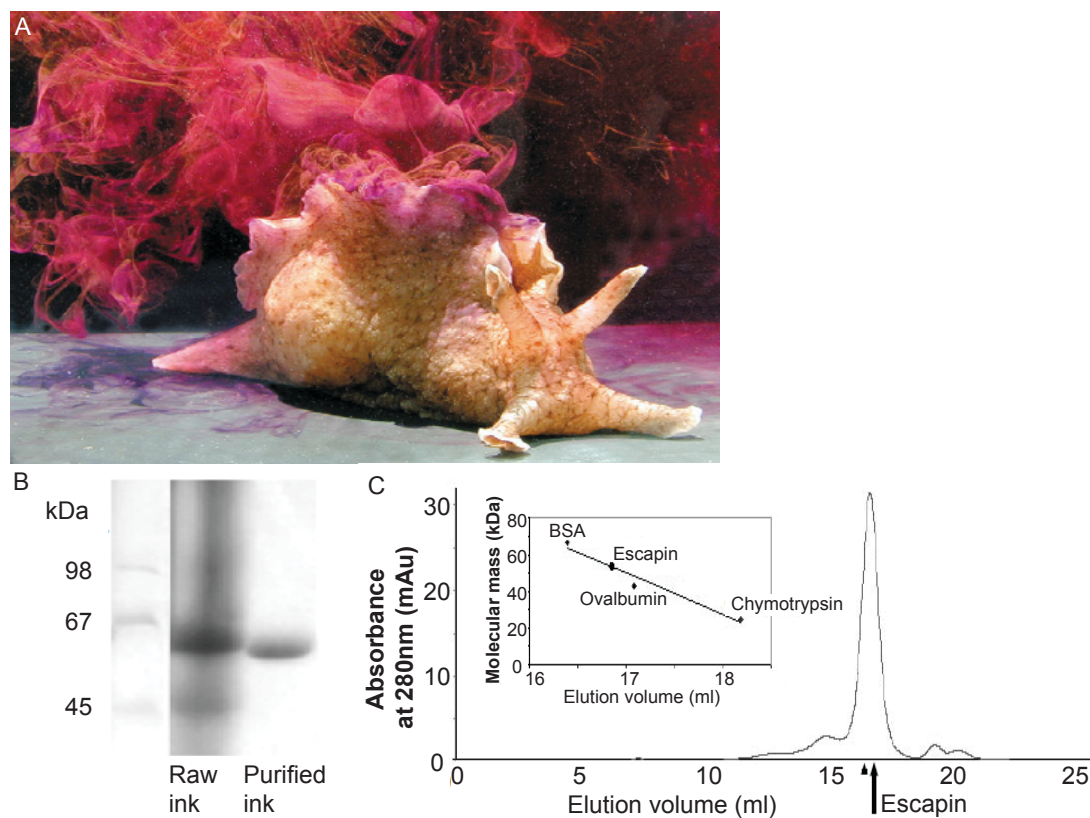
In summary, escapin is both bacteriostatic and bactericidal, and its bactericidal effect does not require protein synthesis or cytolysis. A more complete understanding of the cellular mechanism of escapin's bactericidal effects awaits further investigation.

## **2.6 Acknowledgement**

The isolation and sequencing of the escapin protein, and the cloning and identification of the escapin gene were performed by H. Yang and PM Johnson. We thank Chanda Alexander, Giovanni Gadda, Amy Horner, Ying-Ju Huang, Liang-Ping Jiang, Paul Katz, Kerry Maxwell, Bing Na, Manfred Schmidt, and Wil-

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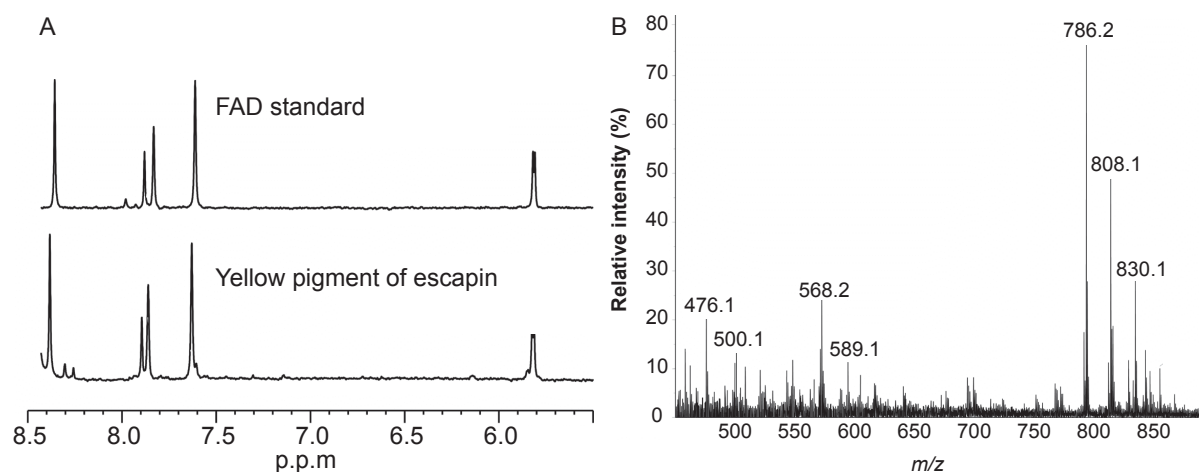




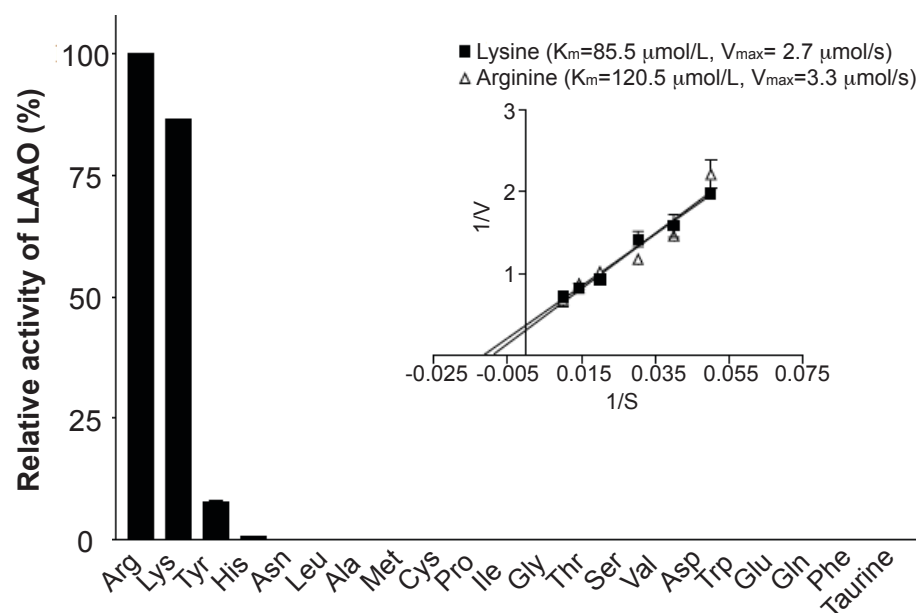
**Figure 2.1. Isolation of escapin.** (A) The ink of *Aplysia californica* containing escapin, a 60kDa protein. (B) SDS-PAGE of raw purple ink (left lane) and ink purified as described in the Materials and methods to yield escapin, a 60 kDa protein (right lane). Molecular mass standards are also shown. (C) Analytical gel filtration shows that escapin elutes as a single peak. Absorbance is expressed on a relative scale; mAu, milliabsorbance units. Arrow and arrowhead indicate elution times for escapin and BSA, respectively. The inset shows elution volumes of molecular mass standards: BSA, 67kDa; ovalbumin, 43kDa; chymotrypsinogen A 25kDa, demonstrating that native (non-denatured) escapin has molecular mass of ca. 60kDa, similar to that of the denatured escapin, as shown in B. The photograph in A is courtesy of Genevieve Anderson.

Escapin	1	MSSAFLACALVISVHADG-VCRNRRCNK-----VCGSSYDVAIVGAGPGGANSAYMLRESGLDIAV	64
APIT	1	MSSAYLLACALVISVHADG-ICRNRRCNK-----VCGSTYDVAIVGAGPGGANSAYMLRDSGLDIAV	64
CyplasinL	1	MAVRFLAPGLLTATLVSGRTVCESKQECDA-----QCDKTLDAIVGAGAGGAYSAYLLRNKGQNIIGV	65
Aplysianin-A (Ac)	1	MAVRFLAPGLLTATLVSGRTVCESKQECDA-----QCDKTLDAIVGAGAGGAYSAYKMRYSKGKDVIGV	65
Aplysianin-A (Ak)	1	MAVRFLAPGLLTATLVSGRTVCESKQECDA-----QCDKTLDAIVGAGAGGAYSAYKMRYSKGKDVIGV	65
Achacin	1	MLLNSALFILCLVWLPPTSSSRVLTREGP-----QSRSDVAIVGAGPGGANSAYMLRDSGLDIAV	65
Apoxin I	1	-MNVFMFSLFLAALGSCAHRNPLLEEFRDTEYEEFLEIAKNGLTATSNPKRVYIVGAGMAGLSAAVLAGAGHMTV	79
Escapin	65	FEYSDRVGGRLFTYQLNPTPDVNLGGMRFIEGAMHRRWKVISELGLTPKVFKEGFGKEGRORFYLRGOSLTKKQVKS6	144
APIT	65	FEYSDRVGGRLFTYQLNPTPDVNLGGMRFIEGAMHRRWKVISELGLTPKVFKEGFGKEGRORFYLRGOSLTKKQVKS6	144
CyplasinL	66	FEFCDRVGGRLFTYQLNPTPDVNLGGMRFITGAHNLLQEGVYRGLGTPVVFTEGFGKLSRTRYYLROOSLTFOEVLIG	145
Aplysianin-A (Ac)	66	FEYSDRVGGRLFTYQLNPTPDVNLGGMRFITGAHNLLQALTKELGLESVPFTEGFGRRPRTFFLRGOSLTIDEVVKFG	145
Aplysianin-A (Ak)	66	FEYCDRVGGRLFTYQLNPTPDVNLGGMRFITGAHNLLQEVTKELGKSVLFTTEGFGRRPRTFFLRGOSLTIDEVVKFG	145
Achacin	66	FEYSNRIGGRFLTTHLPNVPDNLGGMRFIFKNHKKIFGVLYKELNLSNKEFTEGFGRRPRTFFLRGOSLTIDEVVKFG	145
Apoxin I	80	LEASERVGGRLVTRTKK--DWAYNLGPMRLPT-KHRIVREYIKKFDLKLNEFSQ--ENENAWYFIKNIRKRVREYKNN	152
Escapin	145	DVPYDLSPEEKANQGRLEVEYYLEKLTGLQNGGPKREVALKLTVPDGRFLYDLTFDEALDLVASPEGKEFARDTHVFTS	224
APIT	145	DVPYDLSPEEKANQGRLEVEYYLEKLTGLQNGGPKREVALKLTVPDGRFLYDLTFDEALDLVASPEGKEFARDTHVFTS	224
CyplasinL	146	DVPYNLTVAEKQNDNIFAFYLYKELTRFDVGGDFVTRDOLLKURVSGRRLYQLTFDEALDLVASPEGKEFARDTHVFTS	225
Aplysianin-A (Ac)	146	DVPYNLTVAEKQNDNIFAFYLYKELTRFDVGGDFVTRDOLLKURVSGRRLYQLTFDEALDLVASPEGKEFARDTHVFTS	225
Aplysianin-A (Ak)	146	DVPYNLTVAEKQNDNIFAFYLYKELTRFDVGGDFVTRDOLLKURVSGRRLYQLTFDEALDLVASPEGKEFARDTHVFTS	225
Achacin	146	DVPYNLTVAEKQNDNIFAFYLYKELTRFDVGGDFVTRDOLLKURVSGRRLYQLTFDEALDLVASPEGKEFARDTHVFTS	225
Apoxin I	153	PGLLEYPVKPSSEEGKSAAGLYVSLR-----KVVKELKRTNCKYLDKDYDTYSKYLKKEGNSLSPGAVDMIGD	223
Escapin	225	EVTLDASALISIFDDHLGEDYYSSEYILEEGMSSYPGGLQTLDAKKS-NEFFPNNHLLKALRRRTNGOYVLYFEPITSK	303
APIT	225	EVTLDASALISIFDDHLGEDYYSSEYILEEGMSSYPGGLQTLDAKKS-NEFFPNNHLLKALRRRTNGOYVLYFEPITSK	303
CyplasinL	226	EVSDDANALSYFDDHLGEDYYSSEYILEEGMSSYPGGLQTLDAKKS-NEFFPNNHLLKALRRRTNGOYVLYFEPITSK	305
Aplysianin-A (Ac)	226	EVSDDANALSYFDDHLGEDYYSSEYILEEGMSSYPGGLQTLDAKKS-NEFFPNNHLLKALRRRTNGOYVLYFEPITSK	305
Aplysianin-A (Ak)	226	EVSDDANALSYFDDHLGEDYYSSEYILEEGMSSYPGGLQTLDAKKS-NEFFPNNHLLKALRRRTNGOYVLYFEPITSK	305
Achacin	224	EFIEGVSAVNYFLVELGER--EEEILLTLDGMSALPQALADALKSSTS-HALTLLRKLOSLSKTDNGLYLLEFLEINTH	300
Apoxin I	222	LLNEGSGGYVVSFIESLKHDFIFGYEKRFDEIVGGMDQLPTSMYEAIKEK-VGVHFNARVIEIQNDREATVTNOTSANEM	300
Escapin	304	DGOTTINYLEPLKVVCAQRVILAMPVYALRQLDWSQLRNDRAQAYRAVRPMPASKVFMFTDGPWWLON--ERKSWVTK	380
APIT	304	DGOTTINYLEPLKVVCAQRVILAMPVYALRQLDWSQLRNDRAQAYRAVRPMPASKVFMFTDGPWWLON--ERKSWVTK	380
CyplasinL	306	DGKTTILKFEPLQVCTRQILALPVPALMQVDWPPLEENRAQAKAYAVRTIPASKVFMFTDGPWWLON--ERKSWVTK	385
Aplysianin-A (Ac)	305	DGKTTILKFEPLQVCTRQILALPVPALMQVDWPPLEENRAQAKAYAVRTIPASKVFMFTDGPWWLON--ERKSWVTK	385
Aplysianin-A (Ak)	305	DGKTTILKFEPLQVCTRQILALPVPALMQVDWPPLEENRAQAKAYAVRTIPASKVFMFTDGPWWLON--ERKSWVTK	385
Achacin	301	GGYTESNIT-DLVCAKQVILAIPOSALIHLDWKPLRSETVNEAFNAVKFIPTSKVFLTFPTAWWLSQ-AVKNPAFVVK	377
Apoxin I	301	SS-----VTADYVIVCTTSRAARRIKKEPPLPPKKAHALRSVHYRSGTKTFLCKKKEWEDD--GIRGGKS	364
Escapin	381	SDALFSOMYDWOKEASGDYILIASYADGKLAQYLRLELKNQEDIPGSDPGYNOVTVPLKDAIEHLTEAYGVERDSIPE	460
APIT	381	SDALFSOMYDWOKEASGDYILIASYADGKLAQYLRLELKNQEDIPGSDPGYNOVTVPLKDAIEHLTEAYGVERDSIPE	460
CyplasinL	386	GDTPFSOMYDWKSNVSGDYILIASYADGNNTLVQKVLRDQGGPINSSEACAHIVSEPLKNIIDHLSEAYGVERDSIPE	465
Aplysianin-A (Ac)	385	GDTPFSOMYDWKSNVSGDYILIASYADGNNTLVQKVLRDQGGPINSSEACAHIVSEPLKNIIDHLSEAYGVERDSIPE	464
Aplysianin-A (Ak)	385	GDTPFSOMYDWKSNVSGDYILIASYADGNNTLVQKVLRDQGGPINSSEACAHIVSEPLKNIIDHLSEAYGVERDSIPE	464
Achacin	378	STSPFSOMYDWKSNVSGDYILIASYADGNNTLVQKVLRDQGGPINSSEACAHIVSEPLKNIIDHLSEAYGVERDSIPE	464
Apoxin I	365	TTDLPSRFIYYPNHFISGVGVIIAYGIGDANFQALDFKD-----CADIVINDLSLIHQPLKQT	428
Escapin	461	PV-TAASQFWTDYFPFGCGWI-TWRAGYHFDVVISMRRRPSLKDEVYVVGSDYSWGLISSWIEGALETSENVINDYFLL--	535
APIT	461	PV-TAASQFWTDYFPFGCGWI-TWRAGYHFDVVISMRRRPSLKDEVYVVGSDYSWGLISSWIEGALETSENVINDYFLL--	535
CyplasinL	466	PK-TAVSKFWTDYFPFGCGWI-TWRAGYHFDVVMNTRRRPSLKDEVYVVGSDYSWGLISSWIEGALETSENVINDYFLL--	544
Aplysianin-A (Ac)	465	PK-TAVSKFWTDYFPFGCGWI-TWRAGYHFDVVMNTRRRPSLKDEVYVVGSDYSWGLISSWIEGALETSENVINDYFLL--	543
Aplysianin-A (Ak)	465	PK-TAVSKFWTDYFPFGCGWI-TWRAGYHFDVVMNTRRRPSLKDEVYVVGSDYSWGLISSWIEGALETSENVINDYFLL--	543
Achacin	458	PK-SGTISQFWSSYPFEGDWTWKAGYHCEYTYQYIETRRPSLIDDFVYVVGSDYVNCIENAWTESAFLSVENVFEKYF--	531
Apoxin I	429	FCRPSMTDRWSLDKYAMSGITTFTPMDFQHFSEALTAPKRIYFAGEYTAQFHWIDSTIKSGTAAARDVNRASENPSGI	508
Escapin	535	-----	535
APIT	535	-----	535
CyplasinL	545	HNVPPPSHMASHVQ	550
Aplysianin-A (Ac)	544	D-FPNNNHLGSHMA	556
Aplysianin-A (Ak)	544	K-PPSVDHLDHMA	556
Achacin	531	-----	531
Apoxin I	509	HLSNDNEF-----	516

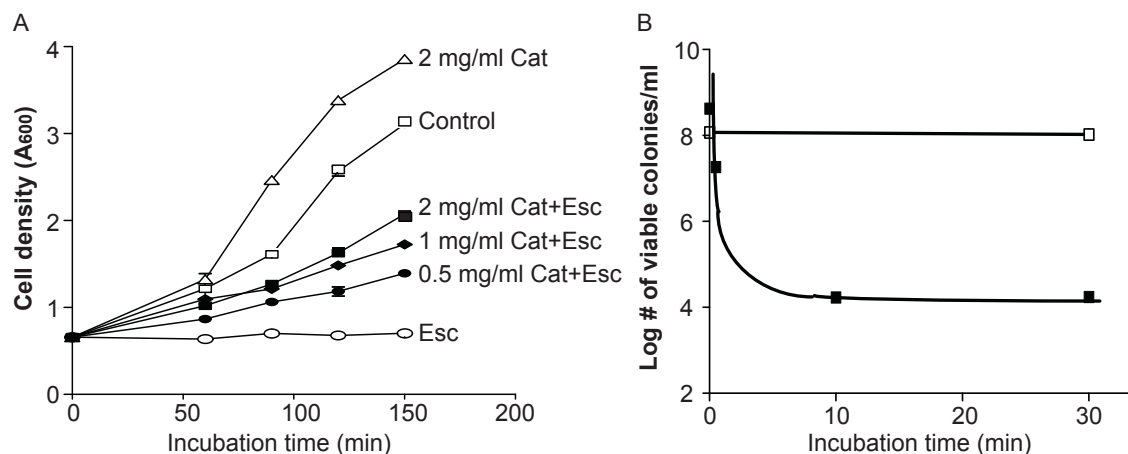
**Figure 2.2. Amino Acid sequence alignment of escapin and related proteins.** Solid and broken underlines indicate DMB and GG motifs, respectively (see text for details). \*Predicted signal sequence cleavage site at A 18 and D 19. \*\* Predicted glycosylation site at Thr 463. Boxed areas indicate regions of homology.



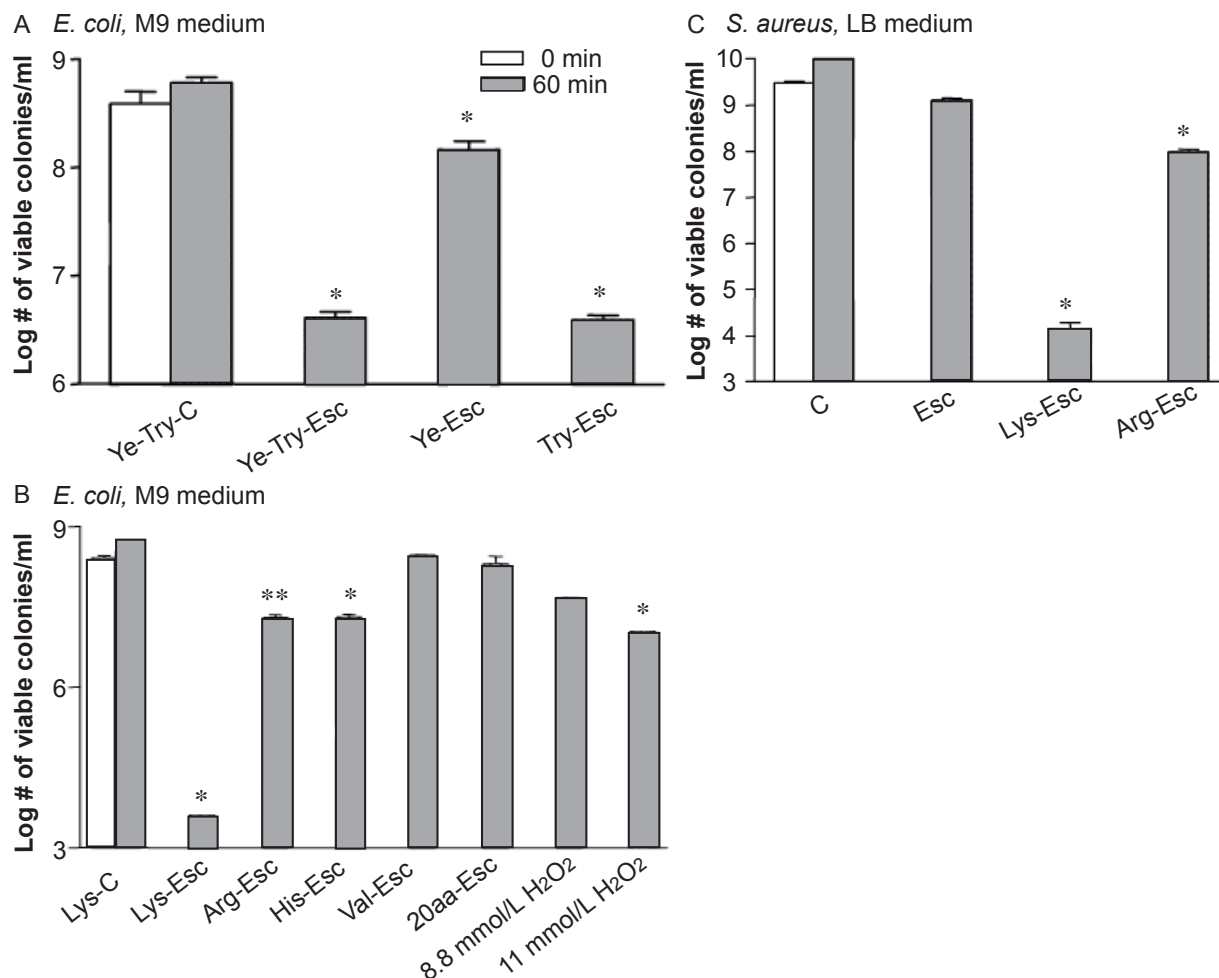
**Figure 2.3. The yellow pigment associated with escapin is FAD.** (A) Aromatic region of  $^1\text{H}$  NMR spectrum for FAD standard and the yellow pigment from escapin, showing identical signals. (B) Positive ion ESI-TOF mass spectrum of the yellow pigment from escapin. See text for explanation of these signals.



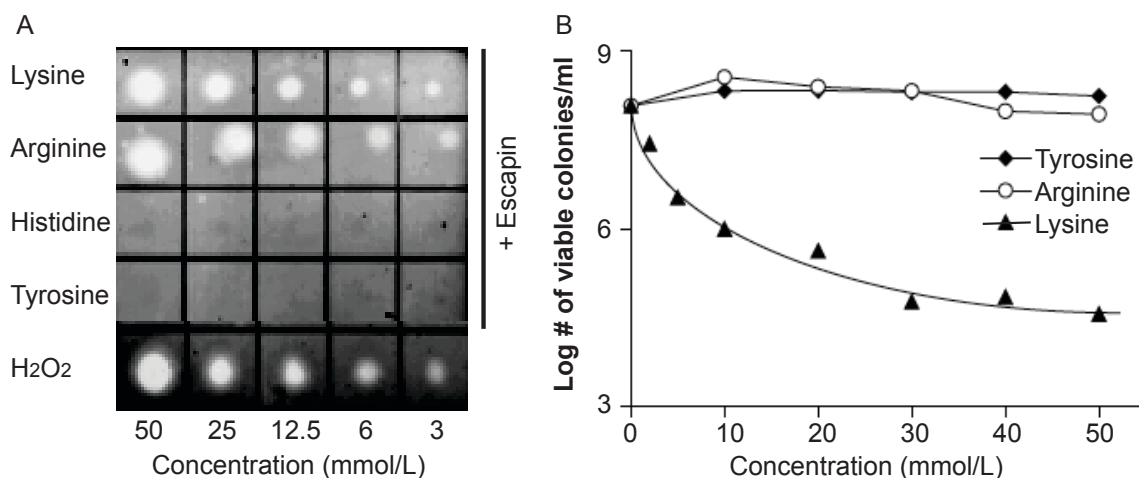
**Figure 2.4. LAO enzyme activity of escapin and its substrate specificity.** 0.6  $\mu\text{g}$  escapin in 100  $\mu\text{l}$  was incubated at 22°C for 1 min in 2 mmol  $\text{l}^{-1}$  of each L-amino acid and taurine. LAO activity was measured by absorbance at 436 nm and normalized to the value for arginine. Values are mean  $\pm$  S.E.M.,  $N=2$ . Inset shows a Lineweaver-Burk plot of data for LAO activity at different concentrations for lysine and arginine. Values are mean  $\pm$  S.E.M.,  $N=2$ .  $K_m$  and  $V_{\max}$  values calculated from this experiment are shown.



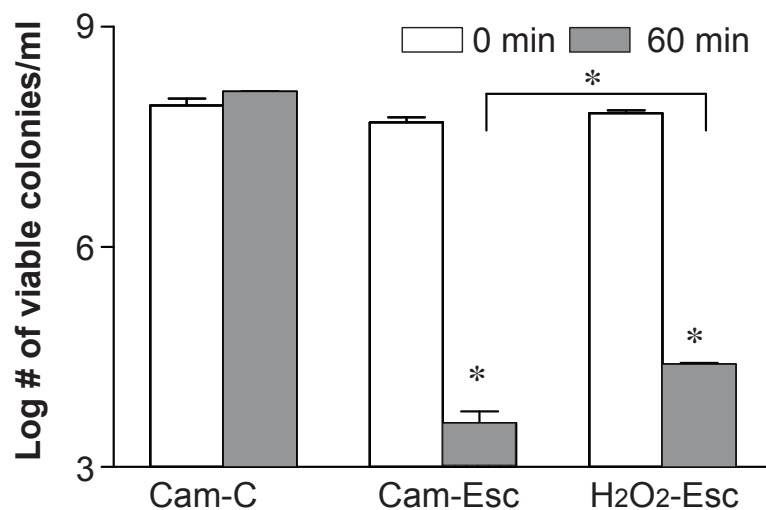
**Figure 2.5. Escapin can be bacteriostatic or bactericidal.** Escapin can be bacteriostatic or bactericidal. (A) Inhibition of growth. *E. coli* cells were incubated in LB medium at 37°C with 10  $\mu\text{g ml}^{-1}$  escapin (Esc) or without escapin (Control: buffer added instead), and with or without 0.4-1.6mg catalase (Cat). Absorbance at 600 nm was measured at the indicated incubation times to determine cell density. (B) Bactericidal effect. *E. coli* cells were grown in LB medium containing  $3 \times 10^8$  cells  $\text{ml}^{-1}$ , then incubated in escapin (10  $\mu\text{g ml}^{-1}$ ) in LB medium at 37°C (closed squares) or 0°C (open squares). Values are means  $\pm$  S.E.M.,  $N=3$ .



**Figure 2.6. Escapin's bactericidal effect was greatest in the enriched growth medium and L-lysine.** (A, B) *E. coli* and (C) *Staphylococcus aureus* cells in early-log growth phase were cultured in M9-glucose medium (for *E. coli*) or LB (for *S. aureus*), and 4 samples of equal cell density were resuspended in different growth media in the presence of escapin (Esc: 50  $\mu\text{g ml}^{-1}$ ) or (C) buffer control. Cells were then incubated at 37°C for 60 min. Ye, 1% yeast extract; Try, 1% tryptone Peptone; Lys, 50  $\text{mmol l}^{-1}$  L-lysine; Arg, 50  $\text{mmol l}^{-1}$  L-arginine; His, 50  $\text{mmol l}^{-1}$  L-histidine; Val, 50  $\text{mmol l}^{-1}$  L-valine; aa, amino acid mixture containing 20 L-amino acids each at 50  $\mu\text{mol l}^{-1}$ . In a follow-up experiment, a 10 times higher concentration of Ye or aa was used, and similar results were observed. Values are means  $\pm$  S.E.M.,  $N=3$ . An asterisk indicates a significant reduction in the number of viable clones ( $p < 0.05$ ,  $t$ -test)



**Figure 2.7. Concentration dependence of the effects of amino acids on escapin's antimicrobial activity.** (A) Plate assay of growth inhibition, *E. coli* cells were grown at 37°C on a plate in minimal medium, (M9+glucose) in the presence of amino acids and escapin, or in hydrogen peroxide alone (without escapin) at concentrations from 3 to 50 mmol l<sup>-1</sup>. (B) Bactericidal assay. *E. coli* cells were grown in M9+glucose containing 3x10<sup>8</sup> cells ml<sup>-1</sup>, then incubated at 37°C with escapin (60 µg ml<sup>-1</sup>) in M9+glucose with L-lysine, L-arginine, or L-tyrosine at 3-50 mmol l<sup>-1</sup>. Values are means ± S.E.M., N=3.



**Figure 2.8. Escapin's bactericidal effect does not require protein synthesis.** *E. coli* at 2x10<sup>8</sup> cells ml<sup>-1</sup> were incubated with or without 50 µg ml<sup>-1</sup> chloramphenicol (Cam), an inhibitor of protein synthesis, in the presence of 50 µg ml<sup>-1</sup> escapin (Esc) or buffer control (C) in LB medium at 37°C. Values are means ± S.E.M., N=3. An asterisk indicates a significant reduction in the number of viable clones ( $p < 0.05$ ,  $t$ -test).

**Table 2.1. Antimicrobial activity of wild-type escapin.**

Microbial species	MIC (µg/ml)
Gram-negative Bacteria	
<i>Escherichia coli</i> (MC4100)	0.62
<i>Salmonella typhimurium</i> AA 140	0.62
<i>Pseudomonas aeruginosa</i> PAO1	0.31
<i>Vibrio harveyi</i> BB170	0.25
Gram-positive Bacteria	
<i>Staphylococcus aureus</i> 6835	0.31
<i>Streptococcus pyogenes</i> NZ131	0.62
<i>Bacillus subtilis</i> 168	2.50
<i>Bacillus subtilis</i> WB600	2.50
Yeast	
<i>Candida krusei</i>	5.0
<i>Saccharomyces cerevisiae</i> BY4761	5.0
Fungus	
<i>Cladosporium notitalics</i>	
in SD medium with 0.8 mmol/L lysine	62
in SD medium with 4.9 mmol/L lysine	15
The minimum inhibition concentration (MIC) of escapin against various microbes was determined by measuring inhibition of growth on plates of solid media	

**3 Identification of potent bactericidal compounds produced by Escapin, an L-amino acid oxidase in the ink of the sea hare *Aplysia californica***

This work has been published as: Ko-Chun Ko, Binghe Wang, Phang C. Tai, and Charles D. Derby. (2008). Identification of Potent Bactericidal Compounds Produced by Escapin, an L-Amino Acid Oxidase in the Ink of the Sea Hare *Aplysia californica*. *Antimicrobial Agents and Chemotherapy*. 52, 4455-4462



### 3.1 Summary

The ink of sea hares (*Aplysia californica*) contains escapin, an L-amino acid oxidase that metabolizes L-lysine, thereby producing a mixture that kills microbes and deters attacking predators. This secretion contains hydrogen peroxide, ammonia, and an equilibrium mixture of “escapin intermediate product” (EIP-K) that includes  $\alpha$ -keto- $\epsilon$ -aminocaproic acid and several other molecules. Components of the equilibrium mixture react nonenzymatically with  $\text{H}_2\text{O}_2$  to form “escapin end product” (EEP-K), which contains  $\delta$ -aminovaleric acid and  $\delta$ -valerolactam. The proportions of the molecules in this equilibrium mixture change with pH, and this is biologically important because the secretion is pH 5 when released but becomes pH 8 when fully diluted in seawater. The goal of the current study was to identify which molecules in this equilibrium mixture are bactericidal. We show that a mixture of  $\text{H}_2\text{O}_2$  and EIP-K, but not EEP-K, at low mM concentrations is synergistically responsible for most of the bactericidal activity of the secretion against *Escherichia coli*, *Vibrio harveyi*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Low pH enhances the bactericidal effect, and this does not result from stress associated with low pH itself. Sequential exposure to low mM concentrations of EIP-K and  $\text{H}_2\text{O}_2$ , in either order, does not kill *E. coli*. Reaction products formed when L-arginine is substituted for L-lysine have almost no bactericidal activity. Our results favor the idea that the bactericidal activity is

due to unstable intermediates of the reaction of  $\alpha$ -keto- $\epsilon$ -aminocaproic acid with  $\text{H}_2\text{O}_2$ .

### 3.2 Introduction

Defensive chemical secretions are produced by many animals, including a marine snail, the sea hare *Aplysia californica*. When vigorously attacked by predators, sea hares release an inky secretion (see Fig. 2.1A). This ink secretion is a mixture of the products of two glands: a purplish ink from the ink gland and a whitish, mucousy ink from the opaline gland (1, 57). This secretion protects sea hares through a diverse number of chemicals and mechanisms, some acting on predators to abate their attack and others acting on conspecific sea hares as alarm cues to evoke escape behaviors (3-8, 47, 58).

One of the bioactive ingredients in the ink gland secretion of *A. californica* is escapin, an  $\text{L}$ -amino acid oxidase (LAEO). Escapin has orthologs in the ink of other sea hare species and paralogs in the eggs and other tissues of *A. californica* (reviewed in references (3) and (59)). Substrates of escapin and its homologs include  $\text{L}$ -lysine and  $\text{L}$ -arginine (17). However,  $\text{L}$ -lysine is the major natural substrate, since the concentration of  $\text{L}$ -lysine in the secretion is much higher than that of  $\text{L}$ -arginine. In the *A. californica* secretion,  $\text{L}$ -lysine and  $\text{L}$ -arginine are present at concentrations of 145 and 0.5 mM, respectively.

The chemistry of the escapin/  $\text{L}$ -lysine pathway is summarized in Fig. 3.1. This series of chemical reactions produces products that interact with each other to form a diverse group of molecules at low millimolar concentrations within a few seconds of the release of the secretion (60). The first step is escapin's oxidative deamination of  $\text{L}$ -lysine (compound **1**), which produces an equilibrium mixture of compounds that we call "escapin intermediate products of lysine" (EIP-K); they include  $\alpha$ -keto- $\epsilon$ -aminocaproic acid (compound **2**),  $\Delta^1$ -piperideine-2-carboxylic acid (compound **3**),  $\Delta^2$ -piperideine-2-carboxylic acid (compound **4**), 6-amino-2-hydroxy-hex-2-enoic acid (compound **7**), possibly 6-amino-2, 2-dihydroxy-hexanoic acid (compound **8**), 2-hydroxy-piperidine-2-carboxylic acid (compound **9**),  $\text{H}_2\text{O}_2$ , and ammonium. Three of these compounds, compounds **2**, **3**, and **4**, then react nonenzymatically with  $\text{H}_2\text{O}_2$  to yield a mixture of  $\delta$ -aminovaleric acid (compound **5**) and  $\delta$ -valerolactam (compound **6**), which we call "escapin end products of  $\text{L}$ -lysine" (EEP-K). The pH of *A. californica* ink is  $\sim 5.0$  at full strength, in contrast to a pH of  $\sim 8.0$  for seawater (61). This is significant because pH affects the equilibrium among the escapin reaction products: though the cyclic forms **3** and **6** dominate at any pH, the naturally low pH of the secretion favors the linear forms **2** and **5** (60).

Sea hare LAAOs have been of interest primarily to biomedical researchers in search of antitumor and antimicrobial compounds (reviewed in references (3) and (59)). There is very little known about antipredatory functions of molecules in the pathway of sea hare LAAOs (60, 62). Most studies of sea hare and other gastropod LAAOs identified  $\text{H}_2\text{O}_2$  as the primary antimicrobial product of this

pathway, though other compounds are implicated (reviewed in references (3, 19, 23, 59)). Escapin was tested on 11 types of microbes, and it was found to be bacteriostatic for all (17). Against *Escherichia coli*,  $H_2O_2$  at concentrations below 10mM accounts for most of the bacteriostatic effects of escapin's products, but it contributes relatively little (10x reduction in bacterial number) compared to the powerful bactericidal effects of escapin's products (up to  $10^7$ x reduction) (17). The reaction of escapin with L-arginine produces a mixture with almost no bactericidal activity, even though the product contains more  $H_2O_2$  than the product of the reaction of escapin with L-lysine (17). This demonstrates that compounds other than  $H_2O_2$  are responsible for the bactericidal effects of escapin.

The goal of this study was to identify the bioactive components of the complex equilibrium mixture of the escapin/ L-lysine pathway by examining their bactericidal effects. We show that only a few of these compounds, in specific combinations, are bactericidal.

### 3.3 Materials and Methods

**Animals.** Sea hares (*Aplysia californica* Cooper 1863) were collected in California by Marinus Scientific (Garden Grove, CA). The sea hares were dissected on the day of arrival in our laboratory. Experiments were performed according to Georgia State University regulations and national guidelines.

**Collection of ink and isolation of escapin.** Ink glands were dissected from anesthetized animals and frozen at  $-80^{\circ}\text{C}$  until they were used. Purple ink was collected by gently squeezing dissected ink glands in a Petri dish with the blunt end of a scalpel handle. Escapin (ATCC accession no. AY615888) was isolated and purified by using an ÄKTA 100 automated fast protein liquid chromatography system. A two-step purification process involving an initial size separation followed by a purification using a cation-exchange Mono S column was performed according to the method of Yang et al. (17).

**Preparation of the products of oxidation of L-amino acids by escapin.** To produce EIP-K or EIP-R, we typically incubated 55 mM L-lysine or L-arginine monohydrochloride,  $1 \times 10^{-3}$  mg/ml escapin, and 0.13 mg/ml catalase in deionized water at  $30^{\circ}\text{C}$  on a shaker for up to 24 hours. This solution was filtered using an Amicon Ultra-4 centrifugal filter device (Millipore Corp., Billerica, MA) to remove escapin and catalase and then stored it at  $-80^{\circ}\text{C}$  until it was used further. To produce EEP-K or EEP-R, we performed the same procedure except catalase was not added to it. The concentration of the EIP-K mixture was expressed as the starting concentration of L-lysine.

**Chemicals and the synthesis of components in EIP-K.** L-lysine (compound **1**),  $\delta$ -aminovaleric acid (compound **5**),  $\delta$ -valerolactam (compound **6**), L-arginine, catalase, and  $\text{H}_2\text{O}_2$  were purchased from Sigma-Aldrich (St. Louis, MO). We synthesized a solution containing  $\alpha$ -keto- $\epsilon$ -aminocaproic acid (compound **2**), the de-

hydropipecolinic acid enamine/imine tautomer mixture (compounds **3** and **4**), 6-amino-2-hydroxy-hex-2-enoic acid (compound **7**), 2-hydroxy-piperidine-2-carboxylic acid (compound **9**), and possibly 6-amino-2, 2-dihydroxy-hexanoic acid (compound **8**), as performed by Kamio et al.(60); we call this synthetic EIP-K. Since both synthetic EIP-K and natural EIP-K are dominated by compound **3**, we quantified their concentration using the NMR signal of compound **3** with 50 nmol of 3-(trimethylsilyl) propionic-2,2,3,3-d<sub>4</sub> acid sodium salt (TSP-d<sub>4</sub>; Sigma-Aldrich) as an internal standard and expressed the concentration relative to the starting concentration of L-lysine.

**Bactericidal assay.** *Escherichia coli* strains MC4100 and C921-b2, *Vibrio harveyi* strain BB170, *Staphylococcus aureus* strain ATCC 6835, and *Pseudomonas aeruginosa* strain PAO1 were used in our assays. Bacteria were incubated until they reached a density of  $\sim 3 \times 10^8$  cells/ml in Luria-Bertani medium (LB) at pH 7, and they were then transferred to LB at pH 7 or LB with adjusted pH buffer. Bacteria were treated with chemicals and controls in a Thermomixer (37°C; Eppendorf) for 10 min. Samples were serially diluted and plated onto Petri dishes with solid LB at pH 7 and incubated overnight at 37°C. Viable cell counts were determined by enumeration of CFU with appropriate dilutions. Given that L-lysine is present at  $\sim 145$  mM in full-strength secretion (4) and given the kinetics of escapin under natural conditions, escapin is expected to generate 1 to 10 mM of H<sub>2</sub>O<sub>2</sub> and other intermediate products within 10s (60). Thus, most of our bioassays used low mM concentrations of products. The statistical significance of bac-

tericidal effects was analyzed with paired  $t$  tests or analysis of variance with  $\alpha$  value of 0.05.

### 3.4 Results and Discussion

#### **Bactericidal activity of the products of escapin's intermediate and end products of $L$ -lysine and $L$ -arginine.**

We used *Escherichia coli* MC4100 to test the bactericidal activity of products generated by the oxidative deamination of  $L$ -lysine or  $L$ -arginine by escapin at pH 7 (Fig. 3.2). The mixture of 45 mM EIP-K and 10 mM  $H_2O_2$  produced by far the greatest bactericidal activity, a  $>7$ -logs unit reduction in cell number. Its effect was significantly greater than that of either EIP-K alone (no reduction from the control) or  $H_2O_2$  alone ( $\sim 1$ -log unit reduction, which is significantly greater than that of its control). The mixture of 45 mM EEP-K and 10 mM  $H_2O_2$  produced a  $\sim 2$ -log unit reduction in cell number, which was a greater bactericidal effect than that seen with  $H_2O_2$  but much less than that seen with EIP-K and  $H_2O_2$ . The mixture of EIP-R and  $H_2O_2$  was not bactericidal (the same as EIP-R alone), even though  $L$ -arginine was as effective as  $L$ -lysine as a substrate for escapin. EEP-R was not bactericidal. EEP-R with  $H_2O_2$  was significantly though mildly bactericidal, producing a  $\sim 2$ -log unit reduction in cell number.

The bactericidal effects of EIP-K,  $H_2O_2$ , and EIP-K +  $H_2O_2$  were then compared for four bacteria: *Escherichia coli* C921-b2, a non-virulent form of a pathogenic strain of this gram-negative species; *Vibrio harveyi*, a Gram-negative ma-

rine species; *Staphylococcus aureus*, a gram-positive and pathogenic species; and *Pseudomonas aeruginosa* PAO1, a gram-negative pathogenic species (Fig. 3.3). These four species were used because escapin was previously shown to be inhibitory against each of them (17). Our results show that EIP-K alone up to 27.5 mM (Fig. 3.3 a1 to d1) or H<sub>2</sub>O<sub>2</sub> alone up to 5 mM (Fig. 3.3 a2 to d2) was not bactericidal against any of them. However, when mixed, EIP-K and H<sub>2</sub>O<sub>2</sub> were potently and significantly bactericidal, in a concentration-dependent manner ( $p < 0.05$ ; analysis of variance). The effect was strongest for *P. aeruginosa* and weakest for *S. aureus*. The bactericidal effect on *E. coli* was lower at the highest concentration, 27.5 mM EIP-K + 5 mM H<sub>2</sub>O<sub>2</sub>. Our finding that *P. aeruginosa* is extremely sensitive to these chemicals is of interest, given that this bacterium is a human pathogen that is often highly resistant to antimicrobial agents (63).

We used *E. coli* C921-b2 as a representative species in all subsequent experiments to determine mechanisms of the bactericidal effect.

### **Identification of bactericidal compounds in escapin's pathway.**

We compared the bactericidal activity of natural EIP-K (generated as in the previous experiment using escapin) and the bactericidal activity of synthetic EIP-K (synthesized as performed by Kamio et al. (60) and which contains an equilibrium mixture of compounds **2** to **4** and **7** to **9**). Both are mixtures dominated by compound **3**. The two mixtures, when in the presence of H<sub>2</sub>O<sub>2</sub>, showed similar bactericidal activities (Fig. 3.4a). The bioactivity was concentration dependent up to 20 mM. The similarity of these functions supports the conclusion



that the bactericidal activity of natural EIP-K is due to the interaction of one or more of the components of the equilibrium mixture of compounds **2** to **4** and **7** to **9** with  $\text{H}_2\text{O}_2$ . EIP-K, either natural or synthetic, at high concentrations and in the presence of 5 mM  $\text{H}_2\text{O}_2$  was less bactericidal than EIP-K at some lower concentrations (Fig. 3.4a; see also Fig. 3.3). This type of concentration-response relationship is known as the Eagle effect and has been reported for a variety of microbes and antimicrobial agents (64-66). The mechanism responsible for the Eagle effect in our study is unknown, but it is worth noting that the curve was U-shaped for either a variable concentration of EIP-K in a constant concentration of  $\text{H}_2\text{O}_2$  or a variable concentration of  $\text{H}_2\text{O}_2$  in a constant concentration of EIP-K (Fig. 3.3 a1 and a2). Also, the concentrations at which the inflection in the curve occurred differed, though inflections typically occurred at >20 mM EIP-K, as can be seen by the relatively high variance for this concentration of EIP-K (Fig. 3.3 a1 and a2).

The components of EEP-K, i.e.,  $\delta$ -aminovaleric acid (compound **5**) and  $\delta$ -valerolactam (compound **6**), presented either alone or in combination with 5 mM  $\text{H}_2\text{O}_2$  had very little bactericidal activity or no bactericidal activity (Fig. 3.4b).

These results, together with those shown in Fig. 3.2 and 3.3, demonstrate that the interaction of  $\text{H}_2\text{O}_2$  with one or more of the compounds **2** to **4** or **7** to **9**, but not compound **5** or **6**, is responsible for the bactericidal activity.

### **Influence of pH in the bactericidal activity.**

The compositions of the reaction products of escapin change with pH of the solution (Fig. 3.1). The linear forms **2** and **5** become relatively more abundant under acidic conditions, although the cyclic forms **3** and **6** always dominate (60). We expect that pH changes will affect the bactericidal activity of the mixture if some components are more bactericidal than others. Thus, to determine which components of the escapin pathway are bactericidal, we examined the effect of pH on the bactericidal activities of EIP-K and the synthetic product in the presence or absence of  $\text{H}_2\text{O}_2$  (Fig. 3.5). The intensity of the bactericidal effect of EIP-K +  $\text{H}_2\text{O}_2$  and synthetic product +  $\text{H}_2\text{O}_2$  changed with pH, being greatest at pH 6, intermediate at pH 7, and virtually absent at pH 8 (Fig. 3.5a). This suggests that the linear form **2**, when mixed with  $\text{H}_2\text{O}_2$ , has bactericidal activity and that the cyclic forms **3** and **4**, when mixed with  $\text{H}_2\text{O}_2$ , do not. To determine if the stronger bactericidal effect at a lower pH was caused by attenuation of the viability of bacteria under stress, we treated bacteria with another form of stress, temperature shock, at pH values of 6 to 8 (Fig. 3.5b). Our results showed that pH did not affect bacterial viability at temperatures as high as  $45^\circ\text{C}$ , suggesting that the effect of pH is not due to a nonspecific stress but rather is due to a chemical-specific effect: the mixture of compound **2** +  $\text{H}_2\text{O}_2$  is responsible for most of the bactericidal activity of escapin.

### **Bactericidal effect of EIP-K and $\text{H}_2\text{O}_2$ requires simultaneous presentation.**

The bactericidal effect of the mixture of EIP-K and  $\text{H}_2\text{O}_2$  might result either

from an unstable intermediate of the reaction of  $\text{H}_2\text{O}_2$  with compound **2** or from  $\text{H}_2\text{O}_2$  and compound **2** acting in combination to kill bacteria. To explore the bactericidal mechanism, we presented EIP-K and  $\text{H}_2\text{O}_2$  sequentially rather than simultaneously and also presented them in different orders. If the sequential presentation is bactericidal, this would be evidence against the bactericidal components being unstable intermediates. The results showed that EIP-K and  $\text{H}_2\text{O}_2$  must be simultaneously presented to be bactericidal (Fig. 3.6), thus supporting the idea that the bioactive molecules are unstable intermediates of the reaction of compound **2** and  $\text{H}_2\text{O}_2$ .

#### **Bactericidal effect of EIP-K and $\text{H}_2\text{O}_2$ does not require protein synthesis.**

The bactericidal effect of the mixture of EIP-K and  $\text{H}_2\text{O}_2$  was not affected by exposure to a protein synthesis inhibitor, either puromycin or chloramphenicol (Fig. 3.7). This result is consistent with the results of Yang et al. (17), who used escapin in LB, and supports the conclusion that this bactericidal mechanism does not require new protein synthesis.

### **3.5 Conclusion**

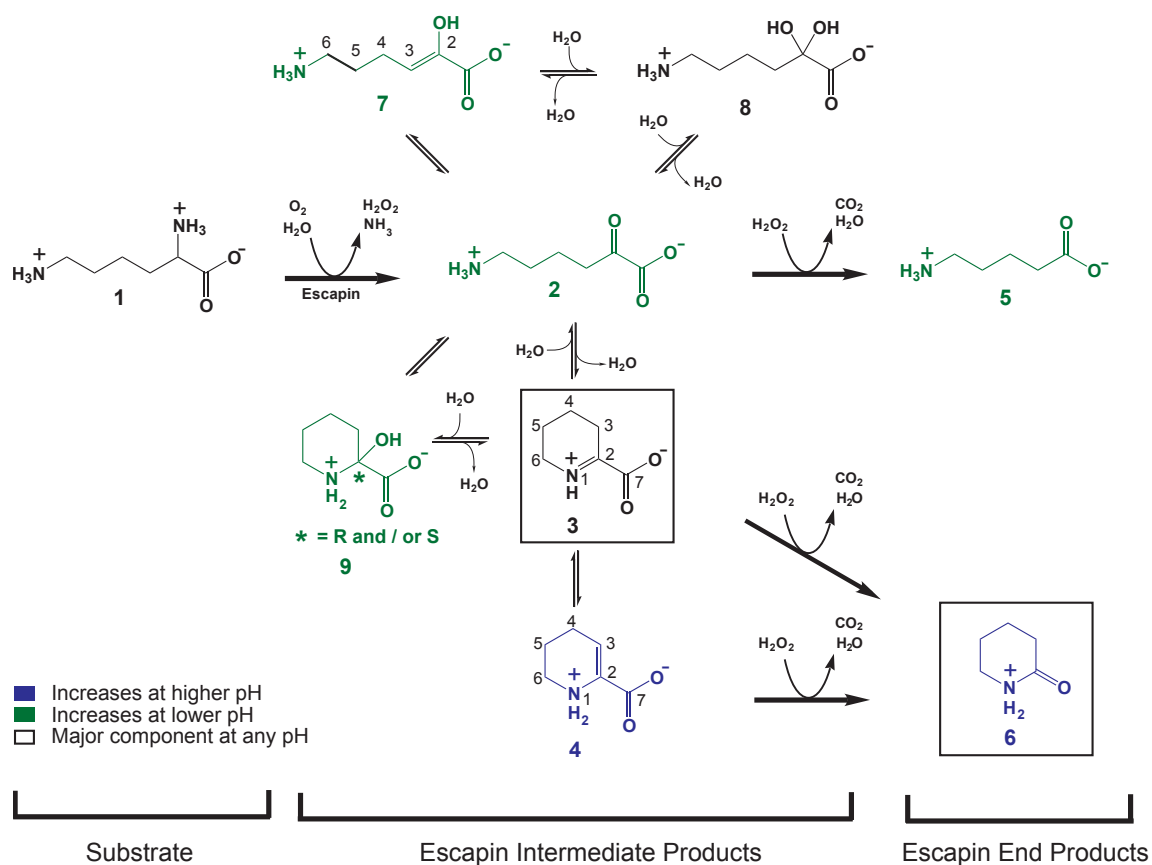
The defensive secretion of sea hares is a complex mixture, of which the molecules generated by escapin, an LAAO that uses L-lysine as its substrate, form an equilibrium mixture whose balance depends on pH, as summarized in Fig. 3.1. These escapin products are both bacteriostatic and bactericidal.  $\text{H}_2\text{O}_2$

accounts for most of the bacteriostatic activity and a significant amount though relatively small portion of the bactericidal activity:  $\text{H}_2\text{O}_2$  at up to 10 mM kills ~90% of the bacteria, but this is much less than the value corresponding to the mixture, which reduces bacterial levels to  $10^{-7}$  times the pre-exposure levels. The bactericidal effect is rapid and does not require protein synthesis. A strong correlation between the effect of pH on the relative concentrations and on the bactericidal activity of molecular species in the equilibrium mixture strongly suggests that the mixture of  $\text{H}_2\text{O}_2$  and  $\alpha$ -keto- $\epsilon$ -aminocaproic acid (compound **2**) accounts for most of the bactericidal activity of escapin, as depicted in Fig. 3.8. Tests using simultaneous or sequential presentation of compounds strongly suggest that the bactericidal effects are unstable intermediates generated by chemical interactions between compound **2** and  $\text{H}_2\text{O}_2$ , but the effects are not due to  $\delta$ -aminovaleric acid (compound **5**), the end product of the reaction of compound **2** and  $\text{H}_2\text{O}_2$ . Our work is consistent with other studies on LAAOs of various types showing that  $\text{H}_2\text{O}_2$  is a potent antimicrobial agent (e.g., references 15 and 16), but it provides the novel finding that other molecules generated by reactions between products of LAAOs are much more potent antimicrobials. Future studies can focus on detailed mechanisms of this unusual synergistic bactericidal effect, identifying anti-predatory components of the escapin pathway, and testing if similar effects occur for other LAAOs.

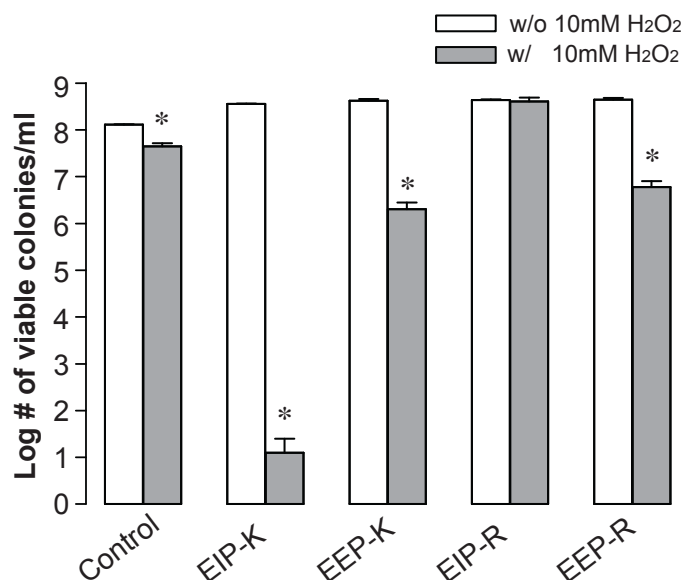
### 3.6 Acknowledgement

We thank Shilong Zheng for synthesizing compounds used in this study, Michiya Kamio for discussions and assistance with preparing Fig. 3.1 and 3.8, Jonathan Beckwith (Harvard Medical School) and June Scott (Emory University) for providing *E. coli* MC4100 and C921-b2, respectively, and Hanan El-Mayas for critical comments on the manuscript.

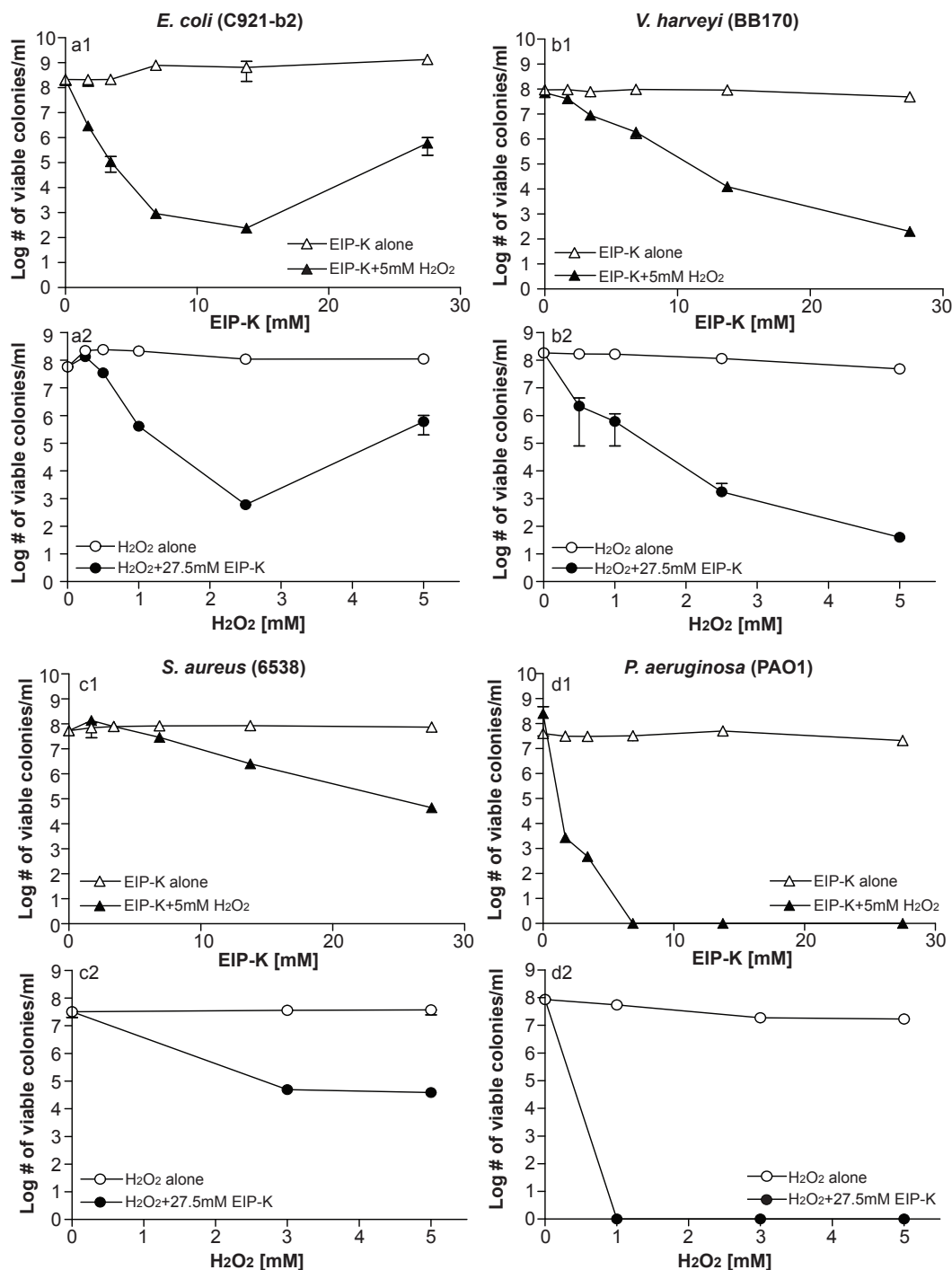
This work was supported by NSF grants IBN-0324435 and 0614685, NIH grant GM-34766, a grant from the Georgia State University Brains and Behavior program, a fellowship to Ko-Chun Ko from the Georgia State University Molecular Basis of Disease Program, and the Georgia Research Alliance.



**Figure 3.1. Summary of the chemistry of reaction of escapin with  $L$ -lysine, including the effects of pH on the relative composition of the molecular species in the equilibrium mixture.**

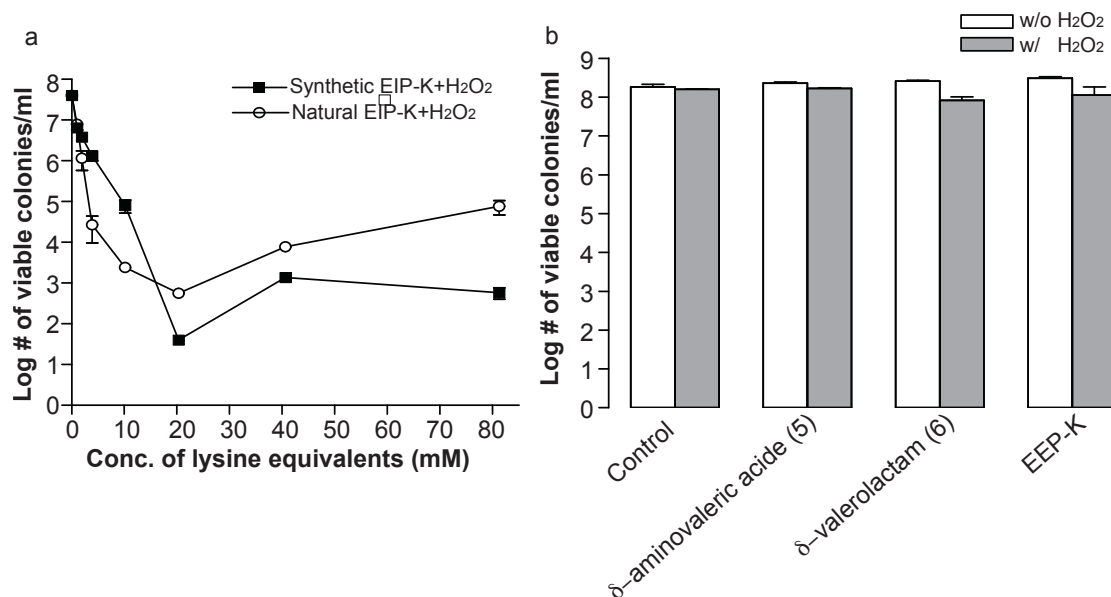


**Figure 3.2. Bactericidal effects of escapin intermediate products and escapin end products in the absence (w/o) or presence (w/) of 10 mM H<sub>2</sub>O<sub>2</sub>, at pH 7, on *E. coli* MC4100.** Concentration of the intermediates and end products are based on a starting substrate concentration of 45mM L-lysine (for EIP-K and EEP-K) or 45mM L-arginine (for EIP-R and EEP-R). Values are means  $\pm$  standard errors of the means for three replicates from a representative experiment. Asterisks indicate that the value for product with H<sub>2</sub>O<sub>2</sub> is significantly lower than that for product without H<sub>2</sub>O<sub>2</sub> ( $p < 0.05$ ; paired  $t$  test).

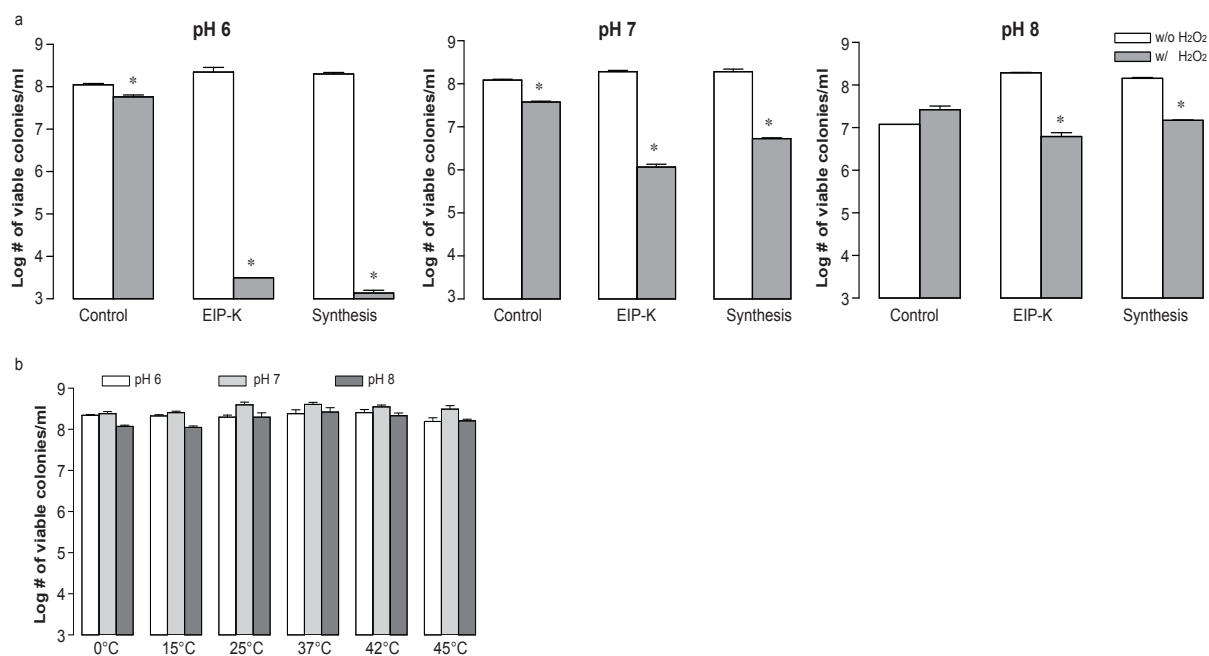


**Figure 3.3. Bactericidal effects of EIP-K, H<sub>2</sub>O<sub>2</sub>, and EIP-K+ H<sub>2</sub>O<sub>2</sub>, for four species of bacteria: *Escherichia coli* strain C921-b2, *Vibrio harveyi* strain BB170, *Staphylococcus aureus* strain 6825, and *Pseudomonas aeruginosa* strain PAO1. Each figure depicts results of one to four experiments, with three replicates per experiment. Panels a1 to d1 depict concentration-response functions for EIP-K alone or EIP-K with 5mM H<sub>2</sub>O<sub>2</sub>. Panels a2 to d2 depict concentration-response functions for H<sub>2</sub>O<sub>2</sub> alone or H<sub>2</sub>O<sub>2</sub> with 27.5mM EIP-K. The antimicrobial effect was greater for the mixture of EIP-K and H<sub>2</sub>O<sub>2</sub> than for either individual component.**

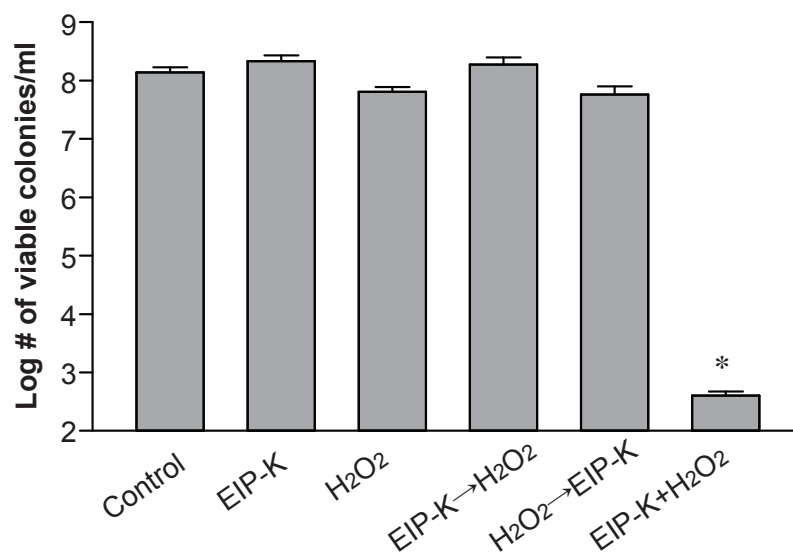




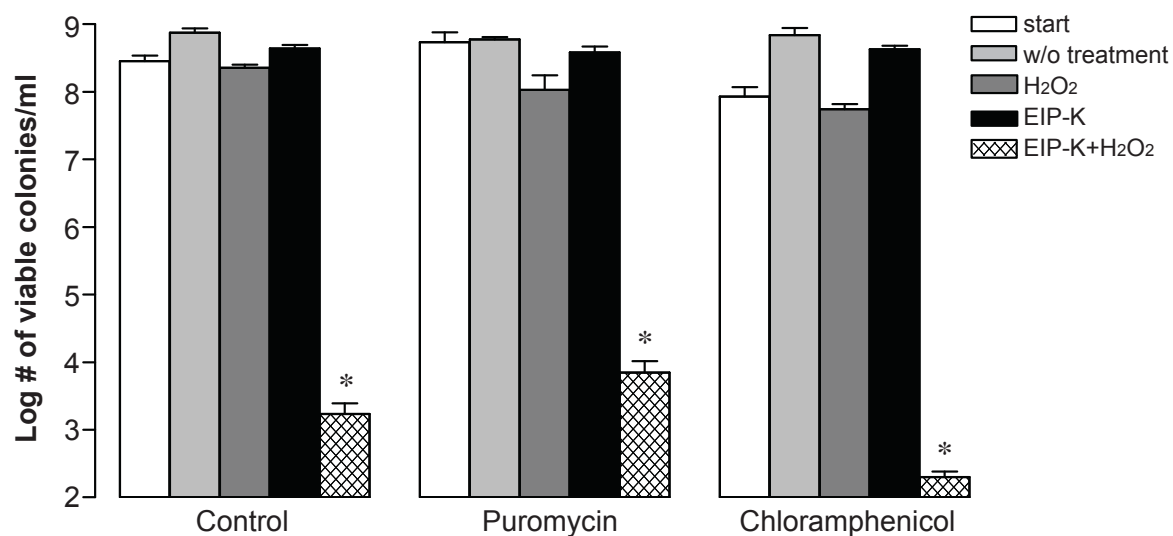
**Figure 3.4. Bactericidal effect of escapin products on *E. coli* C921-b2.** (a) Effects of naturally produced intermediates of action of escapin on  $\alpha$ -lysine versus synthesized lysine intermediate products, tested in the presence of 5 mM H<sub>2</sub>O<sub>2</sub> at pH 7. Both mixtures are dominated by compound 3, so concentrations are defined in lysine equivalents using the nuclear magnetic resonance signal of compound 3 and TSP-d<sub>4</sub> as a standard (see materials and Methods). Conc., concentration. Values are means  $\pm$  standard errors of the means (two to four experiments, each run in triplicate). (b) Bactericidal effects of 55 mM EEP-K and identified end products,  $\delta$ -aminovaleric acid (compound 5) and  $\delta$ -valerolactam (compound 6), whose concentrations are the same as those in EEP-K in the absence (w/o) or presence (w/) of 5 mM H<sub>2</sub>O<sub>2</sub> at pH 7. Values are means  $\pm$  standard errors of the means (two to four experiments, each run in triplicate).



**Figure 3.5. Effect of pH on bactericidal activity of EIP-K.** (a) Effect of pH on bactericidal activity of EIP-K and synthetic products (both at 27.5mM) in the absence (w/o) or presence (w/) of 5 mM H<sub>2</sub>O<sub>2</sub>. Asterisks indicate that the value for the product with H<sub>2</sub>O<sub>2</sub> is significantly lower than that for the product without H<sub>2</sub>O<sub>2</sub> ( $p < 0.05$ ; paired  $t$  test). (b) Viability of bacteria at pH values of 6 to 8 at temperatures from 0 to 45°C. Values are means  $\pm$  standard errors of the means (two to four experiments, each run in triplicate).



**Figure 3.6. Bactericidal effect of EIP-K and H<sub>2</sub>O<sub>2</sub> presented in different temporal combinations.** EIP-K (27.5 mM) and H<sub>2</sub>O<sub>2</sub> (5 mM) were presented to bacteria at pH 7 singly for 10 min, simultaneously for 10 min (EIP-K+ H<sub>2</sub>O<sub>2</sub>), sequentially with EIP-K first for 10 min followed by rinsing and then by H<sub>2</sub>O<sub>2</sub> for 10 min (EIP-K→H<sub>2</sub>O<sub>2</sub>), sequentially with H<sub>2</sub>O<sub>2</sub> first for 10 min followed by rinsing with LB then by EIP-K for 10 min (H<sub>2</sub>O<sub>2</sub>→EIP-K), or with neither EIP-K nor H<sub>2</sub>O<sub>2</sub> (Control). Only EIP-K+ H<sub>2</sub>O<sub>2</sub> produced significant and substantial bactericidal activity. Values are means± standard errors of the means (two to four experiments, each run in triplicate). An asterisk indicates that the value for the product with H<sub>2</sub>O<sub>2</sub> is significantly lower than that for the product without H<sub>2</sub>O<sub>2</sub> ( $p < 0.02$ ; pair  $t$  test).



**Figure 3.7. Effect of a protein synthesis inhibitor, chloramphenicol or puromycin, on the bactericidal activity of EIP-K+H<sub>2</sub>O<sub>2</sub>.** Chloramphenicol (50  $\mu$ g/ml), puromycin (100  $\mu$ g/ml), or H<sub>2</sub>O (Control) was presented to bacteria for 60 min and then rinsed with LB (start). EIP-K (27.5 mM), H<sub>2</sub>O<sub>2</sub> (5 mM), EIP-K+ H<sub>2</sub>O<sub>2</sub>, or H<sub>2</sub>O (w/o treatment) was presented to bacteria separately for another 10 min. Values are means  $\pm$  standard errors of the means (two to four experiments, each run in triplicate). Asterisks indicate that the value for the product with H<sub>2</sub>O<sub>2</sub> is significantly lower than that for the product without H<sub>2</sub>O<sub>2</sub> ( $p < 0.05$ ; paired  $t$  test.)

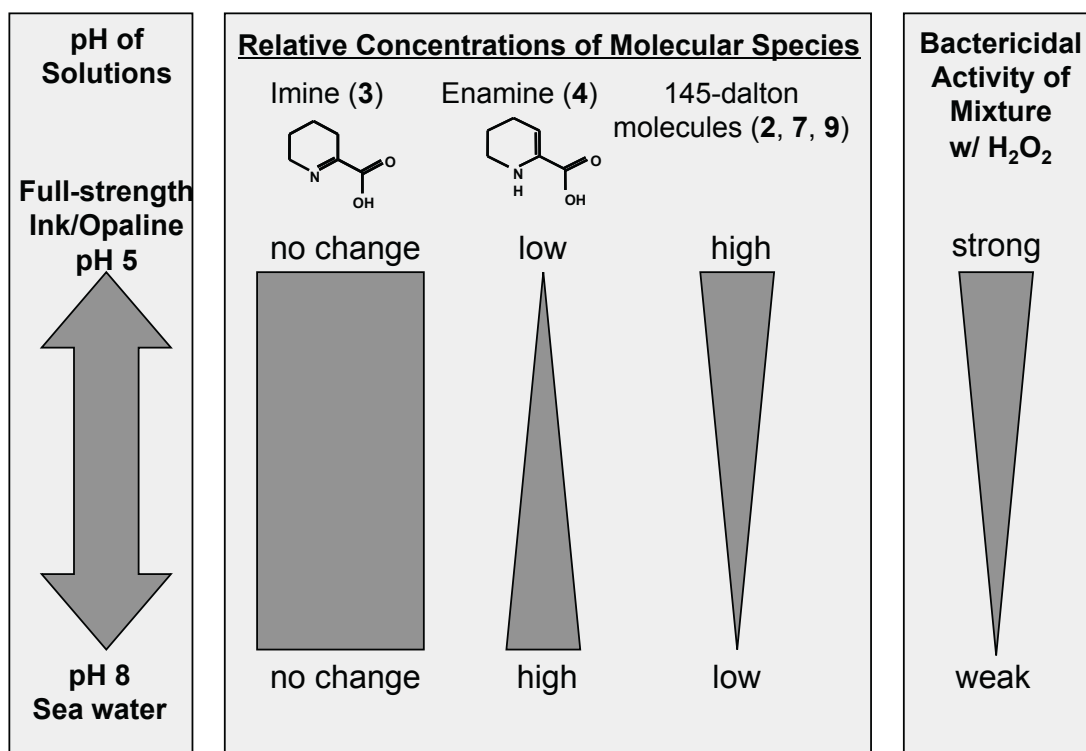


Figure 3.8. Correlation of pH, relative concentration of molecular species in the equilibrium mixture of EIP-K, and bactericidal effect of the mixture.

**4 The chemistry of Escapin: identification and quantification of the components in the complex mixture generated by an L-amino acid oxidase in the defensive secretion of the sea snail *Aplysia californica***

This work has been published as part of: Michiya Kamio, Ko-Chun Ko, Shilong Zheng, Binghe Wang, Stacy L. Collins, Giovanni Gadda, Phang C. Tai, Charles D. Derby. (2009). The chemistry of escapin: identification and quantification of the components in the complex mixture generated by an L-amino acid oxidase in the defensive secretion of the sea snail *Aplysia californica*. *Chemistry: A European Journal* 15: 1597-1603. I performed the NMR analysis collaborating with M. Kamio, who established the chemical shift assignment of each compound (Tables 4.1, 4.2, and 4.3).

## 4.1 Summary

Escapin is an L-amino acid oxidase in the ink of a marine snail, the sea hare *Aplysia californica*, which oxidizes L-lysine (**1**) to produce a mixture of chemicals which is antipredatory and antimicrobial. The goal of our study was to determine the identity and relative abundance of the constituents of this mixture, using molecules generated enzymatically with escapin and also using products of organic syntheses. We examined this mixture under the natural range of pH values for ink—from ~5 at full strength to ~8 when fully diluted in sea water. The enzymatic reaction likely forms an equilibrium mixture containing the linear form  $\alpha$ -keto- $\epsilon$ -aminocaproic acid (**2**), the cyclic imine  $\Delta^1$ -piperidine-2-carboxylic acid (**3**), the cyclic enamine  $\Delta^2$ -piperidine-2-carboxylic acid (**4**), possibly the linear enol 6-amino-2-hydroxy-hex-2-enoic acid (**7**), the  $\alpha$ -dihydroxy acid 6-amino-2, 2-dihydroxy-hexanoic acid (**8**), and the cyclic aminol 2-hydroxy-piperidine-2-carboxylic acid (**9**). Using NMR and mass spectroscopy, we show that **3** is the major component of this enzymatic product at any pH, but at more basic conditions, the equilibrium shifts to produce relatively more **4**, and at acidic conditions, the equilibrium shifts to produce relatively more **2**, **7**, and/or **9**. Studies of escapin's enzyme kinetics demonstrate that because of the high concentrations of escapin and L-lysine in the ink secretion, millimolar concentrations of **3**, H<sub>2</sub>O<sub>2</sub>, and ammonia are produced, and also lower concentrations of **2**, **4**, **7**, and **9** as a result. We also show that reactions of this mixture with H<sub>2</sub>O<sub>2</sub> produce  $\delta$ -

aminovaleric acid (**5**) and  $\delta$ -valerolactam (**6**), with **6** being the dominant component under the naturally acidic conditions of ink. Thus, the product of escapin's action on L-lysine contains an equilibrium mixture that is more complex than previously known for any L-amino acid oxidase.

## 4.2 Introduction

L-Amino acid oxidases (LAAOs; E.C. 1.4.3.2) are enzymes that oxidatively deaminate L-amino acids (67). LAAOs differ in their substrate specificities. LAAOs can function as toxins in defensive or offensive systems. Examples include the venomous enzymes of snakes in which LAAOs function in prey capture and in defense from predators (50), secreted enzymes for allelopathy between bacterial species (68-70), enzymatic chemical defenses in marine algae against parasites (71) and in mucus of fish against microbes (19, 72), and enzymatic antimicrobial and antipredator defenses in secretions of land and marine snails (3, 17, 23, 59, 62). One type of marine snail—the sea hare—has FAD-containing LAAOs in its ink (4, 59). “Escapin” is the LAAO in the ink of the sea hare *Aplysia californica* (17). When attacked by predators, *A. californica* co-releases escapin in ink and escapin's substrate L-lysine in opaline. This mixture contains escapin's products and acts as a chemical defense against predators such as California spiny lobsters and wrasses (62). The preferred substrates of escapin in vitro are L-lysine and L-arginine, similar to that of LAAOs in many other species of sea hares (4, 5, 17). But since L-lysine is ~300 times more concentrated than L-



arginine in *A. californica*'s opaline (~145 vs. ~0.5 mM), L-lysine is escapin's primary substrate in situ (4). Sea hares also have LAAOs in their egg masses, which may protect the eggs from microbes (4, 17, 59).

A goal of our work is to identify bioactive compounds produced by the escapin pathway, and by extension, those of other LAAOs. Previous work on LAAOs suggested and in some cases demonstrated the identity of molecules in this pathway (73-82). This scheme is summarized in the portion of Fig. 4.1 highlighted in yellow. This shows that LAAOs oxidize L-lysine (**1**) to form  $\alpha$ -keto- $\epsilon$ -aminocaproic acid (**2**), and in the process produce  $\text{H}_2\text{O}_2$  and  $\text{NH}_3$ . Acid **2** can cyclize to yield an equilibrium mixture also containing the imine,  $\Delta^1$ -piperidine-2-carboxylic acid (**3**), and the enamine,  $\Delta^2$ -piperidine-2-carboxylic acid (**4**).  $\text{H}_2\text{O}_2$  may react with **2** to form  $\delta$ -aminovaleric acid (**5**) and with **3** or **4** to form  $\delta$ -valerolactam (**6**). However, the ratio of **5** and **6** is highly variable, possibly due to different conditions of the LAAO reactions. Our preliminary work on escapin suggested that the chemistry of this LAAO, and thus perhaps other LAAOs, may be more complicated than suggested by published studies and as depicted in Fig. 4.1, and that the identity of the molecular species in the equilibrium mixture and which dominate under natural conditions for these enzymes are unknown. One factor that may influence the reactions and equilibrium in the escapin/ L-lysine pathway in vivo is pH, since the pH of sea hare ink is ~5 when secreted and ~8 when diluted in sea water (61), and since pH is known to affect some reactions in the general LAAO pathway in vitro (81, 83). This issue of identifying molecular species in an equilibrium mixture, quantifying their relative abundances, and de-

termining their bioactivity has analogs in other fields, such as in food sciences, where sugars exist as an equilibrium mixture and identifying the molecular species responsible for sweetness is important in product development (84).

The specific aims of this study were threefold. First, we wanted to identify all of escapin's enzymatic reaction products with L-lysine, which we term "escapin intermediate product", as well as the subsequent non-enzymatic reaction products resulting from interactions among these enzymatic products, which we call "escapin end product". Second, we wanted to identify their relative abundance under the natural pH conditions of ink. Third, we wanted to evaluate escapin's enzymatic activity at different pH values to quantify its production toward predicting the concentrations of the products of LAAO activity under natural conditions.

### 4.3 Materials and Methods

**Animals.** Sea hares *Aplysia californica* Cooper 1863 were collected in California by Marinus Scientific (Garden Grove, CA, USA). They were dissected on the day of arrival in our laboratory. Experiments were performed within the university regulations and national guidelines.

**Collection of ink and isolation of escapin.** The ink glands were dissected from anesthetized animals and frozen at -80°C until used. Purple ink was collected by gently squeezing dissected ink glands in a Petri dish with the blunt end of a scal-

pel handle. Escapin was isolated and purified using an ÄKTA 100 Automated FPLC. A two-step purification process involving gel filtration followed by purification using cation exchange was performed according to Yang et al (17).

### **Preparation of the intermediate products of escapin's oxidation of L-lysine.**

The method of Meister (1952) was modified to make “escapin intermediate product for L-lysine” (EIP). L-lysine monohydrochloride (10 mg) was incubated with escapin (3 µg) and catalase (130 µg) in double distilled H<sub>2</sub>O (1 ml) at 30°C on a shaker for up to 20–24 h until L-lysine was completely consumed, as determined by thin layer chromatography. This solution was then filtered using an Amicon Ultra-4 Centrifugal Filter Device (Millipore Corp., Billerica, MA, USA) to remove escapin and catalase, and then stored at -80°C until used later. To make “escapin end product for L-lysine” (EEP), the same procedure was performed except that catalase was not included in the incubation and concentration of escapin (0.6 µg) in H<sub>2</sub>O ml. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

**Spectroscopy.** NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer using conventional pulse sequence, and FID data were processed using MestRe Nova software (Mestrelab Research) on Windows XP. 10% D<sub>2</sub>O/ 90% H<sub>2</sub>O was used as solvent for all experiments except that in Fig. 4.2, bottom spectrum. Chemical shift was referenced for internal TSP. Mass spectra (ESI) were

obtained using a Waters Q-TOF micro mass spectrometer and Applied Biosystems QSTAR XL.

#### 4.4 Results and Discussion

##### Identification of the dominant molecular species in escapin's intermediate products at neutral pH.

Escapin intermediate product of lysine (EIP) is an equilibrium mixture; its major component was identified as **3** by NMR and mass spectroscopy. EIP was prepared by incubating escapin, L-lysine·HCl, and catalase (to prevent the reaction of H<sub>2</sub>O<sub>2</sub> with EIP). The presence of anion peaks at  $m/z$  126 and 144 in ESI-MS experiments using neutral carrier solvents indicated that EIP contains at least two major species in the EIP equilibrium mixture (Fig. 4.2b). Molecular formulae for molecules corresponding to these ion peaks were determined by HR-ESI-TOF-MS as C<sub>6</sub>H<sub>9</sub>NO<sub>2</sub> ( $m/z$  126.0555 [M-H]<sup>-</sup>,  $\Delta$ + 0.5 mmu;  $m/z$  128.07165 [M+H]<sup>+</sup>,  $\Delta$ +0.4 mmu) and C<sub>6</sub>H<sub>11</sub>NO<sub>3</sub> ( $m/z$  144.0660 [M-H]<sup>-</sup>,  $\Delta$ -0.1 mmu). On the other hand, <sup>1</sup>H NMR spectra of this mixture reveal that one of the equilibrium mixture's molecular species dominates this aqueous solution (Fig. 4.2b, second spectrum). Since NMR spectroscopy observes molecules in aqueous conditions, it gives realistic ratios of the molecular species. In D<sub>2</sub>O, the intensity of the mixture's CH<sub>2</sub> signal at 2.98 ppm became smaller and totally disappeared after 24 hours, decoupling the CH<sub>2</sub> signals at 1.88 and 3.76 ppm, indicating that the CH<sub>2</sub> protons at 2.98 ppm are exchangeable with deuterium in D<sub>2</sub>O (Fig. 4.2b, bottom spectrum).

This indicates the existence of equilibrium between **3** and **4**, **2** and **7**. To avoid this exchange and to stabilize this compound, further NMR experiments were performed in 90% H<sub>2</sub>O/ 10% D<sub>2</sub>O under neutral pH conditions. An absence of olefinic protons in <sup>1</sup>H NMR spectra (Fig. 4.2b, second spectrum) indicated that this major compound in EIP is not **4** or the linear enol, 6-amino-2-hydroxy-hex-2-enoic acid (**7**) (Fig. 4.2c). 2D NMR experiments established the connectivity of all proton and carbon atoms in this compound. The C-H connectivity was established by HMQC experiments to be the following: CH<sub>2</sub>-3 ( $\delta_{\text{H}}$ =2.98 ppm;  $\delta_{\text{C}}$ =29.4 ppm), CH<sub>2</sub>-4 ( $\delta_{\text{H}}$ = 1.88 ppm;  $\delta_{\text{C}}$ =19.0 ppm), CH<sub>2</sub>-5 ( $\delta_{\text{H}}$ =1.91 ppm;  $\delta_{\text{C}}$ = 21.5 ppm), CH<sub>2</sub>-6 ( $\delta_{\text{H}}$ =3.76 ppm;  $\delta_{\text{C}}$ =47.5 ppm) (Table 4.1). The ring system was established starting from C3 on the basis of COSY correlation from H3 to H4, H4 to H5, and H5 to H6. Long-range coupling between H3 and H6 ( $J_{3,6}$ = 2.7 Hz) is consistent with a structure that has an imine group in the six-membered ring structure **3**. This spin system is extended on the basis of HMBC correlations from H3 and H6 to imine carbon C2 ( $\delta_{\text{C}}$ =184.5 ppm) and carboxylic carbon C7 ( $\delta_{\text{C}}$ =164.9 ppm) (Table 4.1), thus establishing that **3** is the major component in EIP. Assignment of the imine carbon was confirmed based on a down field shift of the imine carbon with protonation on the imine nitrogen atom (174 ppm at pH 10 to 184 ppm at pH 7 and pH 1) (85, 86) (Table 4.1). A small pH effect on the carboxyl carbon might occur because of the formation of an intramolecular salt bridge to inhibit protonation on the carboxyl group under acidic conditions.

### Effect of pH on the equilibrium of escapin's intermediate products.

The effect of pH on the equilibrium mixture of EIP is summarized in Fig. 4.2c. Compound **3** is the dominant species in the equilibrium at any pH. Basic pH shifts the equilibrium towards **4**. Acidic pH shifts the equilibrium towards molecular species with an anion peak at  $m/z$  144, which include **2**, **7**, and **9**. These results were obtained by NMR and mass spectroscopy at different pH values. 2D NMR analysis showed that under all pH conditions, **3** was the major component, although the chemical shifts of the dominant proton signals in EIP at pH 9 were different from those at pH 1 and 7 (Fig. 4.2b). An olefinic proton at  $\delta$  5.70 ppm was observed as minor components at pH 9 (Fig. 4.2b, third spectrum), indicating that **4** or **7** was produced in greater relative amounts under higher pH conditions. HMQC and COSY experiments assigned partial structure for **4** or **7** (Table 4.2). However, the likelihood for this being **7** is low because the cyclic forms dominate in this mixture based on mass spectral data (Fig. 4.2a, c). An  $\alpha$ -keto carbon at 190–204 ppm (87), an expected key signal for **2**, the direct product of escapin, was not observed in  $^{13}\text{C}$  and HMBC experiments at any pH. Small proton signals from 1.7–1.9 ppm at pH 1 and pH 7, 2.0–2.1 ppm at pH 9, 3.2–3.4 ppm at pH 1, 7, and 9 were observed (Fig. 4.2b, Table 4.3). However, these protons could not be assigned because their signals were low intensity and overlapping. MS analysis was performed using three different carrier solvents at different pH values. As pH decreased, the intensity of the ion peak at 144, corresponding to **2**, **7**, or the cyclic aminol, 2-hydroxy-piperidine-2-carboxylic acid (**9**), increased relative to the intensity of the peak at 126, corresponding to **3** or **4** (Fig. 4.2a, c).

Signals ~3.5 ppm might be methylene protons next to nitrogen atoms of **2**, **7**, **9**, and/or the  $\alpha$ -dihydroxy acid, 6-amino-2, 2-dihydroxy-hexanoic acid (**8**). Signals at 2.3 ppm and 1.65-1.85 ppm might be the other methylene protons of **2**, **7**, **8**, and/or **9**. However, full assignment was not possible because of overlapping signals and lack of sufficient HMBC cross peaks due to low concentrations of these molecular species. Although it is not possible to give clear assignment for these molecular species, **7** might not be present under the acidic and neutral conditions because the olefinic proton was not observed in  $^1\text{H}$  NMR spectra under acidic pH conditions when mass spectra show ions corresponding to **7**. Ion signals corresponding to **8** were not observed at any pH in mass spectra, indicating that **8** might not readily ionize under our mass spectrometric conditions; however, it might exist in aqueous solutions.

### **Synthesis of putative escapin intermediate products.**

We synthesized the putative compounds in EIP to verify our identifications of their presence in the escapin pathway. We used a non-enzymatic synthetic pathway starting with pipercolinic acid ethyl ester, as described by Lu and Lewin (81).  $^1\text{H}$  NMR spectra and mass spectra show that **3** is the major product of this synthetic EIP and **4** is the minor one, as expected. Comparison of  $^1\text{H}$  NMR spectra of natural EIP and synthetic EIP revealed identical signals corresponding to **3** (Fig. 4.3).

### Determination of escapin end products.

To determine the molecules in the escapin end products for  $\epsilon$ -lysine (EEP), a solution of EEP prepared at pH 7 was compared with candidate compounds using  $^1\text{H}$  NMR spectroscopy (75, 78).  $^1\text{H}$  NMR spectra of EEP (Fig. 4.4a, top) showed two sets of spin systems, corresponding to **5** and **6** (Fig. 4.4). These two compounds were purified by HPLC from EEP mixture and molecular formulae were confirmed by HR-ESI-TOF-MS as  $\text{C}_5\text{H}_{11}\text{NO}_2$  ( $m/z$  116.0710  $[\text{M}-\text{H}]^-$ ,  $\Delta$ -0.2 mmu) for **5** and  $\text{C}_5\text{H}_9\text{NO}$  ( $m/z$  100.0767  $[\text{M}+\text{H}]^+$ ,  $\Delta$ +0.5 mmu) for **6**. Experiments in which EEP was spiked with standards confirmed the identity of these compounds (Fig. 4.4a, second to fifth). Thus, the conversion of the components of EIP to their respective components of EEP involves a reaction of **2** with  $\text{H}_2\text{O}_2$  to form **5** and a reaction of **4** with  $\text{H}_2\text{O}_2$  to form **6**, respectively. Additionally, we believe that **3** can be converted directly to **6** by a mechanism similar to the conversion of **2** to **5**.

An equilibrium between **5** and **6** is theoretically possible. We tested this by incubating of either **5** (1.5 mg) or **6** (1.5 mg) in  $\text{H}_2\text{O}$  (50  $\mu\text{l}$ ) at pH 2, 7, 9, or 11, for 4 hours at room temperature. We did not find any conversion between the two forms in  $^1\text{H}$  NMR spectra. We conclude that there is no conversion between **5** and **6** within a biological context.

In previous work on LAAOs, **5** is described as the only or major product in some reactions and **6** the only or major product in others (73, 75, 78, 79). Which product occurs or dominates may depend on experimental conditions, such as purity of the enzymes, pH, concentrations of enzyme and substrate, temperature,



and other factors that may influence the equilibrium. In the next sections, we explore some of these factors, such as pH and chemical background, for escapin.

### **Effect of pH on conversion of escapin intermediates to escapin end products.**

A pH-dependent change in the position of the equilibrium in the EIP mixture was observed using ESI-MS and NMR (Fig. 4.2). Since the 145-Dalton component increased at acidic condition, **2** and/or **9** were expected to have stronger signals in the NMR spectra under acidic conditions. However, we could not identify the molecular species as **2** or **9** in the NMR spectra of EIP because of overlapping of signals. So, we employed an indirect method—comparing the ratio of EEP at different pH values. This solution was prepared by mixing EIP and H<sub>2</sub>O<sub>2</sub> in phosphate buffered solutions of pH 6, 7, or 8. The relative amount of **5** in this mixture increased at lower pH values and decreased at higher pH values (Fig. 3.4). These changes may reflect an increased relative amount of **2** at lower pH. Fig. 4.1 provides a final summary of escapin's reaction with L-lysine.

## **4.5 Conclusion**

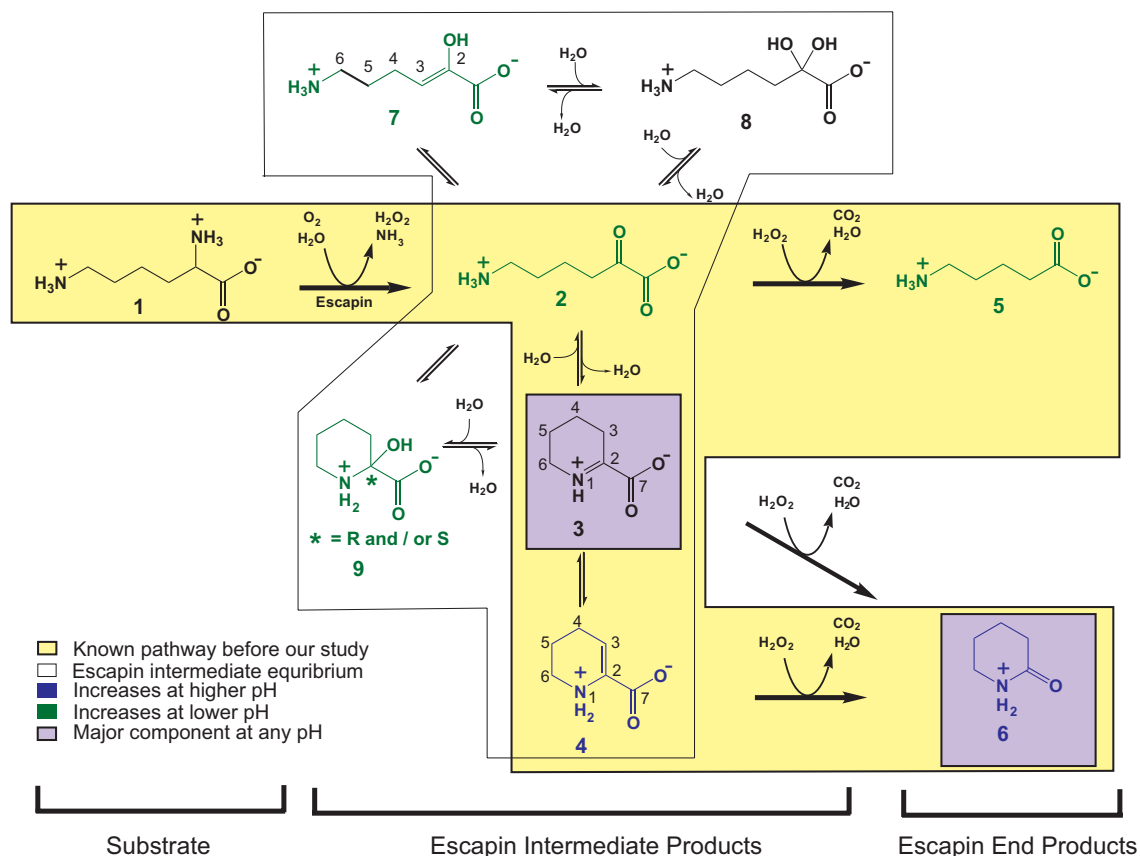
The mixture resulting from the escapin/lysine reaction in the ink secretion of sea hares is complex. It is generated by an initial enzymatic step that generates an equilibrium mixture of components, and followed by non-enzymatic reac-

tions between these components and  $\text{H}_2\text{O}_2$ . The balance within the equilibrium mixture is dependent on the mixture's pH, which ranges from ~5 in full strength ink to ~8 when diluted in sea water. The enzymatic step of the escapin pathway generates  $\Delta^1$ -piperidine-2-carboxylic acid (**3**) and  $\text{H}_2\text{O}_2$  as the major components, regardless of the pH value. Minor forms include  $\alpha$ -keto- $\epsilon$ -aminocaproic acid (**2**), 6-amino-2-hydroxy- hex-2-enoic acid (**7**), and aminol 2-hydroxy-piperidine- 2-carboxylic acid (**9**), which increase in abundance at lower pH, and  $\Delta^2$ -piperidine-2-carboxylic acid (**4**), which increases in abundance at higher pH. The non-enzymatic reaction of  $\text{H}_2\text{O}_2$  with these intermediate forms results in the end products  $\delta$ -aminovaleric acid (**5**) and  $\delta$ -valerolactam (**6**), with **6** dominating under all pH conditions but the relative abundance of the latter increasing under acidic conditions. The complexity of the mixture generated by escapin in aqueous solution is greater than previously realized for any L-amino acid oxidase. The pH effect on the equilibrium in EIP and on the production of end products shows that pH affects the activity of EIP and EEP. Our results with escapin may indicate equally complex mixtures are generated by other L-lysine oxidases. The products of the escapin pathway can now be tested in biological assays to determine which are antipredatory chemical defenses or antimicrobial agents.

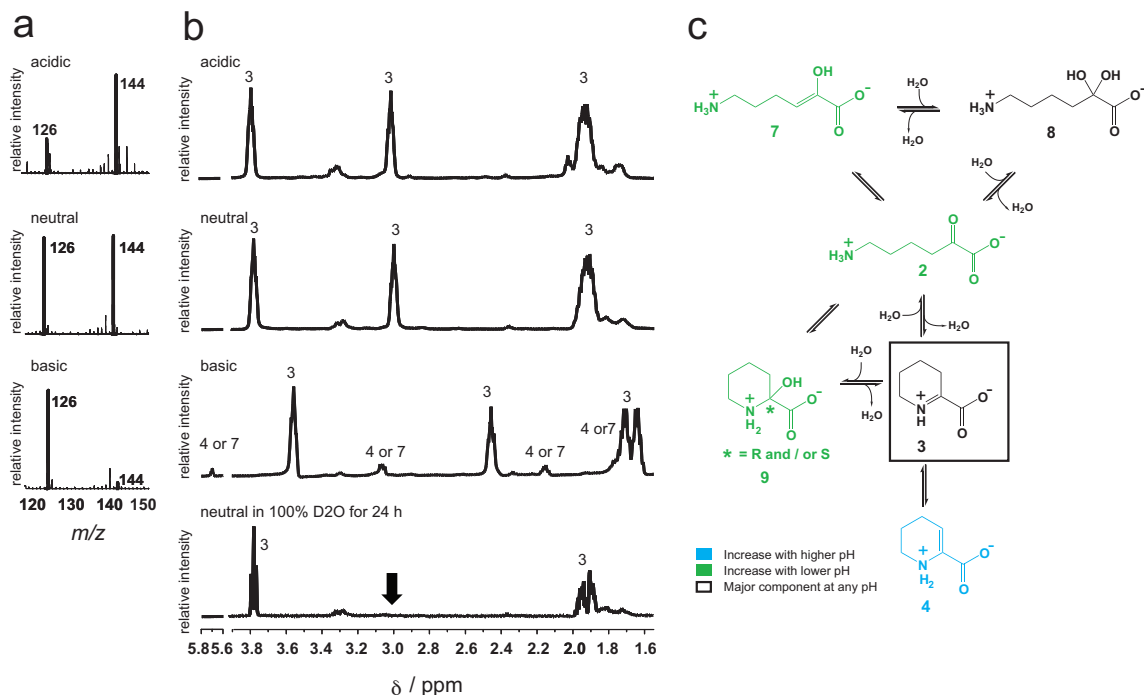
#### 4.6 Acknowledgement

We thank Shilong Zheng and Binghe Wang for producing the chemical synthesis of escapin products. Supported by NSF IBN-0324435 and 0614685 (to

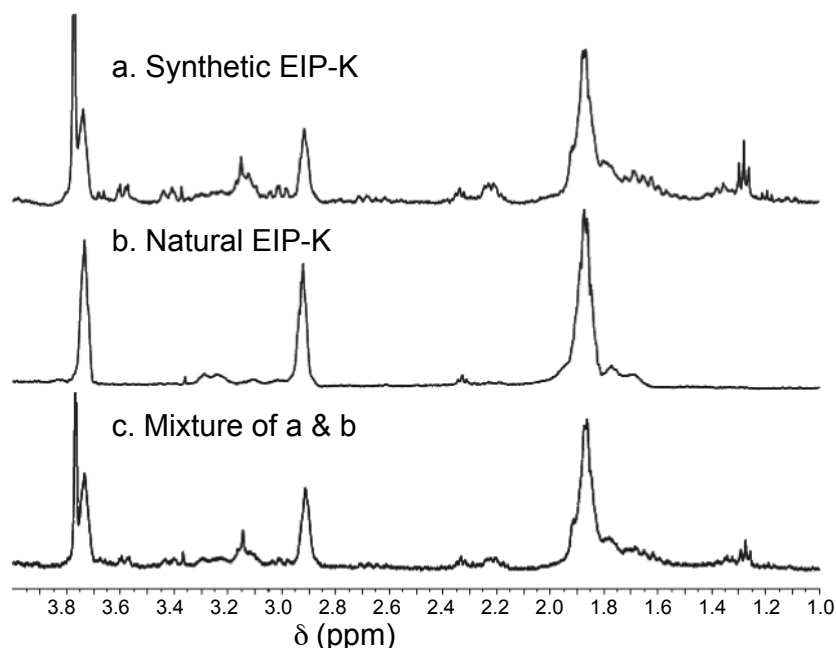
C.D.D.), NIH GM-34766 (to P.C.T.), NSF MCB-0545712 and American Chemical Society PRF 47636-AC4 (to G.G.), a grant from GSU's Brains Behavior program, Core Facilities of the Center of Biotechnology and Drug Design, and the Georgia Research Alliance. K.C.K is a Fellow of GSU's Molecular Basis of Disease Program. We thank Dr. Masaki Fujita, Dr. Julia Kubanek, Dr. Yoichi Nakao, and Dr. Toshiyuki Wakimoto, for valuable discussions on chemical shift assignments of NMR data and Dr. Siming Wang and David Bostwick for operation of mass spectroscopy.



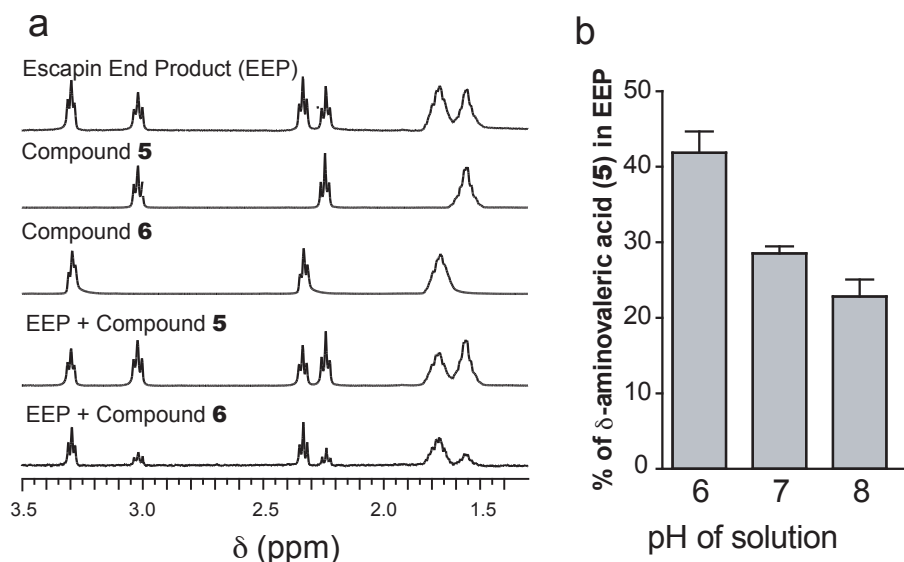
**Figure 4.1. Summary of the compounds of the escapin/  $\text{L}$ -lysine pathway in the ink and opaline secretion of sea hares.** The portion of the pathway highlighted in gray summarizes the state of knowledge of the generic  $\text{L}$ -amino acid oxidase/  $\text{L}$ -lysine pathway from the literature before our study.  $\text{L}$ -lysine (1),  $\alpha$ -keto- $\epsilon$ -aminocaproic acid (2),  $\Delta^1$ -piperidine-2-carboxylic acid (3),  $\Delta^2$ -piperidine-2-carboxylic acid (4),  $\delta$ -aminovaleric acid (5),  $\delta$ -valerolactam (6), 6-amino-2-hydroxyhex-2-enoic acid (7), 6-amino-2, 2-dihydroxyhexanoic acid (8), and 2-hydroxy-piperidine-2-carboxylic acid (9).



**Figure 4.2. Identification of the molecular species in the equilibrium mixture of escapin intermediate products, and the effect of pH on the equilibrium mixture.** (a) Changes in ESI-TOF mass spectrum in negative mode for escapin intermediate products. Aqueous carrier solvents with different pH values were used. Top: acidic conditions, using 50% MeOH+0.1% HCOOH. Middle: neutral conditions, using 50% MeOH. Bottom: basic conditions, using 50% MeOH+0.5% NH<sub>3</sub>. This shows that basic conditions favor the 126-Dalton form and acidic conditions favor the 144-Dalton form. (b) Effect of pH and D<sub>2</sub>O on <sup>1</sup>H NMR spectrum of escapin intermediate products generated by dissolving 5 mg of freeze-dried EIP in 0.5 mL of a 90% H<sub>2</sub>O/10% D<sub>2</sub>O mixture (except bottom spectrum). Top: acidic conditions with 0.4% TFA (pH 1); second: neutral conditions with double distilled H<sub>2</sub>O (pH 7); third: basic conditions with 100 mM Na<sub>2</sub>CO<sub>3</sub> (pH 9-10), **3** is the major molecular species at any pH, and **4** (or **7**) is observed only at pH 9. \* denotes signals that could not be assigned; these might be signals for **2** or **8-9**. We have no experimental evidence for the existence of **8**, but it is theoretically possible. Bottom: neutral condition with 100% D<sub>2</sub>O. After this mixture was held for 24h in D<sub>2</sub>O at room temperature, the CH<sub>2</sub> signal at 2.98ppm indicated by solid arrow disappeared, causing decoupling of the CH<sub>2</sub> signals at 1.88 and 3.76ppm and thus indicating that the CH<sub>2</sub> protons at 2.98 ppm are exchangeable with deuterium in D<sub>2</sub>O. Imine-enamine conversion between **3** and **4** and/ or keto-enol conversion between **2** and **7** makes this proton of **3** exchangeable with deuterium in the NMR solvent. (c) Summary of the effects of pH on the equilibrium of EIP. **2**, **7**, and **9** correspond to 144 (*m/z*) and **3** and **4** correspond to 126 (*m/z*) in mass spectra (see Fig. 4.2a). **8** corresponds to 162 (*m/z*), but it was not observed in mass spectra under these experimental conditions.



**Figure 4.3.  $^1\text{H}$  NMR spectra of escaping intermediate products and organic synthetic compounds.** (a) Synthetic escapin intermediate product. (b) Natural escapin intermediate product. (c) Mixture of a & b. All spectra indicate that (a), (b), and (c) contain  $\Delta^1$ -piperidine-2-carboxylic acid (3) as the major component.



**Figure 4.4. Identification of escapin end product as  $\delta$ -valerolactam (6), and the effect of pH on the ratio of two forms.** EEP was generated by adding  $\text{H}_2\text{O}_2$  (50  $\mu\text{mol}$ ) to EIP generated from lysine-HCl (13.7  $\mu\text{mol}$ ).  $\text{D}_2\text{O}$  was added to make a final concentration of 10%. (a)  $^1\text{H}$  NMR spectra. Top spectrum: Escapin end product (EEP); Second spectrum: **5**; Third spectrum: **6**; Fourth spectrum: mixture of EEP+**5**; Bottom spectrum: mixture of EEP+**6**. (b) Change in ratio of integral value of  $^1\text{H}$ NMR signal of 5 to (**5**+**6**) in EEP as pH changes from 6 to 8. Values are means  $\pm$  S.E.M.,  $N=5$ . **5** and **6** were purchased from Sigma-Aldrich.

**Table 4.1. NMR data of imine (3) at three different pH conditions in 90% H<sub>2</sub>O/ 10% D<sub>2</sub>O.<sup>[a]</sup>** (by Dr. Michiya Kamio)

Position	$\delta_{\text{H}}$	pH 1 $\delta_{\text{C}}$	HMBC (H to C)	COSY	$\delta_{\text{H}}$ ( <i>J</i> in Hz)	pH 7 $\delta_{\text{C}}$	HMBC (H to C)	COSY	$\delta_{\text{H}}$	pH 10 $\delta_{\text{C}}$	HMBC (H to C)	COSY
2		184.4				184.5				174.0		
3	2.96	29.5	2, 4, 5	4, 6	2.98	29.4	2, 4, 5, 7	4, 6	2.47	29.4	2, 4, 5	4, 6
4	1.87	19.1	2, 3, 5, 6	3, 5	1.88 (2.7)	19.0	2, 3, 5, 6	3, 5	1.71	20.8	3, 5	3, 5
5	1.89	21.6	3, 4, 6	4, 6	1.91	21.5	3, 4, 6	4, 6	1.66	23.4	4	4, 6
6	3.75	47.6	2, 4, 5	3, 5	3.76 (2.7, 5.8)	47.5	2, 3, 4, 5, 7	3, 5	3.56	50.3	2, 4	3, 5
7		164.9				164.9				163.7		

[a] All proton signals are multiplets. *J* value in pH 7 was analyzed by decoupling caused by deuterium exchange (Fig. 3.2b, bottom spectrum)

**Table 4.2. NMR data of a spin system reasonable for enamine (4) or enol (7) in 90% H<sub>2</sub>O/10% D<sub>2</sub>O at pH10.<sup>[a]</sup>** (by Dr. Michiya Kamio)

Position	$\delta_{\text{H}}$ mult ( <i>J</i> in Hz)	$\delta_{\text{C}}$	COSY
2		N/A	
3	5.70 t (4.1)	112.7	4
4	2.16 td (4.1, 6.5)	24.8	3, 5
5	1.75 m (N/A)	24.0	4, 6
6	3.06 t (5.55)	44.5	5
7		N/A	

[a] N/A: Cross peaks were not observed because of low sensitivity or overlap of signals in HMQC experiment. HMBC data were not available because of low sensitivity and signal overlap as well.

**Table 4.3. List of candidate NMR signals for 2, 8, and 9 at three different pH conditions in 90% H<sub>2</sub>O/ 10% D<sub>2</sub>O.<sup>[a]</sup>** (by Dr. Michiya Kamio)

Serial number of the signals	$\delta_{\text{H}}$	pH 1 $\delta_{\text{H}}$	COSY	$\delta_{\text{H}}$	pH 7 $\delta_{\text{H}}$	COSY	$\delta_{\text{H}}$	pH 10 $\delta_{\text{H}}$	COSY
1	1.7 m	N/A	2, 3, 5	1.69 m	N/A	3	1.76 m	28.50 or 24.13	3
2	1.8 m	19.0	1, 3, 5	1.78 m	21.1	3	1.86 m	24.0	4
3	2 m	34.5	1, 2	2.32 t	N/A	2	2.32 t	21.8	1
4	2.44 t	N/A	3	3.26 m	40.7	1, 2	3.29 t	44.4	2
5	3.27 m	43.5	1, 2						

[a] These spin systems are candidates for structures 2, 8 and 9 but could not be assigned in this equilibrium mixture. N/A: Cross peaks were not observed because of low sensitivity or overlap of signals in HMQC experiment. HMBC data were not available because of low sensitivity and signal overlap as well.

**5 Escapin, a bactericidal agent in the ink secretion of the sea hare *Aplysia californica*, acts through irreversible DNA condensation**

Manuscript to be submitted for publication by: Ko-Chun Ko, Phang C. Tai, and Charles D. Derby. "Escapin, a bactericidal agent in the ink secretion of the sea hare *Aplysia californica*, acts through irreversible DNA condensation"



## 5.1 Introduction

Many natural and synthesized chemicals have been identified as bactericidal agents that target and irreversibly inactivate essential metabolic pathways. One class of bactericidal agents is the reactive oxygen species (ROSs). ROSs are highly active free radicals containing oxygen, and they include peroxide and superoxide. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is a relatively weak peroxide of ROSs, but it generates a powerful ROS, hydroxyl radical ( $\text{HO}\bullet$ ), through the Fenton reaction.

$\text{H}_2\text{O}_2$  and other ROSs can have a diversity of deleterious effects on cells. They can interact with membrane lipids and proteins, resulting in membrane fluidity and loss of membrane structural integrity, which in turn can affect cell integrity and function (88). Hydroxyl radicals can also damage DNA and cause single strand breaks that accumulate in cells (89, 90). Such DNA damage can block DNA replication and transcription (88).

Bacteria have evolved mechanisms to protect themselves from ROSs. They decompose ROSs using enzymes such as catalase and glutathione peroxidase or by antioxidants such as ascorbic acid. For example, *Escherichia coli* treated with a non-lethal dose (10  $\mu\text{M}$ ) of  $\text{H}_2\text{O}_2$  induced production of 30 proteins (12 early proteins and 18 late proteins) and a tolerance to higher doses of  $\text{H}_2\text{O}_2$  (91). Nine of these 12 early proteins belong to the *oxyR* regulon, including catalase.  $\text{H}_2\text{O}_2$  also induces regulation of *oxyR* to evoke reactive oxidative stress response. Bacteria are highly sensitive to  $\text{H}_2\text{O}_2$  in the *oxyR* deletion mutant (91).

ROSs are produced in a variety of contexts. They are generated during cellular respiration associated with normal metabolism. Stressors such as starvation and induced oxidative stress can cause bacteria to produce and accumulate high levels of ROSs, which they can use in competitive interactions (92, 93). ROSs are also produced by other organisms as natural antimicrobial agents. For example, a marine snail, the sea hare *Aplysia californica*, produces an L-amino acid oxidase, aplysianin A (41), which is likely used to prevent bacterial fouling of the sea hare's egg capsules (4).

A paralog of aplysianin A, called escapin, has been purified from the ink gland of sea hares. Escapin normally functions as an anti-predatory chemical defense (Derby 2007), but it also has broad antimicrobial activity, both bacteriostatic and bactericidal (17), indicative of its evolutionary roots (4). Escapin uses L-lysine as its primary substrate to produce a diversity of products (17, 60). Escapin deaminates L-lysine to generate  $\alpha$ -amino- $\epsilon$ -caproic acid,  $H_2O_2$ , and ammonia ( $NH_3$ ).  $\alpha$ -Amino- $\epsilon$ -caproic acid forms an equilibrium mixture of several compounds, which we collectively call escapin intermediates products of L-lysine (EIP-K). EIP-K reacts with  $H_2O_2$  to produce a mixture of decarboxylation products called escapin end products of lysine (EEP-K). Interestingly, the mixture of EIP-K +  $H_2O_2$ , but not EIP-K, EEP-K,  $H_2O_2$ , or EEP-K +  $H_2O_2$ , shows a rapid, powerful, and long-lasting bactericidal activity, much more than  $H_2O_2$  alone (94). The composition of EIP-K equilibrium mixture changes with pH level (60). A cyclic deaminated product,  $\Delta^1$ -piperidine-2-carboxylic acid, dominates at any pH level, but the linear form,  $\alpha$ -amino- $\epsilon$ -caproic acid, becomes more prevalent at lower pH values

(60), which is biologically significant because sea hare ink is acidic (61). This increase in the relative amount of  $\alpha$ -amino- $\epsilon$ -caproic acid in EIP-K at low pH values is correlated with an increase in bactericidal activity (94). Furthermore, the synergistic bactericidal effect of EIP-K and  $\text{H}_2\text{O}_2$  requires their simultaneous, not sequential, presence (94). Together, these results suggest that the powerful bactericidal effect of EIP-K +  $\text{H}_2\text{O}_2$  is due to a synergy involving  $\alpha$ -amino- $\epsilon$ -caproic acid and  $\text{H}_2\text{O}_2$  (94).

The goal of this study is to understand mechanisms underlying the bactericidal effect of EIP-K +  $\text{H}_2\text{O}_2$ . We pursued two lines of experiments. In the first set, we followed up on results of that showed that EIP-K +  $\text{H}_2\text{O}_2$  causes DNA condensation. DNA condensation is commonly associated with antimicrobial agents that inhibit protein synthesis, most notably chloramphenicol (95). If DNA condensation is long-lasting, it can be bactericidal (96). Consequently, we hypothesized that DNA condensation is a major cause of the bactericidal effect by EIP-K+  $\text{H}_2\text{O}_2$ . To test this idea, we examined the time course of DNA condensation to treatment with EIP-K+  $\text{H}_2\text{O}_2$ , and examined whether DNA condensation and bactericidal activity occurred under the same treatment conditions. Our second line of investigation into the bactericidal mechanism of EIP-K +  $\text{H}_2\text{O}_2$  was to isolate mutant bacterial strains resistant to EIP-K +  $\text{H}_2\text{O}_2$ , followed by comparison of the mutants' genotype relative to the wild-type strain.

## 5.2 Materials and Methods

**Preparation of oxidative products of L-amino acids by escapin.** To produce EIP-K, 55 mM L-lysine monohydrochloride (Sigma-Aldrich, MO, USA), 1 mg/ml escapin, and 0.13 mg/ml catalase (Sigma-Aldrich, MO, USA) in double deionized water (ddH<sub>2</sub>O) were incubated at 30°C with vigorous shaking for up to 24 hr. This solution was filtered using an Amicon Ultra-4 centrifugal filter device (Millipore Corp., Billerica, MA, USA) to remove escapin and catalase and then stored it at -80°C until it was used further. The purity of EIP-K was checked by nuclear magnetic resonance (60). The concentration of EIP-K is expressed as the starting concentration of L-lysine monohydrochloride (60, 94).

**Bacteria strains.** Bacterial strains used in our experiments include the following: *E. coli* strain MC4100; *E. coli* resistant strains RS1 and RS2 (prepared from *E. coli* strain MC4100 as described below); *E. coli* strain NT3 (MC4100 *lac*<sup>+</sup>), individual *E. coli* mutant strains of DNA associated proteins (derived from NT3) including  $\Delta$ Fis,  $\Delta$ HupA,  $\Delta$ Hns,  $\Delta$ HimA, and  $\Delta$ MukB; *E. coli* strain ZK126 (W3110 *tna-2*  $\Delta$ *lacU169*); and its mutant *E. coli* strain of the stationary phase DNA binding protein,  $\Delta$ Dps (derived from *E. coli* strain ZK126).

**Bacterial culture preparation.** *E. coli* MC4100 was used as a wild-type test strain and also as a parental strain for the generation of two strains resistant to EIP-K + H<sub>2</sub>O<sub>2</sub>. Cells were stored as a -80°C stock. For culturing cells, stocks were

incubated at 37°C overnight in Luria-Bertani medium (LB medium), and the overnight culture was diluted 100 times to re-grow until it reached log phase (density  $\sim 3 \times 10^8$  cells/ml,  $OD_{600} \sim 0.5$ ). After washing with phosphate buffered saline (PBS, containing 8 g NaCl, 0.2 g KCl, 1.44 g  $Na_2HPO_4$ , and 0.24 g  $KH_2PO_4$  in 1 L solution, pH~7.3), bacteria were re-suspended to a density of  $6 \times 10^8$  cells/ml. Experiments on mutant strains,  $\Delta HupA$ ,  $\Delta Fis$ ,  $\Delta HimA$ ,  $\Delta Hns$ , and  $\Delta MukB$ , and their parental strain (NT3) were performed at 30°C.

Resistant strain 1 (RS1): *E. coli* MC4100 cultured cells were treated with 13.75 mM EIP-K + 3 mM  $H_2O_2$ , which is the most effective condition of bactericidal activity (94), and spread onto solid LB plates. Surviving colonies were taken from plates and re-inoculated until they reached a density of  $\sim 3 \times 10^8$  cell/ml (log phase,  $OD_{600} \sim 0.5$ ) in LB medium. Cells were washed with PBS and then treated with EIP-K +  $H_2O_2$  and spread onto solid LB plates as before. This process was repeated four times until it yielded a colony that was insensitive to treatment with EIP-K +  $H_2O_2$ , as measured by < one-log reduction in the number of bacterial colony-forming units (CFUs). This insured resistance rather than persistence.

Resistant strain 2 (RS2): Bacteria from the culture preparation were treated for 40 min with a mutagen, 2% ethyl methanesulfonate (EMS, Sigma-Aldrich, MO, USA). This was followed by repeated treatment with EIP-K +  $H_2O_2$  as described above for isolation of RS1.

**Nucleoid staining to evaluate DNA condensation.** To stain DNA, bacteria were washed with PBS and incubated for 10 min in 10  $\mu$ g/ml DNA staining agent,

Hoechst 33342 (Molecular Probes, Oregon, USA). Bacteria were then placed between a microscope slide (Superfrost, Fisher Scientific, Massachusetts, USA) and a cover glass (Fisher Scientific, Massachusetts, USA) with mounting solution, glycerol, and anti-fading agent, DABCO (triethylenediamine, Sigma-Aldrich, MO, USA). Images were captured using a Nikon ECLIPSE 80i microscope under 1000x magnification. Images from stained cells were captured in transmitted light to observe the shape and location of cells and/or in UV light to observe the distribution of DNA in the cells. When images were taken from samples 1.5 to 70 hr after treatment, samples were kept at room temperature.

The size and shape of nucleoid staining, and thus DNA condensation, were analyzed using Cellprofiler software (Broad Institute, [www.cellprofiler.org](http://www.cellprofiler.org)). The length of the major axis and form-factor (which represents shape, with 0.0 indicating a line and 1.0 indicating a perfect circle) of the nucleoid of each cell were quantified. Data from > 50 cells were used for each treatment, and treatments were statistically analyzed using ANOVA, followed by Scheffé post-hoc tests.

**Bactericidal assay.** Bacteria prepared as described above were treated with chemicals, 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub>, for different time periods (0, 2, 4, 6, 8, and 10 min) in the time course experiments and for 10 min in all other experiments. Experiments were performed at 37°C in a Thermomixer (Eppendorf, Hamburg, Germany) for all strains except for  $\Delta$ MukB and its parental strain (NT3), which were performed at 30°C. Samples were serially diluted in log steps

with PBS, and serial dilutions were plated onto Petri dishes with solid LB agar. Plates were incubated overnight at 37°C, and counts of viable cells were determined by enumeration of CFUs with appropriate dilutions.

**Genomic DNA preparation.** Genomic DNA of RS1, RS2, and the parental strain MC4100 were purified by QIAGEN Genomic-tip 100/G and Genomic DNA Buffer Set (QIAGEN Inc., CA, USA), using the protocols in the manufacturer's instruction guide book. To remove high affinity DNA binding proteins and ensure better DNA quality for whole genome sequence, the DNA solution was purified in two washing steps, by 25% phenol/ 25% of chloroform: isopropanol (24:1) mixture and 50% chloroform: 100% isopropanol (24:1 mixture), sequentially. After purification, DNA was precipitated using 66% ethanol with 0.1 M NaOAc (pH 5.2), and the pellet was washed with 70% ethanol. DNA was reconstituted in 10 mM Tris buffer (pH 8.0) for sequencing.

**Localization of mutations.** Whole genome sequencing was conducted by the Interdisciplinary Center for Biotechnology Research of the University of Florida, using a 454 Genome Sequencer GS-FLX (Roche). The results for RS1 and RS2 were mapped against the genome sequence of the parental strain, MC4100. 15 mutations were detected in RS1 and 22 in RS2 by the fast sequencing of 454 life science. Eleven mutations were common to both RS1 and RS2 but different from parental strain were further tested by high fidelity PCR (Roche, Branford, CT) and DNA sequencing using an ABI sequencer. To avoid bias from a single repli-

cation mistake during the PCR process, the entire PCR reaction product, and not just a single cloning product, was sequenced. This process led to the identification of a single gene mutation in both RS1 and RS2: *oxyR*.

**Cloning of *oxyR* gene.** Primers were designed to amplify *oxyR* of MC4100 (*oxyR<sup>wt</sup>*) and *oxyR* of RS1 (*oxyR<sup>A233V</sup>*) for complementation tests. The 5' primer included an NdeI restriction site to provide a start codon and no redundant nucleotide in N-terminal (5' GGATGGATACATATGAATATTCGT 3'). The 3' end included an XhoI restriction site (5' TTAAACGGTCTCGAGTTAAACCGC 3'). PCR was applied using a high fidelity PCR system (Roche, Bradford, CT). *oxyR<sup>wt</sup>* and *oxyR<sup>A233V</sup>* fragments of expected size were individually cloned into pET29a vector (Novagen, Madison, WI, USA). The mutated fragments were confirmed by DNA sequencing using an ABI sequencer.

**Complementation test.** Plasmids pET29a, pET29a-*oxyR<sup>wt</sup>* (*oxyR<sup>wt</sup>*), and pET29a-*oxyR<sup>A233V</sup>* (*oxyR<sup>A233V</sup>*) were transformed into *E. coli* MC4100 or RS1. A single colony was taken from each transformed plate and cultured to the stationary phase ( $OD_{600} > 2.0$ ). Cultures were diluted to  $OD_{600} \sim 0.1$  and cultured anew. The same treatments were applied as in the bactericidal assay.



### 5.3 Results

#### **EIP-K + H<sub>2</sub>O<sub>2</sub> caused intensive DNA condensation in wild-type *E. coli*.**

The structure of DNA was visualized by examining wild type *E. coli* stained with Hoechst under simultaneous UV and transmitted illumination. Cells that were untreated or treated with either H<sub>2</sub>O<sub>2</sub> or EIP-K for 10 min had nucleoid morphology that was similar to each other but different from cells treated with EIP-K + H<sub>2</sub>O<sub>2</sub> (Fig. 5.1). Cells that were untreated or treated with EIP-K or H<sub>2</sub>O<sub>2</sub> showed a variety of nucleoid morphologies. These include cells with relaxed DNA, in which the nucleoid fills most of cell volume, and cells with condensed DNA, in which the nucleoid has various irregular shapes (Fig. 5.1). The nucleoids of cells treated with EIP-K + H<sub>2</sub>O<sub>2</sub> were nearly uniform sphere and significantly shorter (Fig. 5.2) and rounder (Fig. 5.3) and were thus more condensed than those of the other three conditions. DNA condensation caused by EIP-K + H<sub>2</sub>O<sub>2</sub> is indicated by the observation that for cells under this treatment, ~80% had form factor >0.8 (~50% had a form factor of >0.9) and ~80% were <1 mm long. Only ~20% had form factor >0.8 in untreated cell (~5% had a form factor >0.9) (data not shown). DNA condensation was reported for treatment with antibiotics such as chloramphenicol (Cam) that inhibit protein synthesis(95). In our experiments, either Cam or EIP-K+ H<sub>2</sub>O<sub>2</sub> induced DNA condensation, but of different forms. EIP-K+ H<sub>2</sub>O<sub>2</sub> compacted DNA into a tighter area, with nucleoid diameters around half that induced by Cam (Fig. 5.4). Cam induced a heterogeneously condensation with a

dense ring and a hollow center EIP-K+H<sub>2</sub>O<sub>2</sub> induced a solid and homogenous condensation (Fig. 5.5).

**Brief treatment with EIP-K + H<sub>2</sub>O<sub>2</sub> caused irreversible DNA condensation in wild-type *E. coli*.**

Treatment of cells with EIP-K + H<sub>2</sub>O<sub>2</sub> for 10 min caused long-term changes in nucleoid structure. Cells maintained a similar level of DNA condensation 1.5, 25, and 70 hr after a 10-min treatment with EIP-K + H<sub>2</sub>O<sub>2</sub> (Fig. 5.6). DNA of untreated cells showed a small degree of condensation after 25 and 70 hr, many orders of magnitude less than with EIP-K+ H<sub>2</sub>O<sub>2</sub> treatment (Fig. 5.6). Thus, condensation lasts much longer than treatment, with a 10-min treatment with EIP-K + H<sub>2</sub>O<sub>2</sub> causing condensation lasting at least 70 hr.

Incubation of wild-type *E. coli* in EIP-K + H<sub>2</sub>O<sub>2</sub> caused both rapid killing and DNA condensation. The shortest treatment time, which was 2 min, produced a two-log step bactericidal effect, and incubation for 6-10 min caused a maximal effect – an eight-log step effect (Fig. 5.7). Similarly, a 2-min treatment with EIP-K + H<sub>2</sub>O<sub>2</sub> caused robust DNA condensation at the shortest time point, 2 min (Fig. 5.8).

***E. coli* mutants are resistant to EIP-K + H<sub>2</sub>O<sub>2</sub> treatment.**

Two *E. coli* colonies resistant to EIP-K + H<sub>2</sub>O<sub>2</sub>, designated resistant strains 1 (RS1) and 2 (RS2), were isolated and identified as described in the Ma-

terials and Methods. Both showed similar phenotypes, so only data for RS1 are described here. RS1 displayed only a very weak bactericidal effect ( $< 1$  log reduction in number of colony-forming units) to EIP-K +  $\text{H}_2\text{O}_2$  at various concentrations of either EIP-K (Fig. 5.9A) or  $\text{H}_2\text{O}_2$  (Fig. 5.9B).

The effect of pH on the bactericidal action of EIP-K +  $\text{H}_2\text{O}_2$  was examined for RS1, since pH is an important factor of activity for the parental strain (94). For both RS1 and parental strain, the bactericidal effect of EIP-K +  $\text{H}_2\text{O}_2$  was greater at pH 6 than 8 (Fig. 5.10), although the magnitude of the effect was much greater for the parental strain (94) than for RS1.

Higher concentrations of  $\text{H}_2\text{O}_2$  were also tested on RS1, since these concentrations could be bactericidal against the parental strain. Whereas 3 mM  $\text{H}_2\text{O}_2$  had no effect on the parental strain, 15 mM  $\text{H}_2\text{O}_2$  produced a 4-log bactericidal effect on the parental strain (data not shown). However, neither 15 nor 40 mM  $\text{H}_2\text{O}_2$  was bactericidal against RS1 (Figs. 5.9B, 5.11), and 60 mM  $\text{H}_2\text{O}_2$  produced only a slight bactericidal effect ( $< 0.5$  log reduction in # of CFU). Forty mM  $\text{H}_2\text{O}_2$  + 13.75 mM EIP-K produced a four-log bactericidal effect on RS1 and 60 mM  $\text{H}_2\text{O}_2$  + EIP-K produced the maximum effect (Fig. 5.11). Tolerance of higher concentration of hydrogen peroxide was also found for wild type *E. coli* that were cultured in the presence of a low concentration of  $\text{H}_2\text{O}_2$ , and this culture also showed less sensitivity to EIP-K +  $\text{H}_2\text{O}_2$  (Fig. 5.12).

Treatment with 13.75 mM EIP-K + 3 mM  $\text{H}_2\text{O}_2$  did not cause DNA condensation in RS1 bacteria, unlike the parental strain (Fig. 5.13). Treatment with either EIP-K or  $\text{H}_2\text{O}_2$  alone did not have the same effect as the parental strain.

Of the eleven mutations common to RS1 and RS2 by genomic sequencing, only one mutant was confirmed by high fidelity PCR. This was a single amino acid mis-sense mutation in *oxyR*, with a C-to-T transition that changes the amino acid in position 233 from alanine to valine. A complementation assay was conducted to determine whether *oxyR* is the sole mutated gene responsible for resistance of EIP-K + H<sub>2</sub>O<sub>2</sub> in resistant strains. MC4100 without any plasmid, MC4100 carrying the empty vector plasmid, and MC4100 carrying the *oxyR*<sup>wt</sup> plasmid were not resistant to EIP-K + H<sub>2</sub>O<sub>2</sub>, but MC4100 carrying *oxyR*<sup>A233V</sup> plasmid showed resistance to treatment (Fig. 5.14A). Resistance to treatment also occurred when RS1 was used as a host for various plasmids. But a slight reduction in number of CFUs occurred for RS1 carrying *oxyR*<sup>wt</sup> plasmid (Fig. 5.14B). Since an unknown leakage of expression occurs, we were able to observe a plasmid effect on *E. coli* MC4100, a strain lacking an essential factor to overproduce a gene carried by the T7 promoter. This result indicates that the mutation in *oxyR*<sup>A233V</sup> renders resistance to EIP-K + H<sub>2</sub>O<sub>2</sub>, and a gene dosage effect leads to reduction of resistance on resistant strains carrying *oxyR*<sup>wt</sup>.

**The effect of DNA associated proteins EIP-K + H<sub>2</sub>O<sub>2</sub> is bactericidal and causes DNA condensation in *E. coli* mutant strains of DNA associated proteins.**

We hypothesized that DNA condensation resulted from EIP-K + H<sub>2</sub>O<sub>2</sub> targeting either of two targets: Dps (DNA-binding protein from starved cells), H-NS (histone-like nucleoid-structuring protein) (Fig. 5.17). According to our hypothe-

sis,  $\text{H}_2\text{O}_2$  induces oxidative stress, which triggers OxyR to over-express Dps, which self-aggregates and binds to DnaA, thus causing DNA condensation (97). Also according to our hypothesis, EIP-K stabilizes the DNA-Dps complex by inhibiting the activity of the Dps-dissociating enzyme, ClpX, or by stabilizing the Dps-DnaA complex and thus preventing DNA replication. Based on an examination of the effect of cellular overexpression of H-NS, H-NS could potentially cause DNA condensation and induce the bactericidal effect (98). To test the potential role of H-NS in DNA condensation mediated by EIP-K +  $\text{H}_2\text{O}_2$ , we performed experiments with a  $\Delta hns$  *E. coli* mutant strain. To examine the involvement of Dps, we used a  $\Delta dps$  *E. coli* mutant strain and a strain that overproduced Dps. Our expectation was that the  $\Delta dps$  mutant would be resistant to the bactericidal and DNA condensation effects of EIP-K +  $\text{H}_2\text{O}_2$ , while the strain over-expressing Dps would be sensitive to EIP-K alone since the abundance of Dps would mimic the effect of  $\text{H}_2\text{O}_2$ . Our results showed that treatment of either the  $\Delta dps$  or  $\Delta hns$  *E. coli* strain with EIP-K +  $\text{H}_2\text{O}_2$  caused DNA condensation and bactericidal activity, and that EIP-K treatment of cells overproducing Dps did not cause DNA condensation or bactericidal activity (Fig. 5.16).

We also tested other mutant strains defective in other DNA associating proteins, including Fis (Factor for inversion stimulation,  $\Delta fis$ ), one subunit of Hu (a histone-like protein,  $\Delta hupA$ ), one subunit of IHF (Integration Host Factor,  $\Delta himA$ ), and MukB (chromosome partitioning protein,  $\Delta mukB$ ). None of these mutants was resistant to EIP-K +  $\text{H}_2\text{O}_2$ , and the  $\Delta fis$  strain was more sensitive than the wild type strain (Fig. 5.17).

### **Temperature stress does not substitute for oxidative stress.**

$\text{H}_2\text{O}_2$  generates hydroxyl radicals which are known to damage cellular components (99, 100). We hypothesized that the bactericidal effect of  $\text{H}_2\text{O}_2$  + EIP-K is resulted in part from the oxidative stress associated with hydroxyl radicals. To examine whether the bactericidal effect is specific to oxidative stress or whether it generalizes to other stressor, we examined whether heat shock can have the same effect as  $\text{H}_2\text{O}_2$  in combination with EIP-K in our bactericidal assay. Our results showed that EIP-K alone in any temperature is not bactericidal (Fig. 5.18) and  $\text{H}_2\text{O}_2$  is still required in combination with EIP-K to evoke the bactericidal effect. . Thus, heat shock response does not substitute for oxidative stress from  $\text{H}_2\text{O}_2$ .

## **5.4 Discussion**

A broad bactericidal activity has been found for escapin, an L-amino acid oxidase, and L-lysine, its substrate, both present in the defensive ink secretion of the sea hare *Aplysia californica* (17). The products of the escapin-lysine reaction are identified (60) and amongst them,  $\text{H}_2\text{O}_2$  and the escapin intermediate products (EIP-K) together account for the powerful bactericidal effect (17). In this study, we examined the mechanism underlying this bactericidal activity in *E. coli*.

**Escapin products cause irreversible DNA condensation with a time course similar to that of the bactericidal effect.**

During the cell cycle, DNA dynamically changes its morphology, unwinding to allow DNA replication and transcription, followed by DNA condensation to allow separation and movement of replicated DNA to form the two daughter cells, followed by DNA unwinding. Since cells in our culture system were not synchronized in their growth phase, we observed cells in various stages of DNA condensation. Nonetheless, our results clearly showed that brief treatment with sea hare secretion, specifically EIP-K + H<sub>2</sub>O<sub>2</sub>, causes irreversible DNA condensation, but treatment with either EIP-K or H<sub>2</sub>O<sub>2</sub> alone does not. The effect is rapid, as a 2-min exposure causes maximal DNA condensation and also kills 90% of the cells. The condensation lasts for at least 70 hr after treatment and thus is irreversible for bacteria. The condensation results in the nucleoid of most (> 50%) cells having a circular condensed nucleoid (i.e. form factor >0.9), which is a form present in only 5% percent of control cells.

This irreversible DNA condensation indicates inhibition of DNA replication and transcription, resulting in an arrested cell cycle and cessation of cell division, even long after removal chemical treatment. This bactericidal effect is reminiscent of the *E. coli* toxin-antitoxin system, where arresting cell cycle with toxin for more than 6 hr in the absence of antitoxin causes cell death (96). This irreversible DNA condensation associated with cell death also occurs to other chemicals, such as Cyt1Aa (an insecticidal crystal protein purified from *Bacillus thuringiensis*) (101) and H-NS (a bacterial DNA binding protein) (98). It is interesting to

note that the shape of the condensed nucleoid induced by EIP-K + H<sub>2</sub>O<sub>2</sub> is similar to that of these other chemical agents (102). On the other hand, the antibacterial agent chloramphenicol, which inhibits protein synthesis, produces a nucleoid that is condensed but less so and with a different shape EIP-K + H<sub>2</sub>O<sub>2</sub>: most of the DNA is in the perimeter of the nucleoid and its center is without DNA (103). These results suggest that the bactericidal effect of EIP-K + H<sub>2</sub>O<sub>2</sub> may be similarly mediated by DNA condensation, resulting in inhibition of metabolic reactions, leading to cell death.

### **OxyR<sup>A233V</sup> confers high resistance to EIP-K + H<sub>2</sub>O<sub>2</sub>.**

A change in the amino acid composition or sequence of a protein can alter its structure and consequently lower its affinity to antibiotics, thus giving bacteria containing such proteins more resistance to antibiotics (104, 105). We applied this concept to identify potential targets of EIP-K + H<sub>2</sub>O<sub>2</sub>. In our isolation of strains resistant to EIP-K + H<sub>2</sub>O<sub>2</sub>, bacteria were repeatedly treated with EIP-K + H<sub>2</sub>O<sub>2</sub> to ensure that they lost sensitivity to this treatment and thus to differentiate between persister cells and resistant cells. We identified two resistant strains, RS1 and RS2, and showed that they are resistant to H<sub>2</sub>O<sub>2</sub> under conditions lethal to the parental, wild-type strain. The wild-type strain is killed by >10 mM H<sub>2</sub>O<sub>2</sub>, but RS1 can tolerate up to 40 mM H<sub>2</sub>O<sub>2</sub> with very little cell death. Comparison of whole genome sequences of the parental and mutant strains, coupled with a complementary assay, showed that RS1 and RS2 are both single amino acid mutants (A233V) of *oxyR* and that this gene provides resistance to EIP-K + H<sub>2</sub>O<sub>2</sub>.



OxyR is a dual transcriptional regulator protein that senses oxidative stress and regulates antioxidant genes, including oxidative stress regulator (oxyR) itself, catalase (katG), alkyl hydroperoxide reductase (ahpC, ahpF), glutathione reductase (gorA), and others (106). oxyR is required to sense H<sub>2</sub>O<sub>2</sub> and induce its regulation, and cells with oxyR deletion are hypersensitive to H<sub>2</sub>O<sub>2</sub> (91). An identical single point mutation of *oxyR*<sup>A233V</sup> is reported in *E. coli* K12 strain and named *oxyR2* (Christman et al. 1989). The *oxyR2* mutant strain (also called constitutive strain) over-expresses OxyR-regulated proteins such as catalases and peroxidases, in the absence of oxidative stress. Christman et al. (1989) show that *oxyR2* has the same level of oxyR mRNA production as the wild type (107). Therefore, they hypothesized that the alanine-to-valine mutation causes a significant conformational change that affects its regulatory activity. *oxyR*<sup>A233V</sup> is a constitutive mutant that overproduces anti-oxidative stress genes, including Dps, catalase (a H<sub>2</sub>O<sub>2</sub> scavenger), and peroxidases under non-stress conditions (91). This may explain the resistance of RS1 and RS2 to EIP-K + H<sub>2</sub>O<sub>2</sub>, since H<sub>2</sub>O<sub>2</sub>, an essential component of the bactericidal effect, is removed by over-expression of catalase. This explanation is supported by a lack of bactericidal activity to treatment of the resistant strains with 40 mM H<sub>2</sub>O<sub>2</sub>.

### **The relationship between bactericidal and DNA condensation effects of escapin products.**

The close correlation between the bactericidal and DNA condensation effects of EIP-K + H<sub>2</sub>O<sub>2</sub> suggests that DNA condensation is a potential mechanism

behind the bactericidal effect. Is there a link between *oxyR* gene and DNA condensation? Does the mutant strain simply eliminate  $H_2O_2$  or does it also avoid permanently intensive DNA condensation? Knowing this is important in identifying the targets of EIP-K +  $H_2O_2$ .

Both the bactericidal activity and DNA condensation are absent in the resistant strains, and the complementary assay shows that the single mutated gene, *oxyR*<sup>A233V</sup>, is responsible for conferring resistance to EIP-K +  $H_2O_2$ . This mutation has been widely shown to over-express anti-stress proteins, especially oxidative stress proteins. Previous studies show that those anti-stress proteins can be induced in *E. coli* by incubation in low concentrations of  $H_2O_2$  (91). Indeed, the bactericidal effect of EIP-K +  $H_2O_2$  decreases when bacteria are pre-incubated in 100  $\mu$ M  $H_2O_2$  (Fig. 5.12). This is similar to the *oxyR*<sup>A233V</sup> strain, which overproduces catalase, leading to removal of  $H_2O_2$ , which is essential for the bactericidal effect of EIP-K +  $H_2O_2$ . It is still not clear whether the resistance of this mutant is due to removal of  $H_2O_2$  or if the mutant also over-expresses other stress proteins that prevent DNA condensation. However, mutations of *oxyR2* (*oxyR*<sup>A233V</sup>, labelled as *oxyR*<sup>A234V</sup> in the references) could not be generated by  $H_2O_2$  treatment alone, but can only be generated through random mutagenesis (91). In our experimental conditions,  $H_2O_2$  is not lethal, and the damage caused by stress associated with  $H_2O_2$  is repaired through the bacterial oxidative response. These observations suggest that EIP-K +  $H_2O_2$  produces a strong environmental stress response that causes the bactericidal effect or is fol-

lowed by triggering mutation in genes mediating the global stress regulator *oxyR*. We hypothesize that the strong environmental stress is oxidative stress.

### **Mechanisms underlying the effects of escapin products on *E. coli*.**

What are the molecular causes of EIP-K + H<sub>2</sub>O<sub>2</sub> mediated DNA condensation? Although the concentration of H<sub>2</sub>O<sub>2</sub> that we used in our conditions is not lethal to bacteria, cells may have experienced some degree of oxidative stress. DNA is one target for oxidative stress, and cells have protective mechanisms to ensure DNA fidelity. The most direct protective factors are DNA binding proteins. Under environmental stress, DNA binding proteins play important roles in modifying supercoiling to protect DNA. One DNA binding protein, Dps, is under OxyR regulation in exponential-phase cells when cell experiences oxidative stress and is also overexpressed in the *oxyR* constitutive mutant, *oxyR*<sup>A233</sup> (108). Dps self-aggregates, sequesters DNA, and forms a large DNA-Dps condensate to protect cells from oxidative stress. Dps is thought to be a cell cycle checkpoint. Binding of Dps and DnaA, the DNA replication initiator, inhibits unwinding step and hence interferes with DNA initiation (109). Another DNA binding protein, H-NS, when overproduced, increases the bactericidal activity and causes perfectly spherical DNA condensation (98) as does treatment with EIP-K + H<sub>2</sub>O<sub>2</sub> in our experiments. H-NS is a repressor of many genes and traps RNA polymerase (110), so when H-NS is overexpressed, it represses metabolic pathways. H-NS null mutant affects chromosome partitioning and delays initiation of DNA replication (111). Thus, we hypothesized that Dps or H-NS mediates the effect of EIP-K + H<sub>2</sub>O<sub>2</sub> on

DNA condensation. Our tests of this hypothesis, either by using a mutant *E. coli* strains ( $\Delta dps$  and  $\Delta hns$ ) or by over-expressing Dps in the wild-type strain, showed neither Dps nor H-NS alone is necessary for EIP-K + H<sub>2</sub>O<sub>2</sub> mediated DNA condensation (Fig. 5.14). Our negative results may be because of Dps, H-NS, and other DNA binding proteins such as histone-like proteins or condensin-like proteins, can compromise functions of each other (112). In this case, the function of either a Dps or H-NS null mutant might be substituted by other DNA binding proteins.

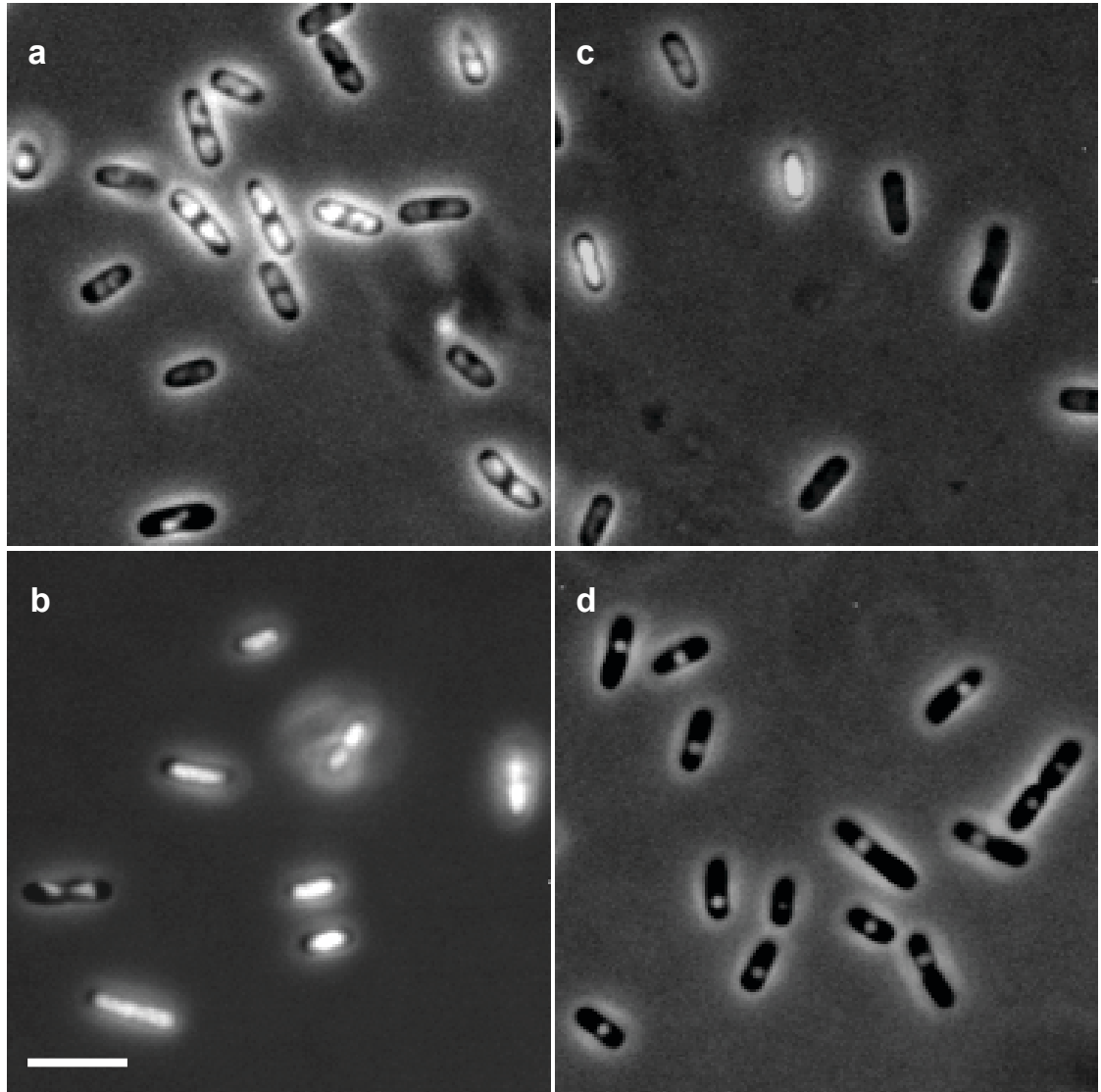
A Fis (Factor for inversion stimulation) deficient strain was more sensitive to EIP-K + H<sub>2</sub>O<sub>2</sub> than was the wild type strain (Fig. 5.17). The Fis null mutant is sensitive to oxidative stress. Fis inhibits DNA condensation induced by Dps, and binding of Fis and DNA also inhibits access of Dps to DNA thus preventing tight condensation (113). The Fis null mutant loses the ability to relax its DNA, which increases the possibility for interactions between Dps and DNA. This observation supports the idea that Dps is a potential component in DNA condensation and a subsequent bactericidal effect.

In conclusion, EIP-K + H<sub>2</sub>O<sub>2</sub> causes bacterial DNA to condense into a tight, spherical nucleoid. We propose that a well-organized compacting mechanism, such as that mediated by DNA binding proteins, is responsible for this effect. This DNA condensation is irreversible, which suggests it is due to damage of DNA unwinding mechanisms. Arresting the initiation of DNA replication, preventing DNA segregation properties, and negatively affecting enzymes that resolve DNA binding protein from DNA are possible reasons to keep DNA con-

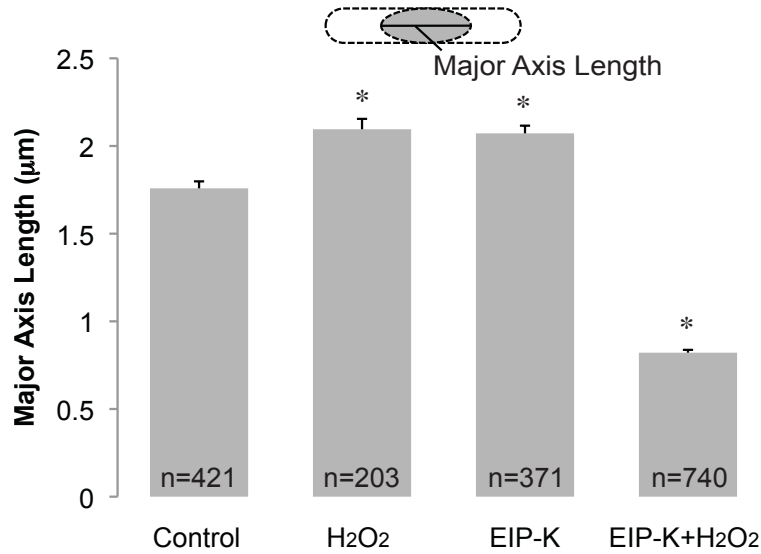
densed. Oxidative stress is well known to induce protection by causing bacteria to overproduce anti-oxidative stress proteins such as catalase and Dps (91, 108). DNA is a favorite target of ROSs, and DNA condensation is a way to avoid attack from ROSs. EIP-K may play a role in stabilizing the oxidative response from H<sub>2</sub>O<sub>2</sub> and in inducing this irreversible DNA condensation. In our model, we speculate that EIP-K may affect ClpXP protease, which regulates Dps degradation (114), and then maintain the self-aggregated form of Dps with DNA or maintain Dps-DnaA binding and thus inhibit DNA replication (109). However, we know very little of the effects of EIP-K and heat shock stress does not induce the same effects as H<sub>2</sub>O<sub>2</sub> when combined with EIP-K. Also, since EIP-K and H<sub>2</sub>O<sub>2</sub> must be present simultaneously to have their effect (94), we hypothesize that the unstable reaction products of H<sub>2</sub>O<sub>2</sub> and EIP-K are the active compounds of this bactericidal effect, and understanding the chemistry of this mixture will be important.

## 5.5 Acknowledgements

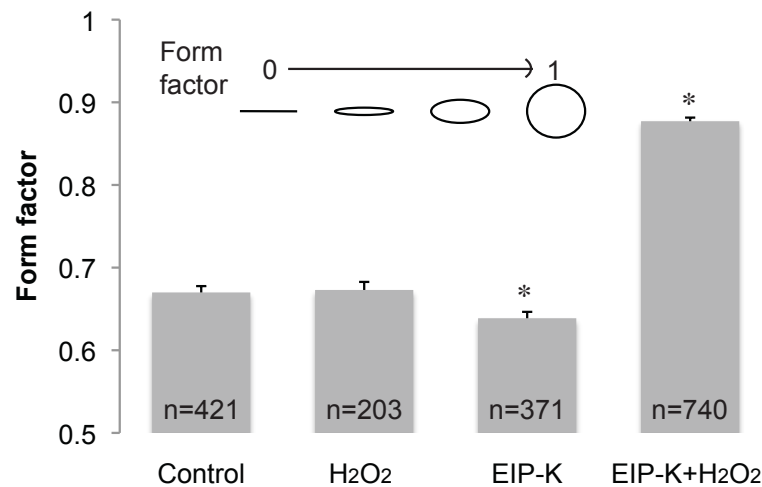
We thank Jonathan Beckwith (Harvard Medical School), Nancy Trun (Duquesne University), and Roberto Kolter (Harvard Medical School) for providing *E. coli* MC4100, DNA associated protein mutants, and Dps null mutant, respectively. This work was supported by NSF grants IOS-0324435, 0614685, and 1036742, NIH grant GM-34766, a grant from the GSU Brains & Behavior program, and a fellowship to Ko-Chun Ko from the GSU Molecular Basis of Disease program.



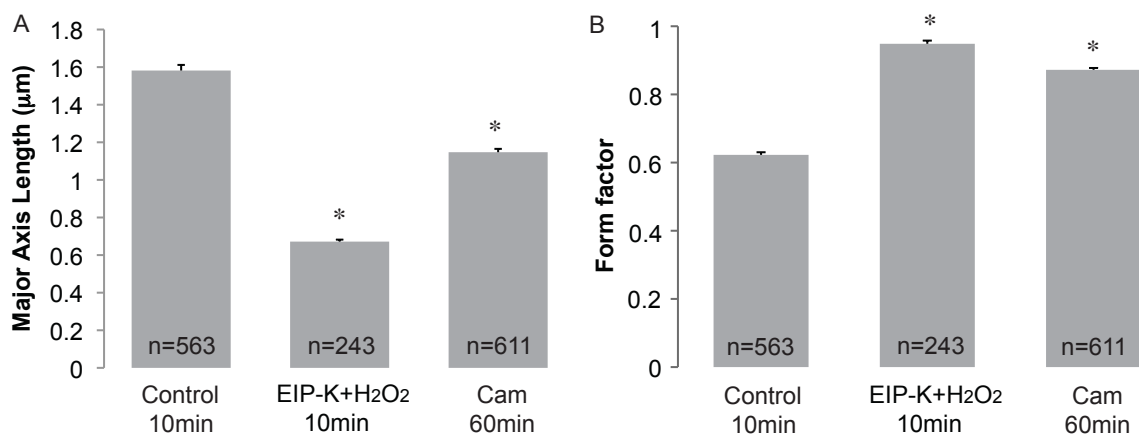
**Figure 5.1. Light micrographs of DNA condensation in wild-type *E. coli* under different treatments.** (a) Control (ddH<sub>2</sub>O, used as solvent in the other treatments); (b) 3 mM H<sub>2</sub>O<sub>2</sub>; (c) 13.75 mM EIP-K; (d) 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub>. Cells are stained Hoechst to label DNA, and images are taken by simultaneously presenting UV and transmitted light. Scale bar, 5  $\mu$ m.



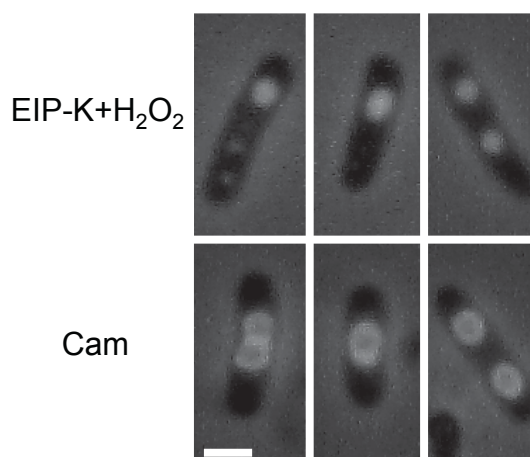
**Figure 5.2. Quantification of DNA condensation in wild-type *E. coli* using length of major axis of nucleoid.** Treatments include: Control (ddH<sub>2</sub>O, used as solvent in the other treatments); 3 mM H<sub>2</sub>O<sub>2</sub>; 13.75 mM EIP-K; 13.75 mM EIP-K + 3mM H<sub>2</sub>O<sub>2</sub>. Values are mean  $\pm$  standard error of the mean, and the number of cells (n) for each treatment is shown in the bars. There is a significant treatment effect (one-way ANOVA,  $F_{[3,1731]} = 389.17$ ,  $p < 0.001$ ), and post-hoc Scheffé tests ( $\alpha = 0.05$ ) show that (H<sub>2</sub>O<sub>2</sub>) = (EIP-K) > (control) > (EIP-K + H<sub>2</sub>O<sub>2</sub>). Asterisk indicates that treatments are significant different from the control.



**Figure 5.3. Quantification of DNA condensation in wild-type *E. coli* using form factor, where 1.0 is a perfect circle and 0.0 is a straight line.** Treatments include: Control (ddH<sub>2</sub>O, used as solvent in the other treatments); 3 mM H<sub>2</sub>O<sub>2</sub>; 13.75 mM EIP-K; 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub>. Values are mean  $\pm$  standard error of the mean, and the number of cells (n) for each treatment is shown in the bars. There is a significant treatment effect (one-way ANOVA,  $F_{[3,1731]} = 355.01$ ,  $p < 0.001$ ), and post-hoc Scheffé tests ( $\alpha = 0.05$ ) show that all treatments differ from each other except the control and H<sub>2</sub>O<sub>2</sub>. Asterisk indicates that treatments are significant different from the control.

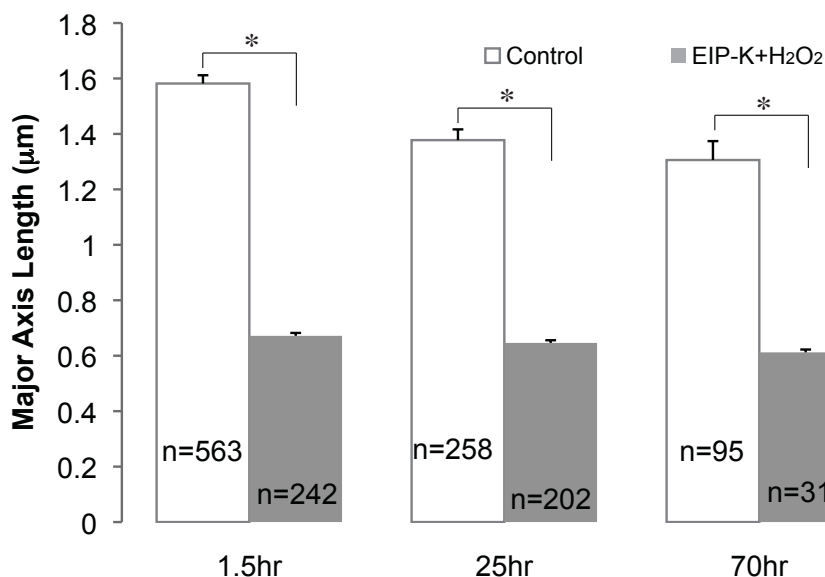


**Figure 5.4. Quantification of DNA condensation in wild-type *E. coli* using length of major axis and form factor of nucleoid.** Treatments include: Control (ddH<sub>2</sub>O, used as solvent in the other treatments); 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub>; 100 μg/ml chloramphenicol (Cam). Values are mean ± standard error of the mean, and the number of cells (n) for each treatment is shown in the bars. There is a significant treatment effect for major axis length (one-way ANOVA,  $F_{[2,1413]} = 526.68$ ,  $p < 0.001$ ) and form factor ( $F_{[2,1413]} = 394.99$ ,  $p < 0.001$ ). Post-hoc Scheffé tests ( $\alpha = 0.05$ ) show that all treatments differ from each other in major axis length as well as in form factor. Asterisk indicates that treatments are significant different from the control.

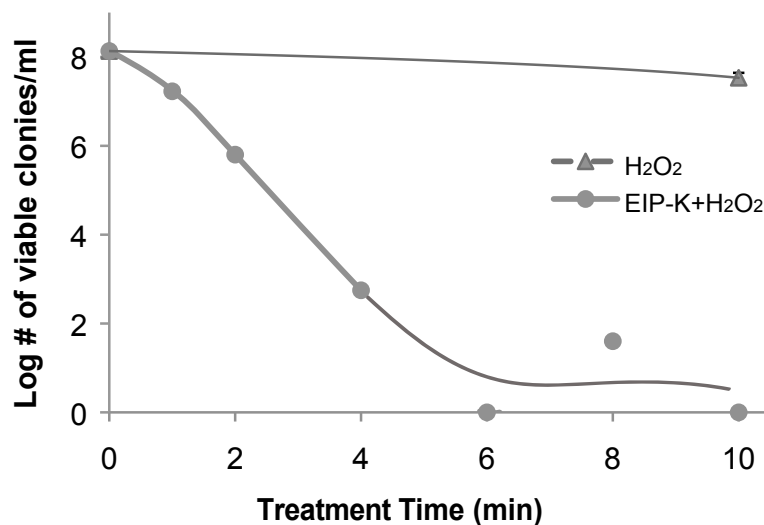


**Figure 5.5. Light micrographs of DNA condensation in wild-type *E. coli* under different treatment.** Treatments include: 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub> and 100 μg/ml chloramphenicol (Cam). Cells are stained with Hoechst to label DNA, and images are taken by simultaneously presenting UV and transmitted light. Scale bar, 1 μm.

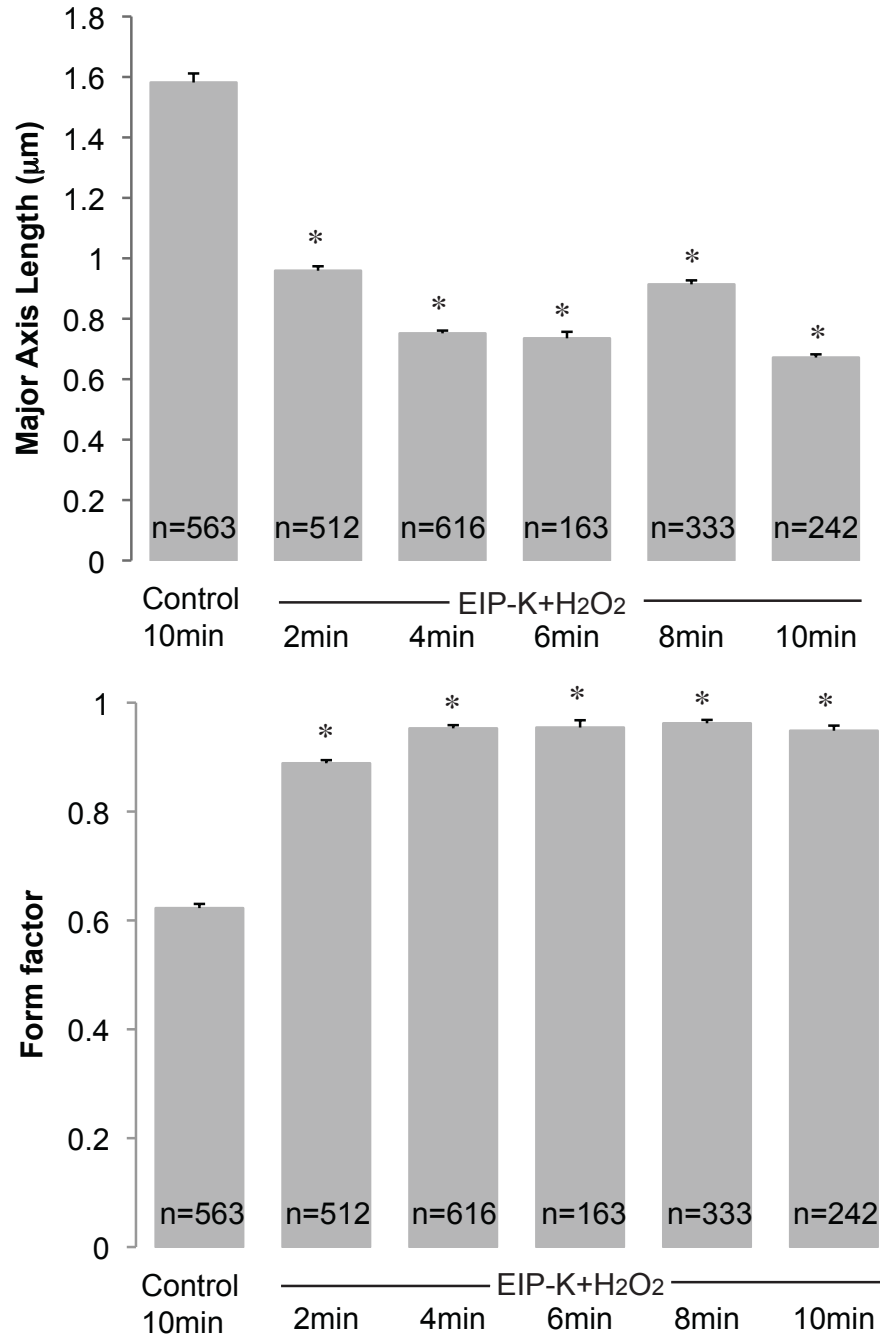




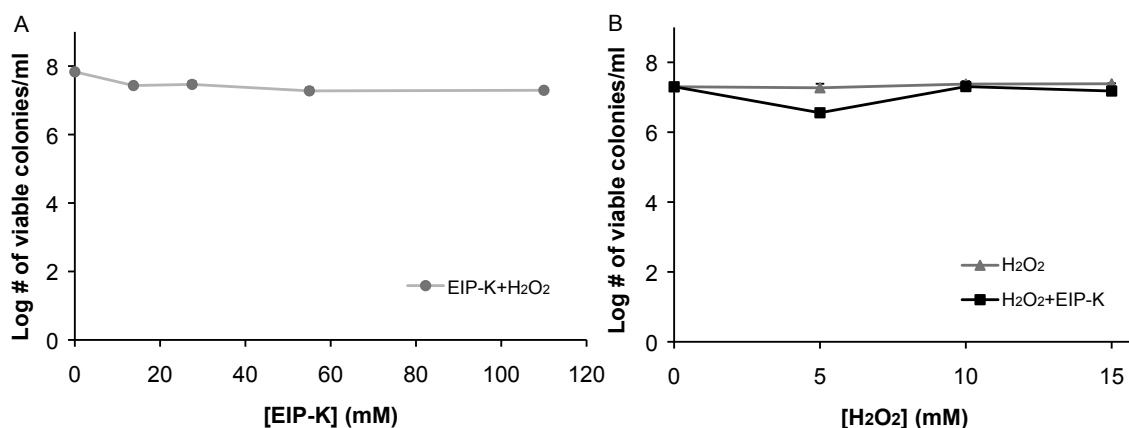
**Figure 5.6. Brief treatment with EIP-K + H<sub>2</sub>O<sub>2</sub> causes irreversible DNA condensation in wild-type *E. coli*.** Treatment was a 10-min incubation in either Control (ddH<sub>2</sub>O, used as solvent in the other treatments), or 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub>, followed by incubation in buffer (PBS) for either 1.5, 25, or 70 hr. Values are mean  $\pm$  standard error of the mean, and the number of cells (n) for each treatment is shown in the bars. For each treatment time, the control values are significantly greater than the EIP-K + H<sub>2</sub>O<sub>2</sub> values, as indicated by an asterisk (Students *t*-tests,  $\alpha=0.05$ ,  $p<0.001$ ).



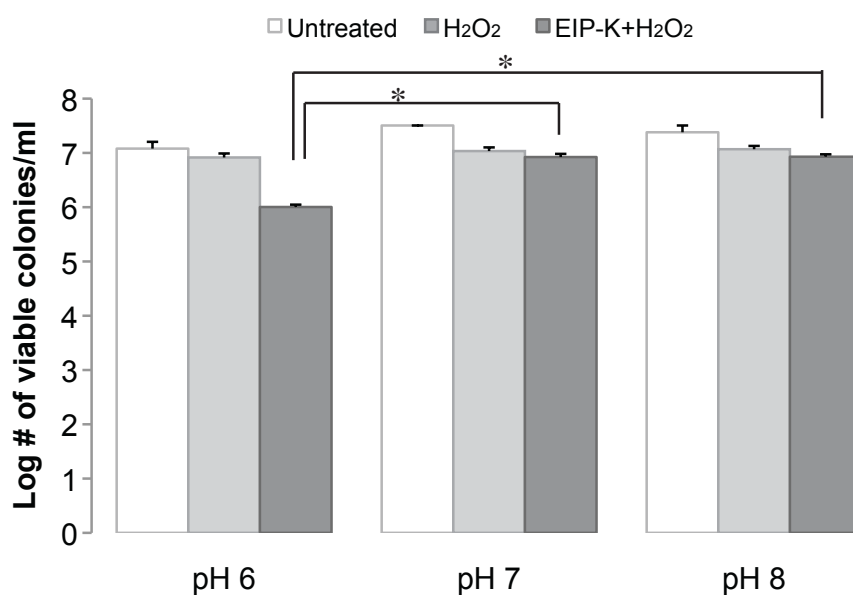
**Figure 5.7. Effective treatment time for bactericidal activity in wild-type *E. coli*.** Values are means  $\pm$  standard error of mean for three experiments each run in duplicate or triplicate.



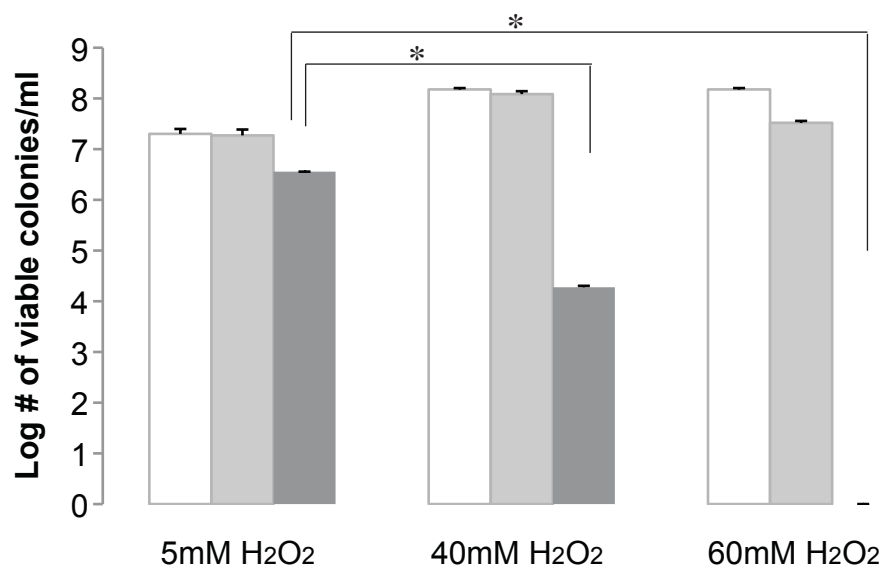
**Figure 5.8. Effective treatment time for DNA condensation in wild-type *E. coli*.** Times of treatment with 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub> are: (a) 2 min; (b) 4 min; (c) 6 min; (d) 8 min; (e) 10 min. Values are mean  $\pm$  standard error of the mean, and the number of cells (n) for each treatment is shown in the bars. One-way ANOVA for nucleoid length (A) shows a significant treatment effect ( $F_{[5,2433]}=320.35$ ,  $p<0.001$ ), and post-hoc Scheffé test ( $\alpha=0.05$ ) shows that the control group is significantly different than the other groups. One-way ANOVA for form factor (B) shows a significant treatment effect ( $F_{[5,2433]}=394.99$ ,  $p<0.001$ ), and post-hoc Scheffé test ( $\alpha=0.05$ ) shows that the control group has nucleoids that are significantly rounder than in the other groups. Asterisk indicates that length and shape in various incubation times are significantly different from untreated cell.



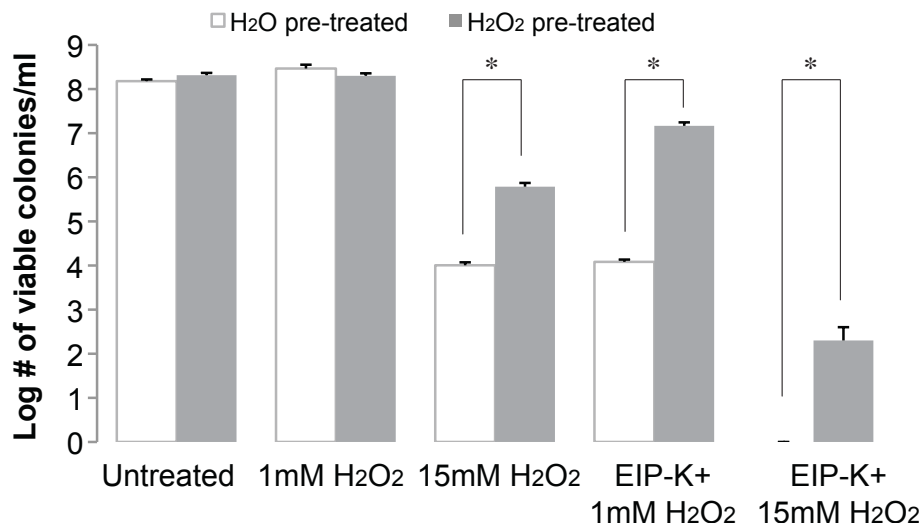
**Figure 5.9. Bactericidal effect in *E. coli* resistant strain 1** with following 10-min treatment with: (A) various concentrations of EIP-K and constant concentration (3 mM) of H<sub>2</sub>O<sub>2</sub>, (B) various concentrations of H<sub>2</sub>O<sub>2</sub> and constant concentration (13.75 mM) of EIP-K. Values are mean  $\pm$  standard error of the mean for two experiments each run in duplicate. (A) One-way ANOVA for each of the three treatment types shows that the treatment effect is non-significant (for EIP-K + H<sub>2</sub>O<sub>2</sub>:  $F_{[4,10]}=0.61$ ,  $p=0.664$ ; for H<sub>2</sub>O<sub>2</sub>:  $F_{[3,6]}=0.30$ ,  $p=0.82$ ; for H<sub>2</sub>O<sub>2</sub>+ EIP-K:  $F_{[3,6]}=7.16$ ,  $p=0.07$ ).



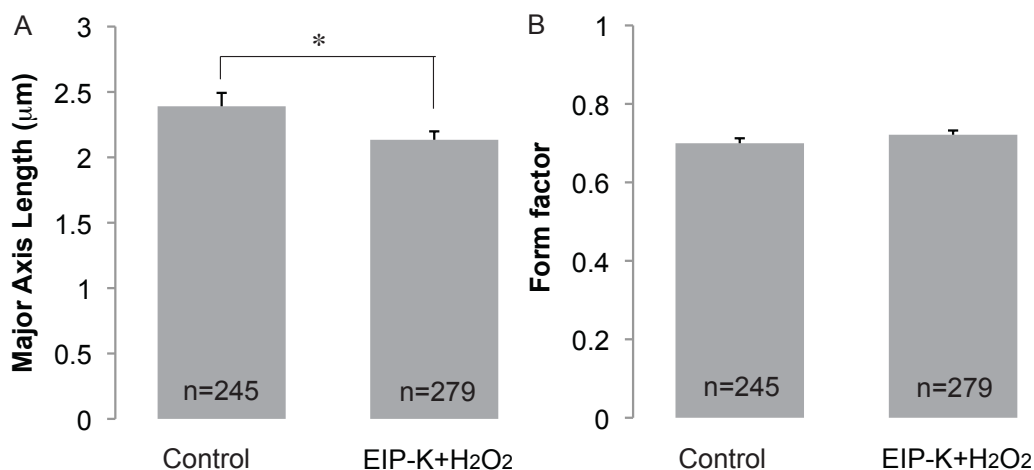
**Figure 5.10. Effect of pH on bactericidal activity using *E. coli* resistant strain 1.** Treatments are 10-min incubation in either 3 mM H<sub>2</sub>O<sub>2</sub> or 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub>. Values are mean  $\pm$  standard error of the mean for two experiments each run in duplicate. Two-way ANOVA shows a significant treatment effect ( $F_{[2,15]}=34.37$ ,  $p=0.000003$ ), a significant pH effect ( $F_{[2,15]}=13.95$ ,  $p=0.0004$ ), and a significant treatment  $\times$  pH interaction effect ( $F_{[4,15]}=3.15$ ,  $p=0.0454$ ). Asterisk indicates that the effect of EIP-K+H<sub>2</sub>O<sub>2</sub> is significantly different at various pH.



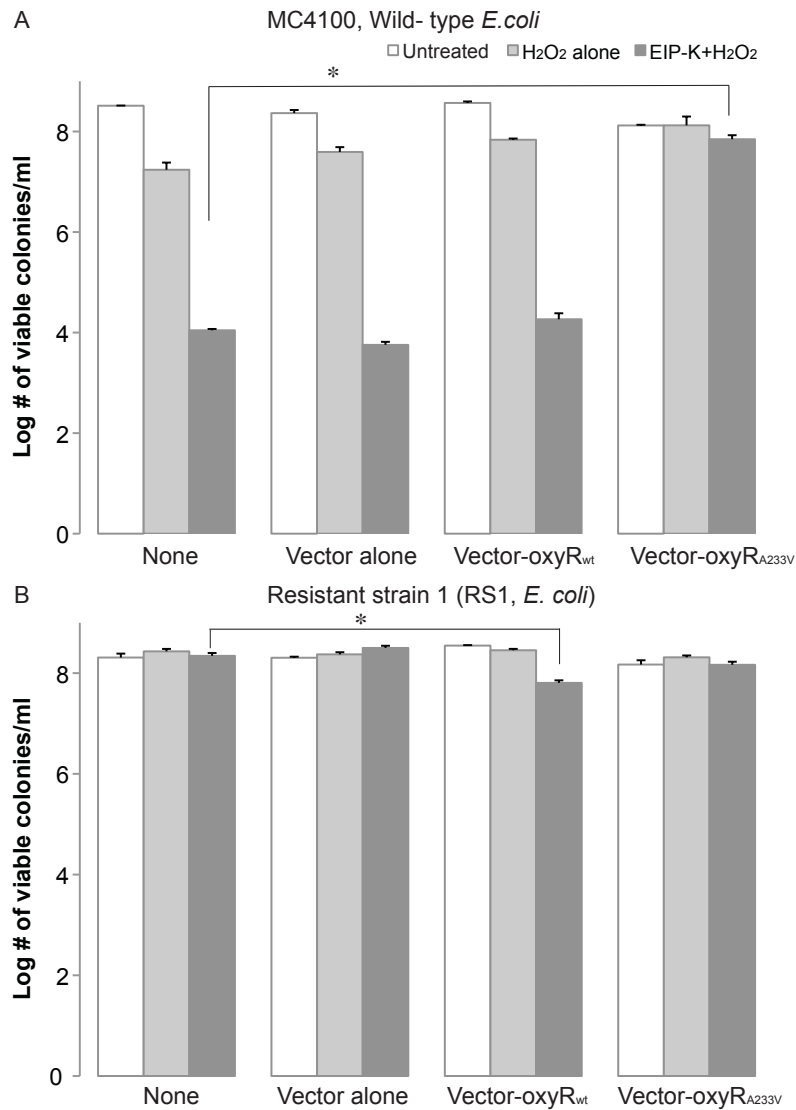
**Figure 5.11. Bactericidal effect of higher concentrations of H<sub>2</sub>O<sub>2</sub> with EIP-K on *E. coli* resistant strain 1.** Gray bars represent values for treatment with H<sub>2</sub>O<sub>2</sub> at the indicated concentration, and black bars represent values for treatment with H<sub>2</sub>O<sub>2</sub> + 13.75 mM EIP-K. Value for 'Untreated' is the number of cells at time 0 (white bar). Values are mean  $\pm$  standard error of the mean for three experiments each run in triplicate. Two-way ANOVA shows a significant treatment effect ( $F_{[2,13]}=96.02$ ,  $p=0.0000001$ ), a significant concentration effect ( $F_{[2,13]}=57.15$ ,  $p=0.0000001$ ), and a significant treatment  $\times$  concentration interaction effect ( $F_{[4,13]}=26.87$ ,  $p=0.000004$ ). Asterisk indicates that the effect of EIP-K+H<sub>2</sub>O<sub>2</sub> on resistant strain is significantly affected by concentration of H<sub>2</sub>O<sub>2</sub>.



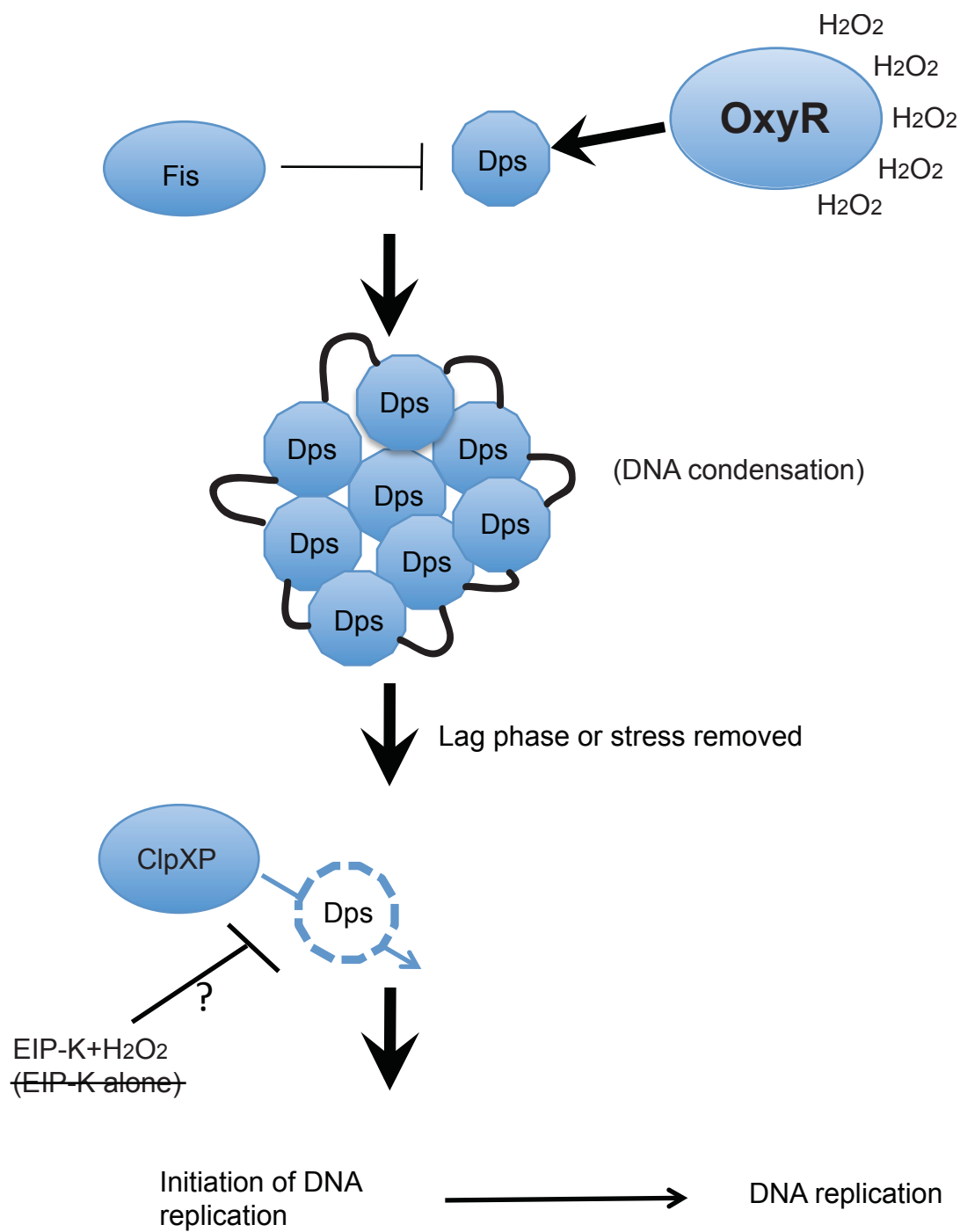
**Figure 5.12. Effect of H<sub>2</sub>O<sub>2</sub> adaption on bactericidal activity of EIP-K + H<sub>2</sub>O<sub>2</sub> in wild-type *E. coli*, MC4100.** Bacteria were pretreated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (or same amount of sterile H<sub>2</sub>O) for 1 hr, followed by a bactericidal assay using various concentrations of H<sub>2</sub>O<sub>2</sub> alone or with H<sub>2</sub>O<sub>2</sub> + 13.75 mM EIP-K. Values are mean  $\pm$  standard error of the mean for two experiments each run in duplicate. Two-way ANOVA shows a significant adaptation effect ( $F_{[1,19]}=16.48$ ,  $P=0.003$ ), a significant treatment effect ( $F_{[2,9]}=16.25$ ,  $P=0.001$ ), and a significant adaptation  $\times$  treatment interaction effect ( $F_{[2,9]}=16.21$ ,  $p=0.001$ ). Asterisk indicates that H<sub>2</sub>O<sub>2</sub> pre-treatment reduces the bactericidal effect of high concentration of H<sub>2</sub>O<sub>2</sub> and EIP-K + H<sub>2</sub>O<sub>2</sub>.



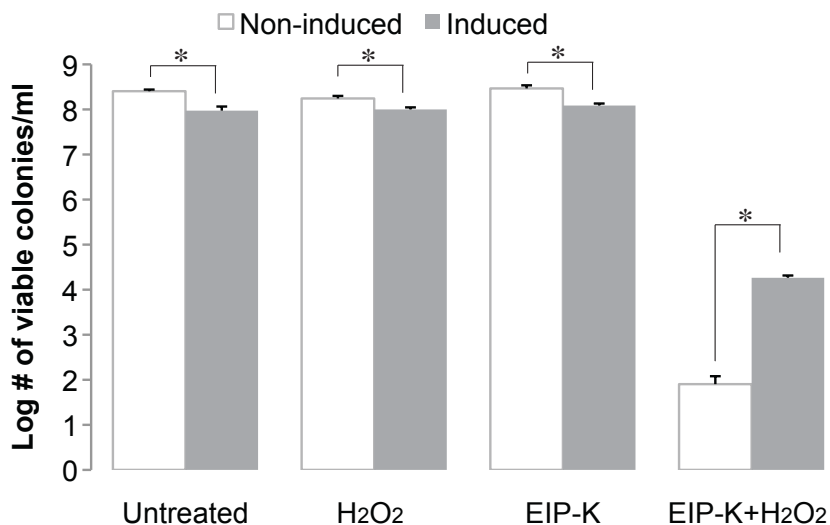
**Figure 5.13. *E. coli* resistant strain 1 does not show DNA condensation to treatment with EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub>.** (A) Measurement of major axis length of nucleoid. (B) Measurement of form factor of the nucleoid. Treatment was a 10-min incubation in either Control (ddH<sub>2</sub>O, used as solvent in the other treatments), or 13.75 mM EIP-K + 3 mM H<sub>2</sub>O<sub>2</sub>. Values are mean  $\pm$  standard error of the mean, and the number of cells (n) for each treatment is shown in the bars. Students *t*-tests show a significant treatment effect for length ( $t_{[522]}=2.17$ ,  $p=0.031$ ) but no significant difference for form factor ( $t_{[522]}=1.31$ ,  $p=0.190$ ).



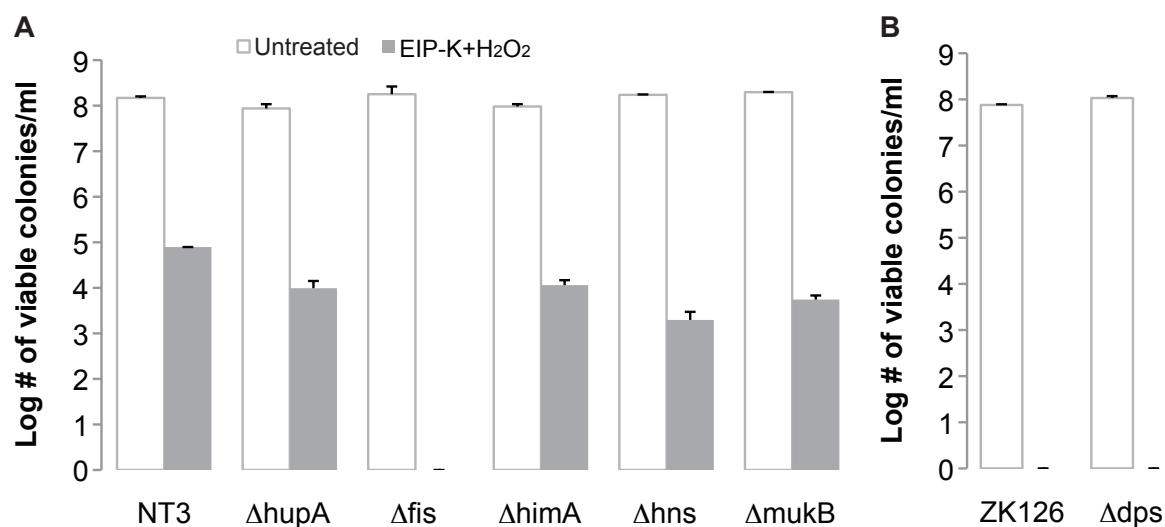
**Figure 5.14. Complementation assay of *E. coli* wild-type MC4100 (MC, A) and Resistant strain 1 (RS1, B) with plasmids containing wild-type or mutant *oxyR* gene.** Complementation assay of *E. coli* wild type MC4100 (MC, A) and Resistant strain 1 (RS1, B) with plasmid pET29a (pET29a), pET29a-oxyR<sup>wt</sup> (oxyR<sup>wt</sup>), pET 29a-oxyR<sup>A233V</sup> (oxyR<sup>A233V</sup>). (None): without carrying any plasmid. Values are mean  $\pm$  standard error of the mean for four experiments each run in duplicate. (A) Two-way ANOVA for the wild type strains shows that the plasmid effect is not significant ( $F_{[3,19]}=2.57$ ,  $p=0.085$ ), the treatment effect is significant ( $F_{[3,19]}=91.01$ ,  $p<0.00000001$ ), the interaction effect is significant ( $F_{[6,19]}=9.02$ ,  $p<0.0001$ ). Given the significant interaction effect, we performed a one-way ANOVA for the EIP-K+H<sub>2</sub>O<sub>2</sub> treatment and found a significant effect ( $F_{[3,7]}=46.64$ ,  $p=0.00005$ ; and Scheffé post-hoc tests show that vector-oxyR<sup>A233V</sup> is different from vector-oxyR<sup>wt</sup>, vector alone, and none. (B) Two-way ANOVA for the resistant strains shows a significant plasmid effect ( $F_{[3,21]}=6.33$ ,  $p=0.0031$ ), a significant treatment effect ( $F_{[2,21]}=7.38$ ,  $p=0.0037$ ), and a significant treatment x plasmid interaction effect ( $F_{[6,21]}=13.85$ ,  $p=0.000002$ ). A subsequent one-way ANOVA for EIP-K + H<sub>2</sub>O<sub>2</sub> shows a significant effect ( $F_{[3,9]}=22.11$ ,  $p=0.00017$ , and Scheffé post-hoc tests show that none is different from vector-oxyR<sup>wt</sup> but not vector alone or vector-oxyR<sup>A233V</sup>. Asterisks indicate significant differences between cells without any plasmid (None) and any of the vector conditions, under EIP-K + H<sub>2</sub>O<sub>2</sub> treatment.



**Figure 5.15. Model of mechanism of DNA condensation in *E. coli* induced by EIP-K + H<sub>2</sub>O<sub>2</sub>.**

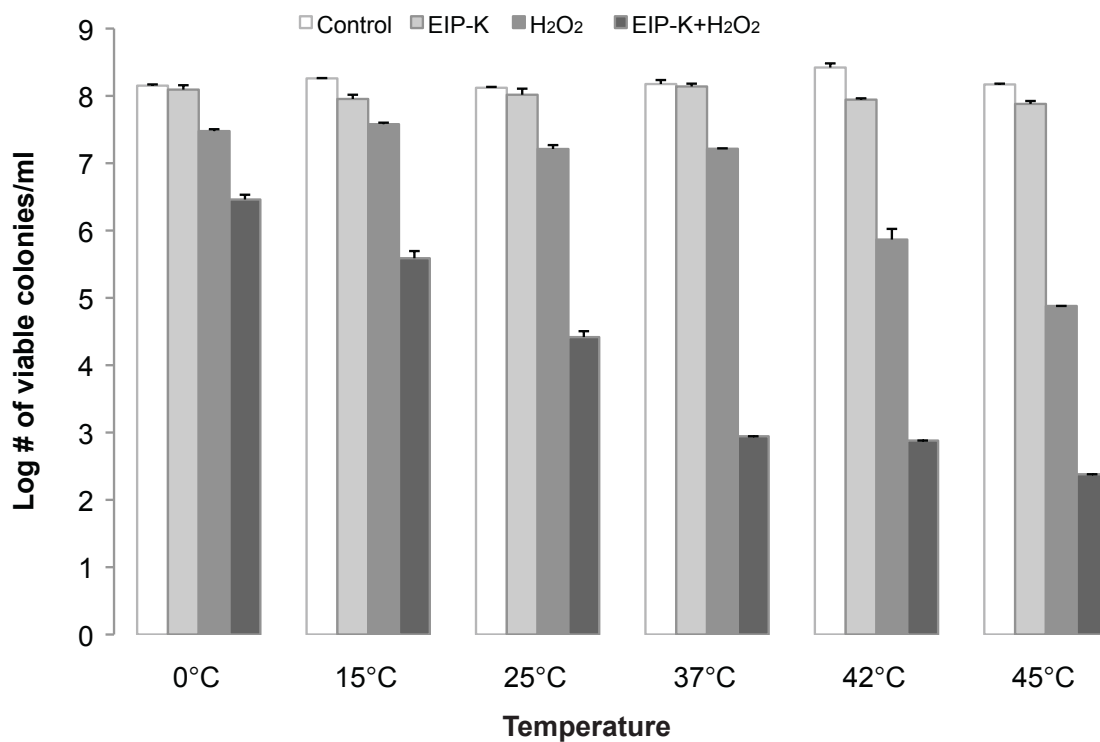


**Figure 5.16. Effect of overexpression of Dps on bactericidal activity.** *E. coli* MC4100 with plasmid *dps* was induced with 0.2% L-arabinose or same volume of ddH<sub>2</sub>O for 1 hr and then treated with 3 mM H<sub>2</sub>O<sub>2</sub>, 13.75 mM EIP-K, or a combination of the two, for 10 min. Values are mean  $\pm$  standard error of the mean for two experiments each run in duplicate. A two-way ANOVA shows a significant induction effect ( $F_{[1,4]} = 33.18$ ,  $p=0.00005$ ), a significant treatment effect ( $F_{[3,14]}=27.77$ ,  $p=0.000004$ ), and a significant interaction effect ( $F_{[3,14]} = 5.24$ ,  $p= 0.0123$ ). Asterisks show data pairs that are significantly different (Students *t*-tests,  $p<0.05$ ). Asterisks indicates that cells overexpressing Dps are significant different from non-induced cells, with an induced increase in resistance only for treatment with EIP-K + H<sub>2</sub>O<sub>2</sub>.



**Figure 5.17. Effect of null mutations of DNA associated proteins on bactericidal activity.** Gray bars represent values for treatment with H<sub>2</sub>O<sub>2</sub> + 13.75 mM EIP-K. Value for 'Untreated' is the number of cells at time 0 (white bar). Values are mean  $\pm$  standard error of the mean for two experiments each run in triplicate. (A) NT3 is the wild type strains for ΔHup, ΔFis, ΔHimA, ΔHns, and ΔMukB. (B) ZK126 is the wild type strain for Δdps. Two-way ANOVA for the mutant strains, ΔFis, ΔHimA, ΔHns, ΔMukB, and wild type strain NT3 shows a significant treatment effect ( $F_{[1,21]}=68.96$ ,  $p=0.0000001$ ), a non-significant mutant effect ( $F_{[5,21]}=1.27$ ,  $p=0.314$ ), and a non-significant treatment x mutant interaction effect ( $F_{[6,21]}=1.269$ ,  $p=0.313$ ). No asterisk indicates that mutant strains show similar effect as wild type under EIP-K + H<sub>2</sub>O<sub>2</sub> treatment.





**Figure 5.18. Effect of temperature on the bactericidal effect of EIP-K + H<sub>2</sub>O<sub>2</sub>.** Bacteria were grown at 37°C to exponential phase and then treated with chemicals as usual at various temperatures as shown. High and low temperature did not show a bactericidal effect with EIP-K alone. The results indicate that the role of H<sub>2</sub>O<sub>2</sub> on bactericidal activity of EIP-K + H<sub>2</sub>O<sub>2</sub> cannot be substituted by other stresses, such as temperature stress. Values are mean  $\pm$  SEM for one experiment run in duplicate.

## 6 CONCLUSIONS

Escapin is an L-amino acid oxidase. Its gene sequence shows two expected conserved motifs of flavin proteins, GG (RxGGRxxS/T) and dinucleotide-binding motifs. Its cofactor, flavin adenine dinucleotide (FAD), was identified by NMR and ESI-TOF mass spectroscopy. The L-amino acids L-lysine and L-arginine are used as specific substrates for escapin. L-Lysine is the major natural substrate, since the concentration of L-lysine in the ink secretion containing escapin is much higher than that of L-arginine: in ink secretion of *A. californica*, L-lysine and L-arginine are present at concentrations of 145 and 0.5 mM, respectively. Escapin has a wide spectrum of antimicrobial activities but different efficacies. Escapin was tested against 11 types of microbes, including bacteria, yeasts, and fungi, and it was found to be bacteriostatic for all. Powerful bactericidal activity occurs in enriched medium containing L-lysine; in media lacking L-lysine, escapin only shows bacteriostatic effects, not bactericidal effects. Most studies of L-amino acid oxidases of sea hares and other gastropods have identified H<sub>2</sub>O<sub>2</sub> as the primary anti-microbial product of this pathway, though other compounds are implicated. H<sub>2</sub>O<sub>2</sub> plays a prominent role in the bacteriostatic effect but only a weak role in the bactericidal effect. Against *Escherichia coli*, H<sub>2</sub>O<sub>2</sub> at concentrations below 10 mM accounts for most of the bacteriostatic effects of escapin's products, but it contributes relatively little (10 times reduction in bacterial number) com-

pared to the powerful bactericidal effects of escapin's products (up to  $10^7$  times reduction).

The reaction of escapin with L-arginine produces a mixture with almost no bactericidal activity, even though the product contains more  $H_2O_2$  than the product of the reaction of escapin with L-lysine. This demonstrates that compounds other than  $H_2O_2$  are responsible for the bactericidal effects of escapin. Escapin's oxidation of L-lysine and L-arginine produces the same amount of  $H_2O_2$ ; the difference in the two reactions is the other molecules produced, so these must account for the different bactericidal effects.

In the second chapter, we measured the bactericidal effects of the oxidized L-amino acids. Two different compounds are generated in this oxidation pathway. The first product is "escapin intermediate product" (EIP), which is an unstable compound and easily reacts with the other product,  $H_2O_2$ , to generate "escapin end product" (EEP). The bactericidal effect only occurs with treatment with EIP of lysine (i.e. EIP-K) +  $H_2O_2$ , but not with EIP-K or  $H_2O_2$  alone, nor with any combination of the EIP of arginine. EIP-K +  $H_2O_2$  is bactericidal against bacteria that cause serious hospital infections, such as *Pseudomonas* and *Staphylococcus*. By testing different concentrations of EIP-K and  $H_2O_2$ , we showed an Eagle effect, in which a maximal bactericidal effect occurred at intermediate concentration. We also showed that the greatest bactericidal effect occurred at pH 6.

The chemistry of the escapin/L-lysine pathway is complex. The first step is escapin's oxidative deamination of L-lysine (compound **1**), which produces an equilibrium mixture of compounds in EIP-K. They include  $\alpha$ -keto- $\epsilon$ -aminocaproic

acid (compound **2**), 1-piperidine-2-carboxylic acid (compound **3**), 2-piperidine-2-carboxylic acid (compound **4**), 6-amino-2-hydroxy-hex-2-enoic acid (compound **7**), possibly 6-amino-2,2-dihydroxy-hexanoic acid (compound **8**), 2-hydroxy-piperidine-2-carboxylic acid (compound **9**),  $\text{H}_2\text{O}_2$ , and ammonium. Three of these compounds, compounds **2**, **3**, and **4**, then react nonenzymatically with  $\text{H}_2\text{O}_2$  to yield a mixture of  $\delta$ -aminovaleric acid (compound **5**) and  $\delta$ -valerolactam (compound **6**), which constitute EEP-K. The pH of *A. californica* ink is  $\sim 5.0$  at full strength, in contrast to a pH of  $\sim 8.0$  for seawater. This is significant because pH affects the equilibrium among the escapin reaction products: though the cyclic forms **3** and **6** dominate at any pH, the naturally low pH of the secretion favors the linear forms **2** and **5**. The composition of EIP-K equilibrium mixture changes with pH level. A cyclic deaminated product,  $\Delta^1$ -piperidine-2-carboxylic acid, dominates at any pH level, but the linear form,  $\alpha$ -amino- $\epsilon$ -caproic acid, becomes more prevalent at lower pH values, which is biologically significant because sea hare ink is acidic. This increase in the relative amount of  $\alpha$ -amino- $\epsilon$ -caproic acid in EIP-K at low pH is correlated with an increase in bactericidal activity. Furthermore, the synergistic bactericidal effect of EIP-K and  $\text{H}_2\text{O}_2$  requires their simultaneous, not sequential, presence. Together, these results suggest that the powerful bactericidal effect of EIP-K +  $\text{H}_2\text{O}_2$  is due to a synergy involving  $\alpha$ -amino- $\epsilon$ -caproic acid and  $\text{H}_2\text{O}_2$ .

The ultimate goal of this study is to understand mechanisms of the bactericidal effect of EIP-K +  $\text{H}_2\text{O}_2$ . We pursued two lines of experiments. In the first set, we followed up on results of our preliminary studies that showed that EIP-K +

H<sub>2</sub>O<sub>2</sub> causes intensive DNA condensation, a different morphology from donut shape condensation induced by chloramphenicol. The long lasting (>70h) condensation is a potential cause of the bactericidal effect. Consequently, we hypothesized that DNA condensation is a major cause of the bactericidal effect by EIP-K + H<sub>2</sub>O<sub>2</sub>. To test this idea, we examined the time course of DNA condensation to treatment with EIP-K + H<sub>2</sub>O<sub>2</sub>, and examined whether DNA condensation and bactericidal activity occurred under the same treatment conditions. Our second line of investigation into the bactericidal mechanism of EIP-K + H<sub>2</sub>O<sub>2</sub> was to generate mutant bacterial strains resistant to EIP-K + H<sub>2</sub>O<sub>2</sub>, followed by comparison of the mutants' genotype relative to the wild strain. A 2-min treatment with EIP-K + H<sub>2</sub>O<sub>2</sub> triggered both the bactericidal effect and DNA condensation. A single mutant on the *oxyR* gene provided resistance to EIP-K + H<sub>2</sub>O<sub>2</sub> and prevented DNA condensation. A similar time course in the bactericidal effect and DNA condensation is consistent with a causal relationship between DNA condensation and the bactericidal effect, though this remains to be experimentally demonstrated.

Future directions might focus on seeking direct evidence that DNA condensation causes the bactericidal effect. One test might be to apply DNA relaxing agents to bacteria with escapin-induced DNA condensation to determine if the bactericidal effect is reversed with DNA relaxation. Another means toward this end might be to identify molecules involved in escapin-induced DNA condensation. Finally, understanding the mechanisms behind EIP-K's synergistic role with H<sub>2</sub>O<sub>2</sub> is an important future direction.

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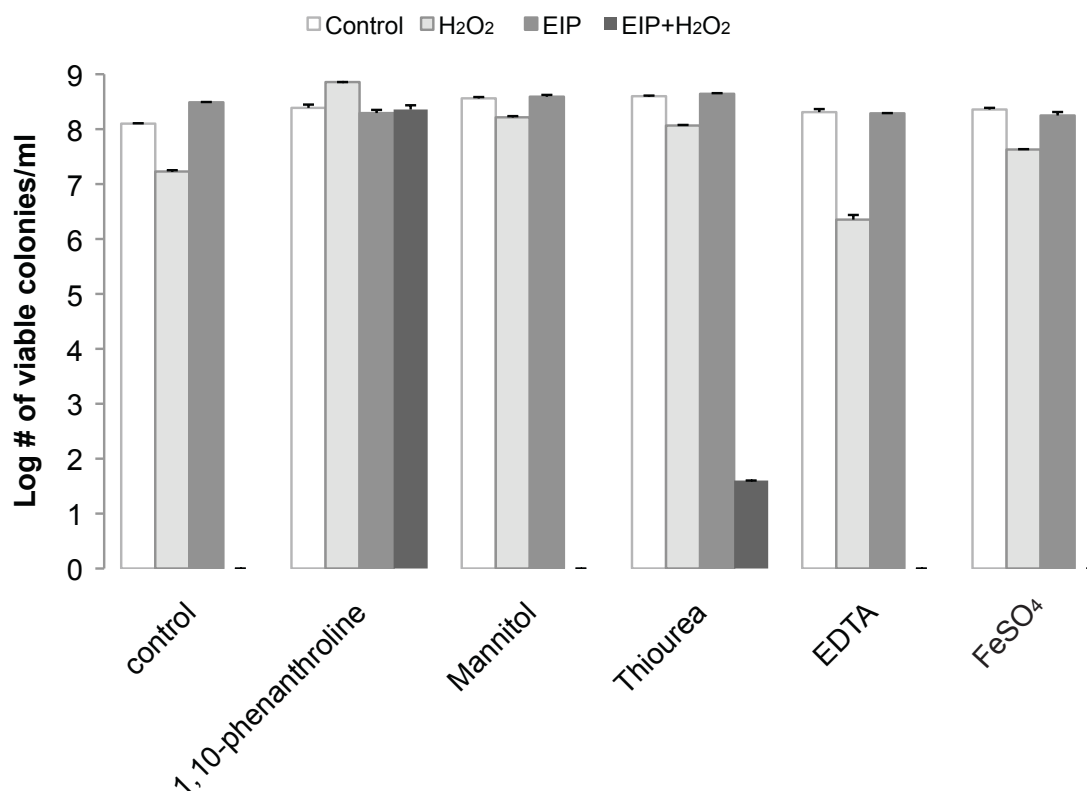
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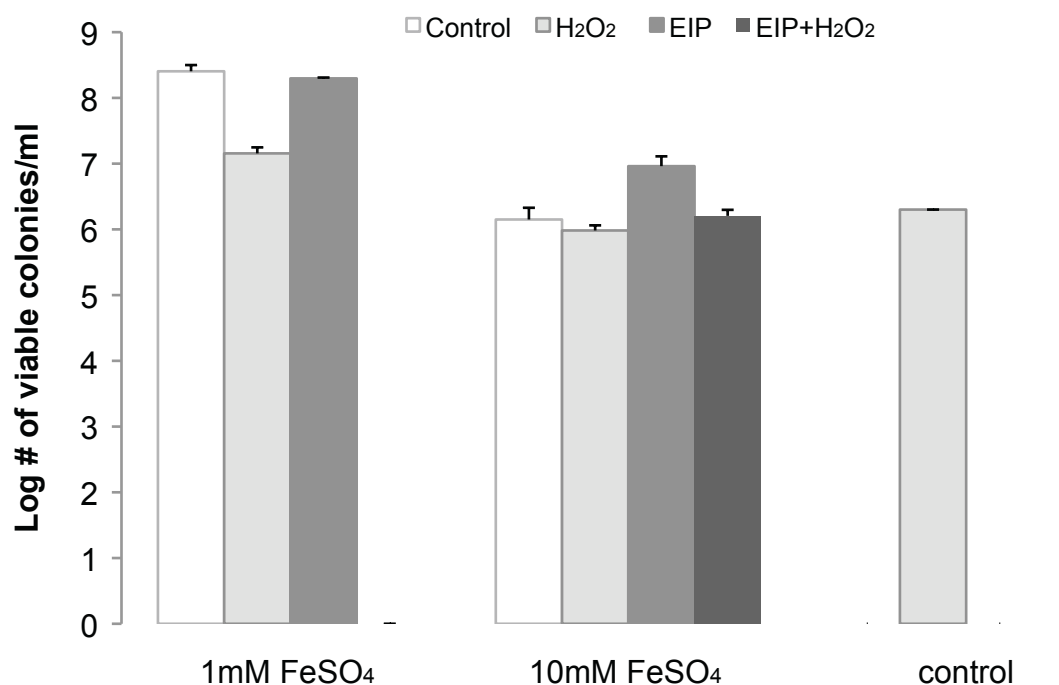
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## 8 APPENDIX

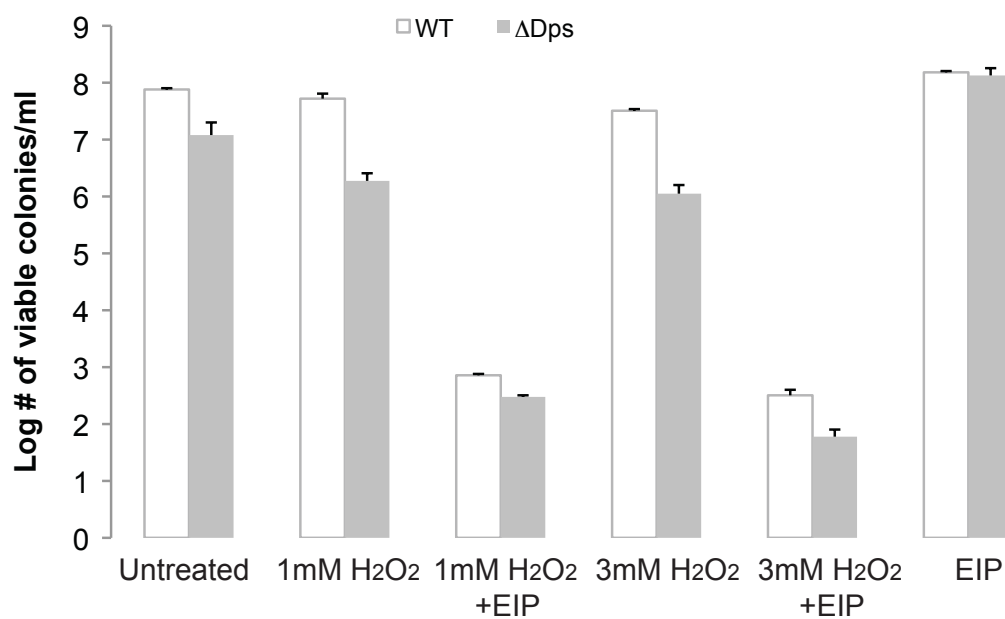


**Figure 8.1. Effect of ferrous chelators, hydroxyl radical scavengers, or ferrous on bactericidal activity of EIP-K + H<sub>2</sub>O<sub>2</sub>.** Bacteria were pre-treated by a ferrous chelator, 1,10-phenanthroline (100  $\mu$ M), and hydroxyl radical scavengers, mannitol (100  $\mu$ M) and thiourea (10 mM), for 30 min, followed by regular treatment protocol of chemicals. EDTA (100  $\mu$ M) and FeSO<sub>4</sub> (10  $\mu$ M) treated bacteria at same time with chemicals. The ferrous chelator, 1,10-phenanthroline, showed strong inhibition of the bactericidal effect of EIP-K + H<sub>2</sub>O<sub>2</sub>. Similar effect was observed in another ferrous chelator, 2,2-bipyridine (250  $\mu$ M), though the inhibition was smaller. Values are mean  $\pm$  SEM for two experiments each run in duplicate.

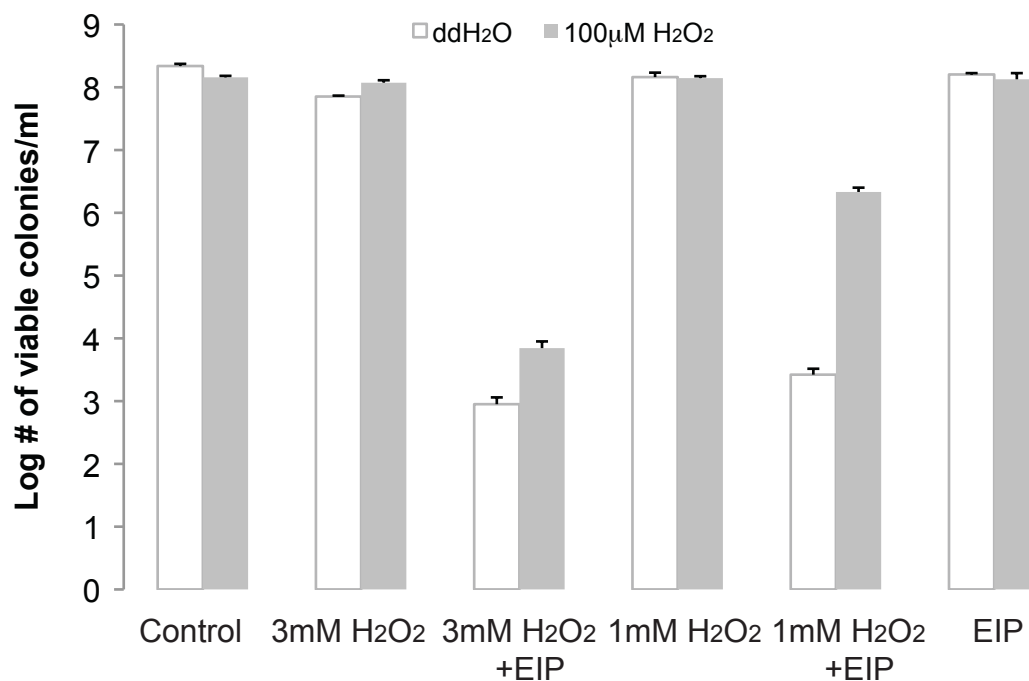


**Figure 8.2. Strong inhibition effect of high concentration of FeSO<sub>4</sub> on the bactericidal effect of EIP-K + H<sub>2</sub>O<sub>2</sub>.** Bacteria were pre-treated with 1 mM or 10 mM FeSO<sub>4</sub> for 30 min, followed by treatment of chemicals. High concentration of FeSO<sub>4</sub> is toxic for bacteria, but a high concentration of FeSO<sub>4</sub> inhibits the bactericidal effect of EIP-K + H<sub>2</sub>O<sub>2</sub>. EIP-K weakens the toxicity of the high concentration of FeSO<sub>4</sub>. Values are mean  $\pm$  SEM for three experiments each run in duplicate.





**Figure 8.3. Bactericidal effect on Dps null mutant strain.** Various concentrations of H<sub>2</sub>O<sub>2</sub> alone or with 13.75 mM EIP-K, and 13.75 mM EIP-K alone were tested on Dps null mutant strain, and compared with wild type strain of Dps null mutant. Values are mean  $\pm$  SEM for one experiment run in duplicate.



**Figure 8.4. Effect of H<sub>2</sub>O<sub>2</sub> adaption on bactericidal activity of EIP-K + H<sub>2</sub>O<sub>2</sub> in wild-type *E. coli*, MC4100.** Bacteria were grown with 100 µM H<sub>2</sub>O<sub>2</sub> (or same amount of sterile H<sub>2</sub>O), followed by a bactericidal assay using various concentrations of H<sub>2</sub>O<sub>2</sub> alone or with H<sub>2</sub>O<sub>2</sub> + 13.75mM EIP-K. Values are mean ± SEM for one experiment run in duplicate.