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The periplasmic serine protease inhibitor ecotin protects bacteria against neutrophil elastase

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Ecotin is a dimeric periplasmic protein from *Escherichia coli* that has been shown to inhibit potently many trypsin-fold serine proteases of widely varying substrate specificity. To help elucidate the physiological function of ecotin, we examined the family of ecotin orthologues, which are present in a subset of Gram-negative bacteria. Phylogenetic analysis suggested that ecotin has an exogenous target, possibly neutrophil elastase. Recombinant protein was expressed and purified from *E. coli*, *Yersinia pestis* and *Pseudomonas aeruginosa*, all species that encounter the mammalian immune system, and also from the plant pathogen *Pantoea citrea*. Notably, the *Pa. citrea* variant inhibits neutrophil elastase 1000-fold less potently than the other orthologues. All four orthologues are dimeric proteins that potently inhibit (< 10 pM) the pancreatic digestive proteases trypsin and chymotrypsin, while showing more variable inhibition (5 pM to 24 μM) of the blood proteases Factor Xa, thrombin and urokinase-type plasminogen activator. To test whether ecotin does, in fact, protect bacteria from neutrophil elastase, an ecotin-deficient strain was generated in *E. coli*. This strain is significantly more sensitive in cell-killing assays to human neutrophil elastase, which causes increased permeability of the outer membrane that persists even during renewed bacterial growth. Ecotin affects primarily the ability of *E. coli* to recover and grow following treatment with neutrophil elastase, rather than the actual rate of killing. This suggests that an important part of the antimicrobial mechanism of neutrophil elastase may be a periplasmic bacteriostatic effect of protease that has translocated across the damaged outer membrane.

Key words: ecotin, Gram-negative bacteria, neutrophil elastase, outer membrane permeability, serine protease inhibitor.

INTRODUCTION

Neutrophils are leucocytes that represent the first line of defence against invading micro-organisms [1]. In a process called phagocytosis, bacteria are engulfed into an intracellular compartment, the phagosome, where an array of antimicrobial agents are released. Two pathways of killing have generally been considered: the oxidative and non-oxidative pathways. Oxidative killing involves the generation, through the action of NADPH oxidase and myeloperoxidase, of oxidants that can be directly toxic to bacteria [2]. The non-oxidative pathway includes a number of proteins with membrane permeabilizing [defensins and bacterial/permeability-increasing protein (BPI)], sugar hydrolysing (lysozyme), and proteolytic [neutrophil elastase (NE), cathepsin G (CatG) and proteinase 3] activities [3].

While the bactericidal nature of neutrophil proteases has been known for some time, recent genetic studies in mice have highlighted their importance in microbial killing. It has recently been proposed that the major role for the oxidative pathway may, in fact, be to cause the release and activation of granule proteases, which are principally responsible for bacterial killing [4]. Mice deficient in NE were found to be more susceptible to infection by the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae*, but not the Gram-positive bacterium *Staphylococcus aureus* [5]. This same species selectivity was seen during killing of bacteria *in vitro* by purified NE, which was found to cleave OmpA (outer membrane protein A). *E. coli* deficient in OmpA were no longer susceptible to killing by purified NE and were therefore no longer affected by whether or not mice were deficient in NE [6]. Furthermore, low concentrations of NE were found to cleave preferentially enterobacterial virulence factors from *Shigella*, *Salmonella* and *Yersinia*, and abrogation of NE activity in neutrophils allowed *Shigella* to escape the phagosome [7].

Mice deficient in CatG were not found to be impaired in their ability to fight off Gram-negative infections [8], but both these mice and their neutrophils appear to be impaired in killing *St. aureus* [4]. The granule proteases therefore appear to have a certain specificity of action, with NE and CatG playing relatively distinct roles in the killing of Gram-negative and Gram-positive bacteria respectively. Although the bactericidal properties of a number of neutrophil components have been described, it is still not clear exactly how bacteria are killed within the phagosome or how bacteria may attempt to defend themselves from this lethal environment. Given the prominent role of proteases in the immune response to bacterial infections, we investigated whether bacteria utilize protease inhibitors to counter host defences.

Protease inhibitors from many plant and animal species have been characterized on the level of protein sequence, structure, inhibitory specificity and kinetics [9,10]. The most well-studied class comprises the 'small' serine protease inhibitors, which inhibit in a 'substrate-like' fashion. The residues flanking the scissile bond are denoted P1 and P1′, with the P1 residue binding in the primary specificity pocket [11]. Although the function of this class of proteins is clearly to prevent proteolysis by binding stably

Abbreviations used: BPI, bactericidal/permeability-increasing protein; CatG, cathepsin G; CFU, colony-forming unit; DOE JGI, Department of Energy Joint Genome Institute; NE, neutrophil elastase; Omp, outer membrane protein; TSA, tryptic soya agar; TSB, tryptic soya broth; uPA, urokinase-type plasminogen activator; WT, wild-type.

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to the active site of a protease, their exact physiological functions have not often been completely elucidated. Even less is known about the role of protease inhibitors from bacteria. *Bacillus brevis* [12,13] and *Prevotella intermedia* [14] both secrete protease inhibitors that are thought to protect the cell against external proteolytic attack. Many of the known periplasmic inhibitors target endogenous proteases. Species such as *Pseudomonas aeruginosa*, *Serratia marcescens* and *Erwinia chrysanthemi* produce a periplasmic inhibitor that is thought to protect periplasmic proteins from serralysin-family metalloproteases prior to their secretion [15,16].

Only one protease inhibitor has been isolated from *E. coli* [17], the periplasmic protein ecotin. Ecotin is a homodimer of 16 kDa subunits that is able to inhibit serine proteases of widely varying substrate specificities, such as trypsin, chymotrypsin and elastase [18]. A crystal structure of ecotin bound to trypsin revealed a heterotetrameric complex in which each trypsin molecule makes contacts with both subunits of ecotin [19]. Ecotin is folded into a β-barrel, with the protease contact surface consisting mainly of four loops connecting the β-strands. The primary binding site of ecotin includes the 80s loop, an extended loop fitting into the protease active site in a substrate-like fashion, and the 50s loop, which stabilizes the 80s loop through disulphide and hydrogen bonds. The same molecule of trypsin makes contacts with the other ecotin subunit through a smaller secondary binding site, consisting of the 60s and 100s loops.

Dimerization of ecotin is mediated primarily through an exchanged C-terminal arm. Dimerization creates an exceptionally large surface for interaction with proteases, and co-operativity between the two binding sites helps ecotin inhibit with extremely broad specificity [20]. In addition to the pancreatic serine proteases, ecotin potently inhibits Factor Xa, Factor XIIa, plasma kallikrein and NE, among others [21,22]. Although its physiological function is unknown, its inhibition of pancreatic digestive proteases has led researchers to postulate a role in protecting the bacterium from external proteolytic attack in the mammalian gut [18].

To help elucidate the function of this inhibitor, we investigated the distribution of orthologues in other bacterial species, which suggested a potential role in protection against NE. To determine whether ecotin variants from different species could share this common target, we cloned three orthologous genes, recombinantly expressed the proteins, and characterized them for oligomerization and for inhibition against a panel of proteases. Finally, we generated an ecotin-deficient strain of *E. coli*, which showed that ecotin protects the bacterium against NE and allowed a closer examination of the mechanism of NE-mediated killing.

EXPERIMENTAL

Compiling sequences of ecotin orthologues

A list of ecotin-containing species was assembled by TBLASTN searches of microbial genomes on the NCBI site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), as well as searches of incomplete genomes at the Sanger Center, the Department of Energy Joint Genome Institute (DOE JGI) and The Institute for Genomic Research microbial database (http://www.tigr.org/tdb/mdb/mdbinprogress.html). DNA sequences for ecotin coding regions were retrieved from published genome sequences and from the following unpublished data. From the Sanger Center (http://www.sanger.ac.uk/Projects/Microbes/), we received the unpublished sequence data for *Yersinia enterocolitica*, *Se. marcescens* (assembled from multiple shotgun reads), *Burkholderia pseudomallei* and *Bu. cepacia*, and searched the unpublished sequence of *Erwinia carotovora*. Preliminary sequence data for *Bu. mallei* were obtained from The Institute for Genomic Research website at http://www.tigr.org. Preliminary data from the unfinished genome sequence of *Salmonella dublin* was obtained from the University of Illinois, Urbana-Champaign, IL, U.S.A. The sequence of *K. pneumoniae* ecotin was obtained from the unpublished genome sequence at the Washington University Sequencing Center. While the native protein sequence was unambiguous, a single base pair sequencing error seemed likely in the signal peptide region, since there was no apparent initiating methionine. Several different single base pair changes would give signal peptides predicted to end at the same point, so the native form of the protein was calculated from this. The *Ps. fluorescens* ecotin sequence was obtained from the genome sequence at the DOE JGI. The previously unpublished ecotin protein sequence from *Pantoea citrea* was obtained from the genome sequence obtained by Genencor International Inc. (Palo Alto, CA, U.S.A.). TBLASTN searches were also conducted on the unpublished sequences at the DOE JGI of *Bu. fungorum* and *Ps. syringae* pv. Tomato DC3000.

Full-length protein sequences were calculated from assembled DNA sequence data. The start of the native sequence was calculated using the signal prediction program SignalP v1.1 (http://www.cbs.dtu.dk/services/SignalP/) [23]. Multiple sequence alignment was performed using ClustalW (version 1.4) in the MacVector 7.0 suite of programs, using the default parameters. A phylogram was constructed from the ClustalW Guide Tree.

Cloning of ecotin orthologues

The DNA encoding the *Ps. aeruginosa* PAO1 strain ecotin gene was obtained in an M13 vector from the University of Washington Genome Center as part of the *Pseudomonas* Genome Project [24]. A PCR product containing the gene was generated using the primers 5′ TAG TGG GAT CCA TCG ATG CTT AGG AGG TCA TAT GAA AGC ACT ACT GAT CGC CGC C 3′ and 5′ TAG TGA AGC TTT TAT TCG CTG ACC GCT TTC TCG AC 3′. After digestion with *Bam*HI and *Hin*dIII, the insert was subcloned into the expression vector pTacTac [25]. The *Ps. aeruginosa* signal peptide was later replaced with that of *E. coli* by PCR (using the forward primer 5′ GCT ACG ACG TCC GCC TGG GCG GCC AAA CTG GAT GAA AAG G 3′) and subcloning into *Aat*II and *Hin*dIII sites of the ecotin expression vector. The free cysteine at position 108 was replaced with arginine (by overlap-extension PCR using the primers 5′ CGC TAC AAC AGC AAG CTG CCG ATC G 3′ and 5′ GGC AGC TTG CTG TTG TAG CGC AGC AGG AAG CCT TCG C 3′) to match the sequence found in the PA103 strain of *Ps. aeruginosa* and all other ecotin orthologues. The *Pa. citrea* gene was amplified from genomic DNA from the sequenced strain, using the primers 5′ GCT ACG ACG TCC GCC TGG GCG GCA ACG GAG ATA ACT GAT GCA CAG 3′ and 5′ ATA AGC TTA ACG AAC CTG TGA CGG GAC AAT CTG 3′. The product was digested with *Aat*II and *Hin*dIII, and inserted into the pTacTac vector after the *E. coli* ecotin signal peptide. The *Y. pestis* ecotin gene was generated by oligonucleotide reconstruction from the published sequence and inserted into pTacTac after the *E. coli* ecotin signal peptide. The inactive ecotin variant, $80A_6$ mEcotin, was constructed in pTacTac by subcloning the *Bam*HI/*Bsm*I fragment from the 81–86A ecotin variant [26] into the monomeric ecotin variant, mEcotin [20]. The sequences of the cloned ecotin orthologues were determined, confirming the absence of mutations arising from DNA manipulations.

Construction of ecotin knockout strains of *E. coli*

A *Sal*I/*Eco*RI restriction fragment from the pBR322 clone used in the original cloning [27], which contained the ecotin gene and

flanking DNA, was subcloned into pUC18. A double-stranded *Bam*HI linker oligonucleotide (CGGATCCG) was inserted into the unique *Bst*Z17I site in the middle of Tyr^{118} of ecotin, as well as the *Hpa*I site immediately downstream of the catIII chloramphenicol resistance gene of plasmid pUC18:IM3 [28]. The *Bam*HI fragment containing catIII and its promoter was inserted into the new *Bam*HI site in ecotin. The entire construct was excised with *Hin*dIII and moved into pBSII, where flanking *Apa*I and *Not*I sites were used to subclone into the pBIP3 suicide phagemid vector [29].

A pBIP3 lysate was prepared by transforming the pBIP3-ecotin vector into JM109 cells and infecting with f1R189 helper phage [29]. X90 cells were infected with the lysate and selected for kanamycin and chloramphenicol resistance. Individual colonies were grown up and plated on 5 % (w/v) sucrose plus 25 μg/ml chloramphenicol to select for cells that had lost the WT (wild-type) gene copy and retained the disrupted copy. Kanamycin sensitivity and PCR analysis confirmed that the strains contained only the insertionally inactivated ecotin gene. P1 bacteriophage transduction, followed by selection for chloramphenicol resistance, was used to move the ecotin disruption into JM101 to give the strain IMΔecoJ, which was used for protein expression. The knockout was moved from IMΔecoJ into the K12 sequence strain MG1655 by three sequential rounds of P1 transduction to generate the strain MG1655Δeco. MG1655 and MG1655Δeco were used in the cell-killing experiments, where they are referred to as WT and *eco*$^-$ *E. coli* respectively. Both strains were verified by immunoblotting (see Figure 2B). MG1655 *E. coli* and P1 bacteriophage were kindly provided by Dr Carol Gross (Department of Microbiology and Immunology, University of California at San Francisco). Mid-exponential cell pellets were boiled in loading buffer and subjected to SDS/PAGE on 10 % tricine gels. Protein was transferred to nitrocellulose and probed with rabbit anti-ecotin polyclonal antibodies.

Protein expression and purification

Expression vectors were transformed into the ecotin knockout strain IMΔecoJ. One-litre cultures were grown at 37 °C in Luria–Bertani broth to $D_{600} = 0.7$ and induced with 0.5 mM isopropyl β-D-thiogalactoside. After overnight culture, the bacteria were pelleted and resuspended in 20 ml of 25 % sucrose/10 mM Tris/HCl, pH 8.0. Chicken egg white lysozyme (2 ml of 15 mg/ml) in 40 mM EDTA were added to the suspension and incubated with rocking for about 1 h. After pelleting the cells, the supernatant was taken as the periplasmic fraction. At this point the four orthologue proteins were treated in separate ways. *E. coli* ecotin was dialysed into 1 mM HCl, centrifuged and the supernatant was brought to a final concentration of 20 mM Tris/HCl/5 mM $MgCl_2$, pH 7.5, by the addition of a $\times 25$ stock. The protein was passed over a DEAE-Sepharose column equilibrated with the same buffer, and the ecotin was collected from the flow-through fractions, pooled and concentrated. The protein was then purified by reverse-phase HPLC as described previously [26]. *Y. pestis* ecotin was precipitated from the periplasmic fraction with 65 % ammonium sulphate and dialysed into 10 mM Tris/HCl, pH 8.0. Protein was purified on a Mono Q HR 10/10 strong anion exchange column (Pharmacia) equilibrated in the same buffer, eluting in a gradient of NaCl at around 10 mM. The *Ps. aeruginosa* ecotin periplasmic fraction was loaded on to a DEAE-Sepharose column equilibrated with 10 mM Tris/HCl, pH 8.0, and eluted by the addition of 120 mM NaCl. *Ps. aeruginosa* ecotin was purified by strong anion exchange in the same manner as *Y. pestis* ecotin. *Pa. citrea* ecotin was purified on a DEAE column in the same way as *E. coli* ecotin. The protein was dialysed into 10 mM Mes, pH 6.0, and loaded on to an SP Sepharose Fast Flow strong cation-exchange column (Pharmacia) equilibrated with the same buffer and eluted with a gradient up to 100 mM NaCl. All ecotin orthologues were concentrated, further purified on a HiLoad 26/60 Superdex 75 preparative gel filtration column (Pharmacia) equilibrated with 20 mM Tris/HCl/150 mM NaCl, pH 8.0, and concentrated again. Protein concentrations were determined at A_{280} based on predicted molar absorption coefficients [30] and by titration with 4-methylumbelliferyl *p*-guanidinobenzoate-quantified trypsin. Protein masses, as measured by matrix-assisted laser-desorption ionization–time-of-flight MS, were consistent with masses predicted from amino acid sequences.

Gel filtration analysis of ecotin orthologues

Oligomerization of ecotin orthologues was analysed on a Pharmacia Superdex 200 10/30 column run at 0.5 ml/min with 20 mM Tris/HCl/150 mM NaCl, pH 8.0. Samples (100 μl) of 10 μM inhibitor, which was either uncomplexed or pre-incubated with 20 μM rat trypsin with a $Ser^{195} \rightarrow$ Ala substitution, were loaded on to the column, and peaks were assigned at A_{280}. Inactive rat trypsin, with a $Ser^{195} \rightarrow$ Ala substitution, was prepared as described previously [20]. Apparent molecular masses were determined from a standard curve of ferritin (440 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), myoglobin (17.6 kDa) and cytochrome *c* (12.3 kDa).

Measurement of protease inhibition

Equilibrium inhibitory constants (K_i values) were determined by incubating varying inhibitor concentrations with a given protease concentration, which ranged from 0.1 to 3 nM, depending upon activity. The length of incubation was determined based on the concentrations used so as to ensure that protease and inhibitor reached equilibrium. All assays were performed in 200 μl of 50 mM Tris/HCl/100 mM NaCl/20 mM $CaCl_2$/0.05 % (v/v) Tween 20, pH 8.0, in a Molecular Devices microplate reader. K_i values greater than 500 nM were fitted to the Michaelis–Menten equation after separate determination of the K_m for the substrate:

$$v = V_{max}[S]/\{[S] + K_m(1 + [I]/K_i)\}$$

For K_i values less than 500 nM, the equation for tight-binding inhibitors was used to fit the apparent equilibrium inhibition constant, K_i^*:

$$v_i/v_o = 1 - \{E_o + I_o + K_i^* - [(E_o + I_o + K_i^*)^2 - 4E_oI_o]^{0.5}\}/(2E_o)$$

For those assays in which inhibitor, enzyme and substrate were all in equilibrium during the course of the assay ($K_i > 2$ nM), the K_i value was calculated from the K_i^* by the equation

$$K_i = (K_i^*[S])/([S] + K_m)$$

Otherwise, the two values were considered equal. Although the K_i values were corrected for substrate-induced dissociation, the inhibitory constant must still be considered an apparent value, since the monomer/dimer equilibrium leads to a more complex situation than a simple binary interaction [20]. Uncertainty values represent the sample S.D. of at least two independent assays.

Enzymes and substrates for kinetic assays were as follows: human uPA (urokinase-type plasminogen activator; American Diagnostica) with Spectrozyme UK (American Diagnostica); human α-thrombin (Haematologic Technologies Inc.) with Spectrozyme TH (American Diagnostica); human Factor Xa (Haematologic Technologies Inc.) with S-2765 (DiaPharma); bovine chymotrypsin (Sigma) with suc-AAPF-pNA (Bachem);

bovine trypsin (Sigma) with Z-GPR-pNA (Sigma); human CatG (Calbiochem) with suc-AAPF-SBzl (Bachem); human NE (Calbiochem) with suc-AAPV-pNA (Bachem) (where suc is succinyl, pNA is *p*-nitroanaline, Z is benzyloxycarbonyl and Bzl is benzyl).

Bacterial cell-killing assays

Because the balance between protease-mediated killing and cell growth was so important, we found that the specifics of the cell preparation and incubation conditions played a large role in the outcome of experiments. MG1655 (WT) and MG1655Δeco (*eco*$^-$) *E. coli* were plated on tryptic soya agar (TSA) from − 80 °C stocks, grown overnight at 37 °C and placed at 4 °C for not more than 5 days. Single colonies were picked into tryptic soya broth (TSB) and grown overnight at 37 °C with shaking. Samples were diluted 1:1000 into 10 ml of 10 mM sodium phosphate plus 5 % (v/v) TSB, pH 7.4, and grown in a 50 ml Corning tube at 37 °C until a D_{540} of 0.3 was reached (approx. 220 min). A 1 ml sample of each strain was spun at 1500 ***g*** for 2 min, washed once and resuspended in 1 ml of 10 mM sodium phosphate plus 1 % TSB, pH 7.4. The absorbance of a 2-fold dilution was taken and the samples were diluted in the same buffer to a D_{540} of 0.01 [approx. 4000 CFU (colony-forming unit)/μl]. Either an equal mix of both strains or individual samples were then added to NE (final concentration 3.4 μM) or its storage buffer (50 mM sodium acetate/150 mM NaCl, pH 5.0). Uncertainty values were calculated as the S.E.M. of the $\log_{10}$ of CFUs for quintuplicate assays. Statistical significance was determined by the unpaired Student's *t* test, with $P < 0.001$ indicated on the graphs by an asterisk. Single time-point experiments were performed with 50 μl volumes in 0.6 ml tubes in a 37 °C shaker. After 6 h of incubation, samples were serially diluted into TSB and plated on to TSA. In experiments using equal mixtures of both strains, samples were plated on both TSA and TSA plus 20 μg/ml chloramphenicol to select for the knockout strain. Time-course experiments were performed in larger volumes (250–520 μl) in 1.5 ml tubes in an end-over-end rotator at 37 °C. For experiments involving bacteriostatic tetracycline, 5 μg/ml of the antibiotic was added to samples at time zero. For experiments monitoring outer membrane permeability, samples were also plated on to TSA plus 5 μg/ml rifampicin, with or without 20 μg/ml chloramphenicol. NE was obtained from Elastin Products Company (Owensville, MO, U.S.A.).

RESULTS

Distribution and sequences of ecotin homologues

The amino acid sequence of *E. coli* ecotin was used as the query sequence to perform a TBLASTN search on the NCBI BLAST server of microbial genomes, as well as the non-redundant database, genome survey sequence and unfinished high-throughput genomic sequences. Individual genomes that were not part of these databases were searched at the Sanger Centre, TIGR, UW Genome Project and DOE JGI, as well as the complete genome sequence of the plant pathogen *Pa. citrea* performed at Genencor.

While ecotin did not appear to have significant homology to any other *E. coli* proteins, orthologous proteins were found in several proteobacteria (Figure 1A). Within the gamma subdivision, ecotin orthologues were found in the genuses *Pseudomonas* and *Shewanella*, in addition to many members of the Enterobacteriaceae family: *Escherichia*, *Shigella*, *Salmonella*, *Yersinia*, *Serratia*, *Klebsiella* and *Pantoea*. The beta subdivision genus *Burkholderia* contained an orthologue, but many other completely sequenced proteobacteria did not, including *Vibrio*, *Haemophilus*, *Pasteurella*, *Helicobacter*, *Campylobacter*, *Neisseria* and *Mesorhizobium*.

In general, those species containing ecotin encountered mammalian hosts, but were not obligate intracellular parasites. In the alpha subdivision, the genus *Rickettsia* appeared to be in the process of removing the gene [31], since *Rickettsia conorii* and *R. sibirica*, but not *R. prowazekii*, contained an ecotin pseudogene. Within the *Enterobacteriaceae* family, neither the plant pathogen *Erwinia*, nor the insect endosymbionts, *Buchnera aphidicola* and *Wigglesworthia glossinidia*, have the ecotin gene. Within the genus *Pseudomonas*, ecotin is present in the species *Ps. aeruginosa*, *Ps. putida*, and *Ps. fluorescens*, but not *Ps. syringae* pv. tomato str. DC3000, which infects tomato and *Arabidopsis*. The genus *Burkholderia*, similar to *Pseudomonas*, represents ubiquitous environmental bacteria that can, in the case of *Bu. cepacia*, *Bu. mallei*, and *Bu. pseudomallei*, become opportunistic mammalian pathogens. Ecotin is present in those species, but not in *B. fungorum*, which belongs to a clade not implicated as pathogenic [32]. A seeming exception to this rule is *Pa. citrea*, the phytopathogen responsible for pink disease in pineapple [33]. The ecotin protein sequences for *Pa. citrea* and *Shewanella oneidensis* are the most different from *E. coli*, even though both species are closer in evolutionary distance than *Pseudomonas* or *Burkholderia*, as measured by the 16 S rRNA sequence. This could signify a divergence in the protease target.

Outside of the proteobacteria, homology to ecotin was found only in the marine unicellular cyanobacterium *Prochlorococcus marinus* and the protozoal parasites, *Trypanasoma brucei* and *Leishmania major*. Within *Pr. marinus*, ecotin is present only in strain MIT9313 and not in the smaller genomes of strains MED4 and SS120, or in the closely related *Synechococcus* sp. strain WWH8102. Comparison of these genomes shows a dynamic process of genetic change involving gene loss, rearrangement and acquisition [34–36]. In MIT9313, the ecotin orthologue flanks one such region in flux that includes a group of nitrogen usage genes, some of which appear to have been gained through lateral gene transfer from proteobacteria [34]. This strain may have only recently acquired ecotin, which may not be expressed or play any functional role in the cyanobacterium. Outside of bacteria, the homologous genes in *T. brucei* and *L. major* lack many of the conserved protease contact residues, as well as the disulphide bond between positions 50 and 87 that stabilizes the substrate-like loop. The sequence conservation is mainly in the adjacent β-sheet residues making up the protein core. Recombinantly expressed and purified *T. brucei* homologue protein had no detectable inhibitory activity (results not shown).

While the presence of ecotin in enterobacteria is consistent with a putative protective function against digestive proteases in the gut, such an explanation seems inadequate for such bacteria as *Pseudomonas*, *Burkholderia* or *Shewanella*. However, a wider number of these species would be expected to encounter the mammalian immune system, suggesting the possibility of a target protease involved in immunity. Consistent with this notion are the reports that NE plays a direct role in the killing of *E. coli*, *K. pneumoniae*, *Shigella flexneri* and *Ps. aeruginosa*, all species with ecotin orthologues [5–7,37].

Alignment of protein sequences

Complete DNA sequences were assembled for each of the orthologues, the signal sequence cleavage sites were predicted using SignalP V1.1 [23], and the native protein sequences were aligned using CLUSTALW (Figure 1B). The *Pa. citrea* ecotin sequence is published here for the first time. The published sequence of the PAO1 strain of *Ps. aeruginosa* contained an unusual

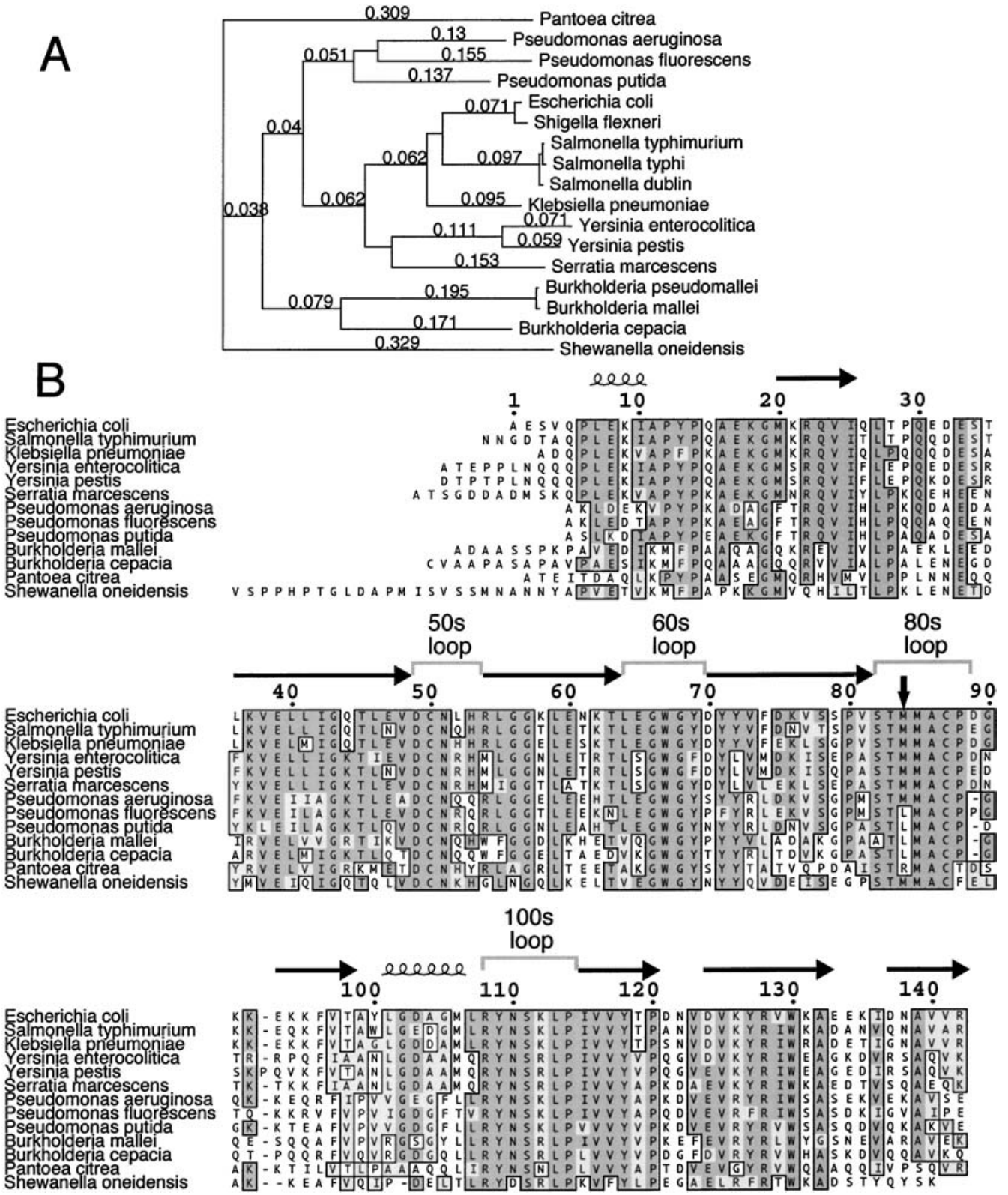

Figure 1 Dendrogram and sequence alignment of proteobacterial ecotin orthologues

(**A**) Species known to contain an ecotin orthologue are shown according to the CLUSTALW alignment guide tree. Numbers approximate fractional differences in protein sequence. (**B**) Ecotin orthologue native protein sequences were aligned, using CLUSTALW. *Sh. flexneri* was omitted due to similarity to *E. coli* (K18T, N61S and V140E), and *Sa. typhi* (V140I) and *Sa. dublin* (G1S) were omitted due to similarity with *Sa. typhimurium*. Numbering is according to *E. coli* ecotin. Secondary structure is shown above the sequence and was taken from the PDB file of uncomplexed ecotin, 1ECY [70]; arrows represent β-sheets and loops represent helices. The four main protease contact loops are labelled, and the P1 position (the primary specificity determinant) is designated by a downward arrow.

free cysteine at position 108, which was arginine in all other species. Cloning the gene directly from PAO1 confirmed the published sequencing results, but sequencing of the cytotoxic PA103 strain of *Ps. aeruginosa* revealed the conserved arginine at that position; this latter sequence is used in the alignment.

Of the 142 residues of ecotin, 28 of them are completely conserved among all species. Many of these are cysteine, glycine and proline residues that play important roles in defining the secondary structure, whereas others have side chains involved in core packing. However, about half of the conserved residues are in the four surface loops that contact target proteases. As expected, the residues showing the most variation were generally solvent exposed in edge strands of the β-sheet or in loops not contacting the protease. Insertions or deletions were present at the N-terminus and in the residues immediately following the substrate-like loop, two regions that were disordered in the ecotin–trypsin structure [19]. While the substrate-like 80s loop was well conserved from the P3 to the P4′ positions (residues 82–88), suggesting a similar protease target, the important P1 residue (residue 84) showed some variation. The P1 methionine of *E. coli* ecotin has been hypothesized to play a role in the pan-specificity of the inhibitor, since it is able to fit into many different primary specificity pockets [27]. Therefore, the P1 leucine of orthologues from some *Pseudomonas* and *Burkholderia* species could indicate a different or more specific target. Likewise, the arginine at the P1 of *Pa. citrea* ecotin could indicate a trypsin-like target.

Searches for functionally linked proteins

Several lines of evidence suggest that ecotin orthologues do not share an endogenous target. First of all, ecotin has only been shown to inhibit trypsin-fold serine proteases in the S1A subfamily of clan PA, of which *E. coli* has no members [38,39]. While *E. coli* does have three periplasmic proteases in the S1C family (DegP, DegQ and DegS), ecotin is known not to inhibit DegP [18], which forms a hexameric complex presumably inaccessible to ecotin [40]. DegQ also is thought to form a large oligomeric complex [41]. Ecotin has been shown not to inhibit the following *E. coli* proteases: Do, Re, Mi, Fa, So, La, Ci, Pi, and proteases I, II, IV, V and VI [18,42]. Furthermore, no interacting *E. coli* proteins were detected when a periplasmic fraction was passed over an ecotin–agarose affinity column (results not shown).

Finally, we utilized a comparative genomics approach to search for proteins with orthologues in the same set of fully sequenced genomes as ecotin, since proteins involved in the same function would be expected to be maintained or eliminated from a genome in a correlated fashion [43]. Because ecotin is not in the COG database, we utilized both the HOBACGEN database of protein families (release #10, Feb, 2002) [44] and the Comprehensive Microbial Resource (www.tigr.org) to conduct searches based on taxonomic relationships. Genes were selected that contained homologues in *E. coli*, *Sa. typhimurium*, *Y. pestis* and *Ps. aeruginosa*, but not in closely related proteobacteria lacking an ecotin orthologue. The amino acid sequences of genes from this subset of candidates were used for individual TBLASTN searches of the microbial database. No genes were found with the same phylogenetic profile as ecotin, suggesting that ecotin orthologues are not functionally linked to a common endogenous protein and strengthening the case for an exogenous protease target.

Cloning, expression and purification of ecotin orthologues

To determine whether mammalian proteases, such as NE, may be targets of ecotin in multiple bacterial species, we cloned the ecotin genes from *Y. pestis*, *Ps. aeruginosa* and *Pa. citrea*. Unlike

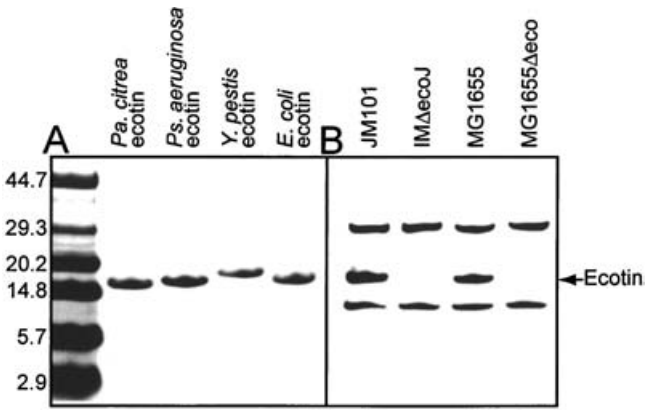

Figure 2 Gel electrophoresis of purified proteins and bacterial strains

(**A**) Coomassie Blue-stained SDS/PAGE gel of purified ecotin orthologues. The apparent molecular masses of protein standards are given in kDa. (**B**) Immunoblot of the utilized strains of *E. coli*, grown to mid-exponential phase and probed with anti-ecotin polyclonal antibodies. The ecotin band is in the middle of two cross-reacting bands.

the enterobacteria *Y. pestis* and *E. coli*, *Ps. aeruginosa* would not be expected to reside in the intestine, but would encounter the mammalian immune system. *Pa. citrea*, although it belongs to the family Enterobacteriaceae, is a plant pathogen and so is expected to inhabit a very different environmental niche. *Ps. aeruginosa* ecotin was cloned from a DNA vector used in the original genomic sequencing of strain PAO1 by the *Pseudomonas* Genome Project [24]. Residue 108 was mutated from cysteine to the arginine found in the PA103 strain, since this was conserved in all other species and was found to be more stable in an oxidative environment. The *Y. pestis* clone was obtained by gene synthesis from oligonucleotides, based on the published sequence. *Pa. citrea* ecotin was cloned from genomic DNA. All orthologues were placed into the standard ecotin expression vector, fusing the native protein sequence to the *E. coli* ecotin signal peptide. The ecotin knockout strain IMΔecoJ (Figure 2B) was used to express the four orthologues, which were purified to homogeneity by treating the periplasmic fraction with different combinations of precipitation, ion exchange chromatography and gel filtration (Figure 2A).

Analysis of inhibition and oligomerization

Purified ecotin from the four species was assayed for inhibitory activity against a panel of serine proteases from neutrophils, the intestine and blood: NE, CatG, trypsin, chymotrypsin, Factor Xa, thrombin, and uPA (Table 1). Ecotin variants from *E. coli*, *Y. pestis* and *Ps. aeruginosa* potently inhibited the neutrophil and pancreatic proteases, with more variable inhibition of the blood proteases. With its arginine primary specificity determinant (P1 residue), *Pa. citrea* ecotin was expected to have a more trypsin-like specificity. Indeed, it inhibited chymotrypsin less well and was about 1000-fold less potent against NE than the other variants, but it was the best inhibitor of the trypsin-like uPA. However, *Pa. citrea* ecotin was the worst inhibitor of thrombin, which also has arginine specificity, highlighting the importance of the many other contact residues in determining the strength of inhibition. The substantially lower sequence conservation in *Pa. citrea* ecotin, compared with its evolutionary distance, does appear to correspond to an altered inhibitory specificity. The kinetic data support the notion that *Pa. citrea* ecotin is not under selective pressure to inhibit NE. While up to 1800-fold differences in K_i values were observed against blood proteases, each of the three orthologues encountering mammalian hosts was at least a

Table 1 Protease inhibition by ecotin orthologues

The K_i inhibitory constant was measured for ecotin orthologues from four species against a panel of serine proteases.

		K_i (nM) of ecotin			
Protease	Organism . . .	*E. coli*	*Y. pestis*	*Ps. aeruginosa*	*Pa. citrea*
NE		0.012 ± 0.004	0.016 ± 0.003	< 0.005	8.8 ± 0.4
CatG		< 0.005	< 0.005	< 0.005	< 0.005
Trypsin		< 0.001	< 0.001	< 0.001	< 0.001
Chymotrypsin		< 0.002	< 0.002	< 0.002	0.005 ± 0.002
Factor Xa		0.0046 ± 0.001	0.23 ± 0.01	0.007 ± 0.001	0.22 ± 0.005
Thrombin		770 ± 80	1400 ± 100	1500 ± 100	$24\,000 \pm 3000$
uPA		670 ± 20	$13\,900 \pm 1500$	7.8 ± 0.4	1.1 ± 0.2

Table 2 Size-exclusion chromatography of ecotin orthologues

The four ecotin orthologues were run on an analytical gel filtration column either alone or complexed with an excess of trypsin. Predicted molecular mass was deduced from the amino acid sequence. Apparent molecular mass was calculated from comparison of elution volumes with known protein standards.

Ecotin orthologue	Predicted mass (kDa)	Uncomplexed elution volume (ml)	Apparent mass (kDa)	Complexed elution volume (ml)	Apparent mass of complex (kDa)
E. coli	16.098	14.89	37.4	13.28	85.5
Y. pestis	16.727	14.78	39.6	13.26	86.4
Ps. aeruginosa	15.507	15.06	34.3	13.36	82.1
Pa. citrea	15.800	15.06	34.3	13.37	81.7

low picomolar inhibitor of neutrophil and pancreatic proteases, indicating their potential as physiological targets.

Analytical gel filtration analysis showed that all four orthologues form the same oligomeric complexes (Table 2). On their own, ecotin variants ran as homodimers, with apparent molecular masses slightly higher than double the predicted monomeric mass due to the elongated shape of ecotin. When ecotin was preincubated with an excess of inactive rat trypsin, the complex ran with an apparent molecular mass equal to that of a heterotetramer.

Ecotin protects *E. coli* from NE

Given the species distribution and inhibitory activities of ecotin orthologues, NE appeared to be a promising candidate for a physiological target of ecotin. To test whether ecotin would protect bacteria from NE, we disrupted, by homologous recombination, the ecotin gene in *E. coli* with a marker for chloramphenicol resistance. The disruption was moved into the K12 sequence strain MG1655 by three rounds of P1 bacteriophage transduction, and this *eco*$^-$ strain was verified by PCR and by immunoblotting (Figure 2B).

NE-mediated killing experiments were performed essentially as described previously [5], with some modification (see the Experimental section). At concentrations of 3.4 μM NE (100 μg/ml), substantial killing of *E. coli* was observed compared with the buffer control, and the *eco*$^-$ strain was significantly more sensitive to NE. After 6 h of incubation with NE, 2.76 ± 0.04 orders of magnitude (570-fold) fewer viable ecotin-deficient *E. coli* cells remained than WT cells (Figure 3A).

Transforming into *eco*$^-$ *E. coli* a plasmid overexpressing either WT ecotin or an inactive ecotin variant showed that this difference in sensitivity was due to inhibition by ecotin, rather than any extraneous strain differences, such as expression of the antibiotic marker. The K_i for NE of this inactive variant, which has a disrupted primary binding site and dimerization interface, was greater than 10 μM, or 10^6-fold worse than WT ecotin. Expression of WT ecotin made the cells more resistant to NE than expression of inactive ecotin, yielding 2.1 ± 0.4 orders of magnitude (140-fold) higher cell counts (Figure 3B). Since the ecotin-deficient strain contained an antibiotic-resistance marker, a direct competition could be performed between the two strains in one test tube, quantifying viable bacteria by plating both with and without chloramphenicol. This procedure assured that each strain was exposed to the exact same environment. Again, the *eco*$^-$ strain yielded 2.6 ± 0.2 orders of magnitude (390-fold) fewer colonies than WT *E. coli* after 6 h incubation with NE (Figure 3C).

The observation that the presence of ecotin has an effect during direct competition of the two strains requires that ecotin plays its protective role while localized to the bacterium producing it, rather than after release into solution, where it would inhibit NE activity against both strains. Since ecotin is found in the periplasm, this strongly suggests that NE is crossing the outer membrane and that a bactericidal or bacteriostatic effect is due to periplasmic NE activity. If this were the case, one would expect an ecotin-independent outer membrane permeability to precede killing. Outer membrane permeability is generally accompanied by sensitivity to detergents, such as SDS, and to certain hydrophobic antibiotics, such as rifampicin and actinomycin D [45]. In order to observe how the presence of ecotin affected the rate of both outer membrane damage and cell killing, we followed a time course of NE treatment of an equal mixture of *eco*$^-$ and WT *E. coli*, monitoring cell viability on agar plates with and without rifampicin as a gauge of outer membrane permeability.

It was immediately apparent that the majority of the effect of ecotin was due to different lengths of time taken to recover and start growing again following NE treatment (Figure 4A). After 30 min, net killing of WT *E. coli* essentially stopped, whereas the ecotin-deficient strain continued to be killed for the first few hours. At this point, both strains began growing at the same rate, even though virtually all of the cells had damaged, permeable outer membranes, as judged by sensitivity to rifampicin. The outer membrane was permeabilized at a faster rate than killing, and sensitivity to 3% SDS showed the same effect (results not shown). Although the greater amount of new growth by WT *E. coli* led to somewhat more rifampicin-resistant bacteria, the fraction of cells resistant to rifampicin decreased at identical rates for

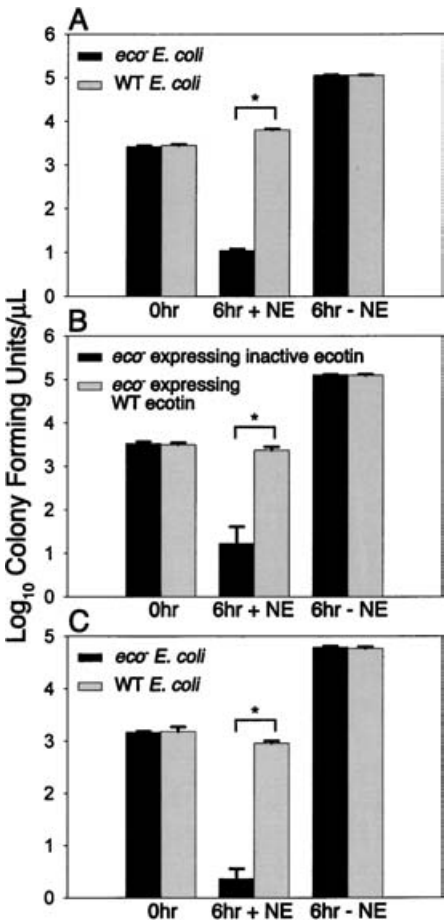

Figure 3 Ecotin protects *E. coli* against NE

WT or *eco*$^-$ *E. coli* were treated either with 3.4 μM NE or with buffer control and incubated for 6 h at 37 °C. Viable cells were quantified as CFUs on TSA. Error bars designate the S.E.M. for quintuplicate assays. An asterisk signifies a statistical significance of $P < 0.001$. (**A**) Each strain was treated in a separate tube. (**B**) *eco*$^-$ *E. coli* were transformed with a plasmid expressing either WT ecotin or an inactive variant and treated as before. (**C**) WT and *eco*$^-$ *E. coli* were mixed in the same tube and treated as before. CFUs for each strain were determined by plating with and without chloramphenicol, since the *eco*$^-$ strain carries an antibiotic resistance marker.

both strains (Figure 4B). These results suggested that ecotin was having its effect not by protecting against the initial damage by NE, but by allowing quicker growth and recovery following NE assault.

To test whether ecotin was inhibiting a bacteriostatic effect of NE, equal mixtures of the two strains were incubated with or without NE in the presence or absence of a bacteriostatic amount of tetracycline to stop new growth (Figure 4C). Outer membrane damage does not sensitize bacteria to tetracycline, which is thought to freely diffuse through pores [45]. Tetracycline (5 μg/ml) was found to stop the growth of control cells without causing cell death. When tetracycline was used to inhibit growth and protein synthesis, there was no difference in bacterial killing between the strains during the first 3 h, when the number of *eco*$^-$ bacteria would otherwise drop far below that of WT *E. coli*. This indicated that the presence of ecotin in the periplasm was facilitating growth and/or repair of WT *E. coli*, rather than affecting killing *per se*. After 3 h, the subset of cells that were initially resistant to NE-mediated killing started to die off in a process that was dependent on ecotin, possibly indicating a late killing effect of periplasmic NE.

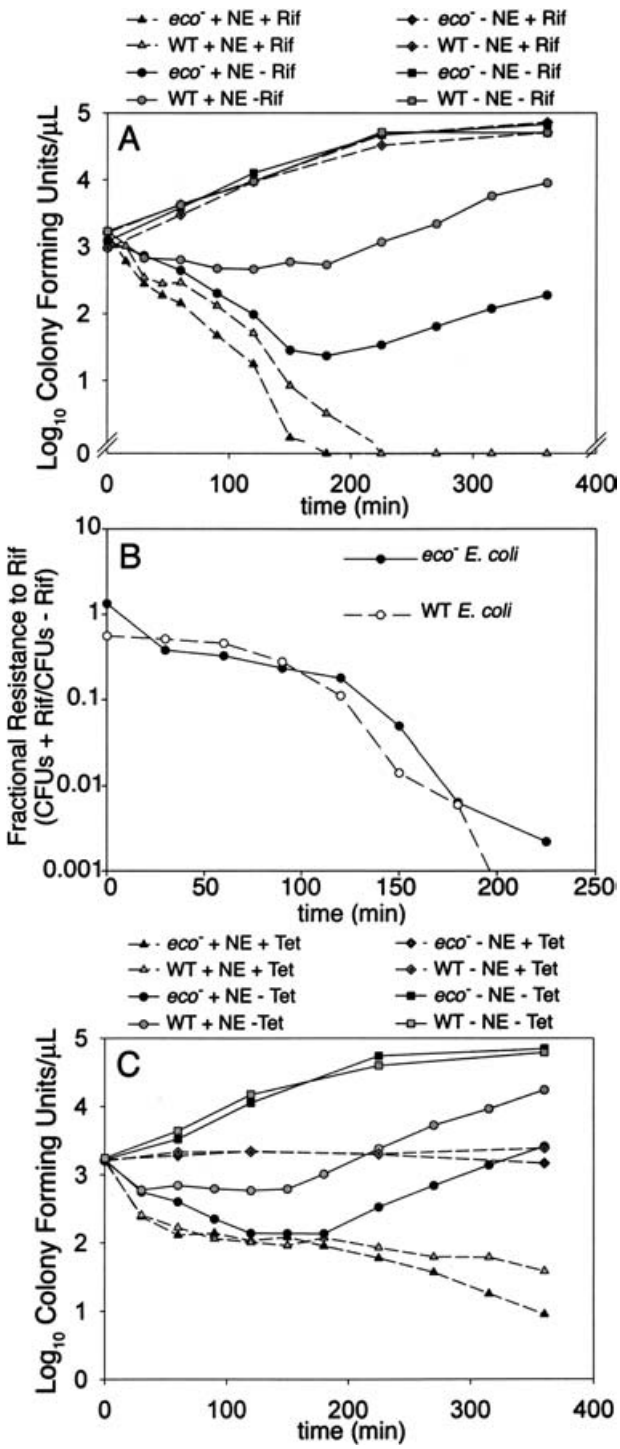

Figure 4 Time courses of NE-mediated killing

(**A**) NE treatment leads to prolonged outer membrane permeability, which precedes cell killing. An equal mixture of WT and *eco*$^-$ *E. coli* was treated with either 3.4 μM NE or buffer control. At time points, dilutions were plated on to TSA with different combinations of antibiotics. Chloramphenicol was used to differentiate between the two strains. Outer membrane permeability to hydrophobic antibiotics was assessed by sensitivity to 5 μg/ml rifampicin (Rif). Numbers given are from duplicate platings of a representative time-course very similar to several other trials. (**B**) Outer membrane damage is independent of ecotin. The ratio of CFUs plated with and without rifampicin for both strains after treatment with NE is plotted from the above data. (**C**) Ecotin inhibits a bacteriostatic effect of NE. A bacteriostatic amount of tetracycline (5 μg/ml; Tet) was used in some assays to prevent new cell growth. An equal mixture of WT and *eco*$^-$ *E. coli* was treated with buffer control, NE, tetracycline, or NE + tetracycline. Time point samples were diluted and plated on to TSA and TSA + chloramphenicol to differentiate the strains.

DISCUSSION

As part of the immune response to bacterial infection, neutrophils direct a large arsenal of antimicrobial agents against invading bacteria. Many of these agents demonstrate specificity in their antimicrobial profiles, showing bactericidal or bacteriostatic effects mainly against certain subsets of bacteria or fungi. The important role of proteases in neutrophil-mediated killing has been demonstrated by recent genetic studies in mice. The serine protease NE has been shown to be important for neutrophil killing of such Gram-negative bacteria as *Escherichia*, *Shigella* and *Klebsiella*. While the exact mechanism of protease-mediated killing remains unknown, NE cleavage of OmpA in *E. coli* appears to be important [6]. OmpA is thought to be important in maintaining outer membrane stability, and OmpA-deficient *E. coli* are more sensitive to environmental stresses [46].

Given the killing specificity of NE, it is interesting to note that those bacteria that have shown sensitivity to NE belong to the small subset of bacteria containing the periplasmic protease inhibitor ecotin. This raises the question of what the physiological target of ecotin is and whether it plays a role in protecting bacteria against NE. Do only bacteria susceptible to NE require ecotin, and what would make certain species naturally resistant to NE? Ecotin orthologues appear to be present mainly in straight-rod Gram-negative bacteria, as opposed to curved, helical, or coccobacillary rods. Certain cell morphologies may be more susceptible to NE cleavage of OmpA and the concomitant breakdown of the cell envelope. It is known that OmpA is important for cell morphology [47], and it is possible that sequence differences in the extracellular loops of OmpA could lead to differential susceptibility to NE.

Bacteria may also be less sensitive to the immune system or exogenous proteases during certain growth phases. For example, in stressful environments, bacteria tend to form biofilms, which are more protected against host defences, such as neutrophils [48–50]. In regulating the formation of biofilms, bacteria appear to monitor the concentration of acetyl phosphate as an indicator of the nutritional status of the cell [51]. Interestingly, ecotin and the periplasmic inhibitor of vertebrate lysozyme, *ivy*, were two of 25 genes significantly upregulated by higher concentrations of acetyl phosphate, which tends to promote the free-living planktonic state that is more susceptible to neutrophils [51].

In order to gain insight into what exogenous targets ecotin and its orthologues may inhibit, we cloned, expressed and purified ecotin from three additional species: *Y. pestis*, *Ps. aeruginosa* and *Pa. citrea*. All four ecotin variants were found to form homodimers and to inhibit trypsin in a heterotetrameric complex (Table 2). By allowing the inhibitor to make contacts with the protease at two distinct protein interfaces, dimerization has the effect of making inhibition more potent and less specific [20]. For that reason, we expected the orthologues to share relatively broad inhibitory profiles. However, *Pa. citrea*, as a plant pathogen, was expected to inhabit a different environment from the other three and so potentially encounter different proteases. Its gene sequence shows a fair amount of divergence in the protease contact loops, especially in having arginine as the primary specificity determinant (P1), rather than the methionine observed in the other three. While all variants were tight inhibitors of the pancreatic proteases trypsin and chymotrypsin and the neutrophil protease CatG, *Pa. citrea* ecotin was about 1000-fold less potent against NE, which the others inhibited tightly (Table 1).

Interestingly, a P1 methionine is also present in the natural physiological inhibitor of NE, α1-proteinase inhibitor (α1-antitrypsin). Imbalance between NE and its inhibitor is seen not only in α1-proteinase inhibitor deficiency, but also in cystic fibrosis, which is typified by chronic respiratory infection in the presence of a vast influx of neutrophils [52]. Degranulating neutrophils lead to very high concentrations of elastase, averaging 100 μg/ml in cystic fibrosis sputum, the same concentration used in our cell-killing assays [53,54]. The vast majority of the NE is free and active, since the physiological inhibitors tend to be degraded or otherwise inactivated [54,55]. The most important pathogens causing chronic infections in adult cystic fibrosis patients are *Ps. aeruginosa* and *Bu. cepacia*, two ecotin-containing species [56,57]. More broadly, such species as *K. pneumoniae*, *E. coli*, *Se. marcescens* and *Ps. aeruginosa* are often responsible for nosocomial respiratory infections, especially in neonatal and elderly populations [58–61]. Many of these pulmonary infections are marked not only by increased numbers of neutrophils, but also by high levels of free uninhibited elastase [62]. Even when excess α1-proteinase inhibitor is present in the lung, release of granules from neutrophils can lead to bursts of very high concentrations of free NE immediately surrounding the neutrophil [63,64]. Therefore, the respiratory system could represent a location where ecotin-containing bacteria encounter not only neutrophils, but also high concentrations of free NE.

Some of the ecotin-containing bacteria are known to be susceptible to NE-mediated killing, and ecotin inhibits NE, but does ecotin localized to the periplasm actually protect the bacterium against external NE? To answer this question, we generated an ecotin-deficient strain of *E. coli* and treated it and an isogenic WT strain with purified NE. After 6 h of incubation with NE, cells producing functional ecotin were present at 140- to 570-fold higher numbers than those without ecotin (Figure 3). This difference was due to the inhibitory activity of ecotin (Figure 3B) and involved protein that was localized, rather than released into bulk solution (Figure 3C). The concentrations of any released ecotin would be so low in our assays that it could not inhibit the activity of the vast excess of NE. This situation probably holds *in vivo*, as well. The number of NE molecules in a single neutrophil is about 3×10^7 [65,66], which is 10000-fold higher than the 3000 ecotin molecules per *E. coli* estimated from immunoblotting (results not shown). Therefore, it seems likely that ecotin in the periplasm is situated to inhibit the small fraction of NE molecules that manage to permeate the outer membrane.

Time courses of bacterial killing demonstrated that the effect of ecotin was in promoting growth after NE assault. The presence of ecotin did not affect the rate of outer membrane damage by NE, as assayed by sensitivity to the hydrophobic antibiotic rifampicin (Figures 4A and 4B). When a bacteriostatic amount of tetracycline was added in order to prevent new growth, the strain difference in sensitivity disappeared, although late ecotin-dependent death did occur (Figure 4C). Taken together, these results suggest the following model for NE-mediated killing of *E. coli* (Figure 5). NE initially cleaves OmpA, leading to outer membrane damage and increased permeability. Such damage can lead directly to loss of cell viability, although in any population of bacteria, not all cells appear to be equally sensitive. Killing by NE is balanced by growth and repair of cells. However, permeability of the outer membrane allows NE to translocate into the periplasm, where it can inhibit these processes, leading to a bacteriostatic effect. Ecotin inhibits this periplasmic activity, allowing quicker recovery following NE treatment.

While the periplasmic targets of NE remain unknown, they could include proteins involved in envelope stress responses. Several pathways exist in *E. coli*, including Sigma(E) and Cpx, for transmitting periplasmic stress signals to promote cell wall biosynthesis and protein folding [67]. The OmpR pathway of osmotic regulation of outer membrane proteins has been shown to be essential for allowing *E. coli* to withstand low doses of BPI and for preventing the progression to lethal damage of the inner

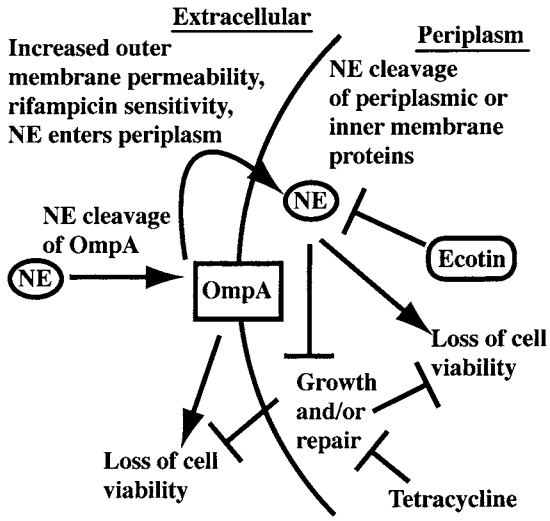

Figure 5 Model describing the role of ecotin in protection against NE

Exogenous NE cleaves OmpA), leading to increased outer membrane permeability and loss of cell viability. NE-mediated killing is reduced by cell growth and repair. NE is able to translocate across the permeabilized outer membrane into the periplasm, where it inhibits this growth and repair and eventually leads to cell death. Ecotin inhibits periplasmic NE activity, allowing quicker recovery after protease treatment.

membrane [68]. BPI is known to attack *E. coli* in a two-stage mechanism, involving first damage to the outer membrane, followed by inner membrane damage, which may be caused by a subpopulation of BPI molecules that penetrates the outer membrane [69]. Likewise, NE appears to have both initial effects from cleavage of Omps and later effects from NE activity within the periplasm. These experiments were done with purified protein, but it is possible that NE may work synergistically with other antimicrobial factors within the neutrophil phagosome. For instance, membrane permeabilizing proteins, such as BPI or the defensins, could help NE enter the periplasm.

While ecotin has been shown to be important in protecting *E. coli* against NE, it seems unlikely that NE is the only target. The potent non-specific inhibition generated by forming tetrameric complexes with target proteases appears well adapted to inhibiting proteases with a wide range of specificities. The fact that ecotin is found widely in enterobacteria suggests that protection against pancreatic digestive proteases could be another important role. The ecotin sequence similarity in *Shewanella* and *Pantoea* is significantly less than would be expected simply from their evolutionary distance from *E. coli*, as measured by 16 S rRNA sequence. This could be indicative of a change in inhibitory target in these species. *Pa. citrea* is mainly known as a pathogen of pineapple, but the family of serine proteases that ecotin inhibits is little known outside animals. Therefore, it is possible that the presence of ecotin may indicate that *Pa. citrea* encounters digestive proteases after ingestion of contaminated fruit by animals. Although *Pa. citrea* is a much weaker inhibitor of NE, it is still a very potent inhibitor of the pancreatic digestive proteases. Neither *Pseudomonas* nor *Burkholderia* tend to inhabit the intestine, but they do encounter the immune system and the often high-NE environment of the respiratory system. *Ps. aeruginosa* ecotin was found to be the tightest NE inhibitor of the four orthologues characterized, with a K_i below 5 pM. In general, ecotin is found in straight-rod Gram-negative bacteria that encounter mammalian hosts at some point, but different species may use it to inhibit different proteases, depending on their specific environment.

Ecotin may represent a way in which the bacterium defends itself against one of the components of the neutrophil antimicrobial arsenal directed against it. Studying the role of ecotin in the periplasm can help clarify the mechanism by which NE attacks bacteria. Our cell-killing assays suggest that NE cleaves proteins in the periplasm that may be responsible for growth and repair following NE assault. Future genetic studies could investigate periplasmic proteins important in withstanding NE treatment and test whether ecotin is important in protecting these proteins. The presence of ecotin in a genome may be a marker for which bacteria are susceptible to a certain type of cell damage. Gaining a greater understanding of how the immune system deals with bacterial infections and how bacteria defend themselves against it may lead to the discovery of bacterial targets that would help in the development of novel antibiotics.

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