A High Capacity Microbial Screen for Inhibitors of Human Rhinovirus Protease 3C

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We have developed a high capacity screen for compounds that inhibit the 3C protease of human rhinovirus-1b. The assay uses a recombinant strain of *Escherichia coli* expressing both the protease and a tetracycline resistance-conferring protein modified to contain the minimal protease cleavage site. Cultures growing in microtiter plates containing tetracycline are treated with potential inhibitors and simultaneously monitored for change in growth over time using an oxygen probe. Most of the cultures, not containing an inhibitor of the 3C protease, show reduced growth due to cleavage of the essential gene product; normal growth is seen only in the infrequent culture that contains an inhibitor. In the present example, we have used the *tetA* gene of plasmid pACYC184 as the modified gene. The system has been validated using inhibitors of protease 3C, and has been used to identify three new inhibitors of the enzyme, active in the micromolar range.

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Proteases have broad potential as targets for therapeutic agents. They have been shown to be involved in pathologies of digestion, inflammation, blood clotting, fertilization, and the complement system, as well as in bacterial, viral, and fungal infection, hypertension, emphysema, malignancy and metastasis[1-5]. In the cardiovascular area, angiotensin-converting enzyme (ACE) has proved to be a fruitful target for inhibition[6] and renin, another protease, acts in the same regulatory cascade as ACE and is the subject of intensive investigation. Similarly, inhibitors of the proteases of HIV-2, adenovirus, bovine leukemia virus, hepatitis A virus, and human rhinovirus (HRV) are also being sought as possible therapeutic agents.

Human rhinoviruses are the infectious agents responsible for more than 40% of cases of the common cold[7], and the great diversity of serotypes has precluded the development of effective vaccines[8]. The HRV genome encodes the production of two viral proteases, 2A and 3C, both of which are required for maturation and infectivity of the virus[9].

Here we present a whole cell screen devised to identify rhinovirus protease 3C inhibitors. The screen relies upon the expression of HRV protease 3C and its activity *in vivo* against a modified bacterial target protein containing the rhinovirus protease cleavage site. The screen was used to identify three new inhibitors, one a novel compound, all with specific activities in the micromolar range.

Results

A derivative of the 3C protease of HRV strain 1b is expressed by *E. coli*. The genome of HRV is a single molecule of positive strand RNA whose sequence consists of one long open reading frame encoding a polyprotein of 2,157 amino acids. Following synthesis, the polyprotein is processed to yield all the constituent enzymatic and structural proteins of HRV. Most of the proteolytic processing sites are cleaved specifically by the 3C protease, whose activity is an absolute requirement for viral development[9].

RT-PCR was used to amplify the protease 3C coding region of HRV strain 1b, along with the small upstream coding region 3B. The amplified DNA was cloned into the pUC18-based expression vector pKB130 (ref. 10), placing expression of the cloned sequence under the control of the tightly regulated *araBAD* promoter (Fig. 1). This construction directs the expression of the 3B-3C portion of the polyprotein as a fusion to the eleven N-terminal amino-acyl residues of *E. coli* β-galactosidase (βG'). The resulting plasmid, pOM99, synthesized an arabinose-inducible protein with a molecular weight of 25 kD as determined by SDS-PAGE (data not shown), consistent with the predicted molecular weight of 23.6 kD for unprocessed βG'-3B-3C product. (The molecular weight of mature 3C protease is 20.5 kD.) The induced protein, whose identity was verified by N-terminal sequencing, constituted approximately 4% of total cellular protein as determined by laser scanning densitometry of coomassie-stained SDS-PAGE gels. The material was found exclusively within the insoluble fraction of whole cell lysates (data not shown).

The protease 3C derivative shows intracellular proteolytic activity. Though insoluble and apparently not capable of significant self-processing, the protease 3C derivative displayed specific proteolytic activity against a modified bacterial target protein supplied *in vivo*. The tetracycline resistance-conferring protein TetA, encoded by the plasmid pACYC184 (ref. 11), was used to construct a target molecule for rhinovirus protease activity. It had been shown previously that small, in-frame insertion mutations of *tetA* can be introduced at the unique SalI site of this plasmid without significant loss of tetracycline resistance[12]. An oligonucleotide encoding the 10 amino acid sequence

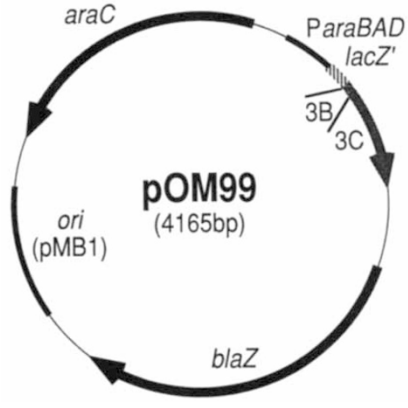

FIGURE 1. Schematic diagram of plasmid pOM99, constructed for the expression of HRV protease 3C in *E. coli*. The 3B-3C coding region of the human rhinovirus genome (strain 1b) was amplified using RT-PCR, and inserted into the expression plasmid pKB130[10] as an SstI-PstI fragment. The translation product contains an initial eleven amino acids derived from the amino terminus of LacZ. Transcription initiates from the strong, tightly regulated *araBAD* promoter, which is controlled by the *araC* regulatory gene also on the plasmid. Expression is induced by the addition of arabinose to the growth medium.

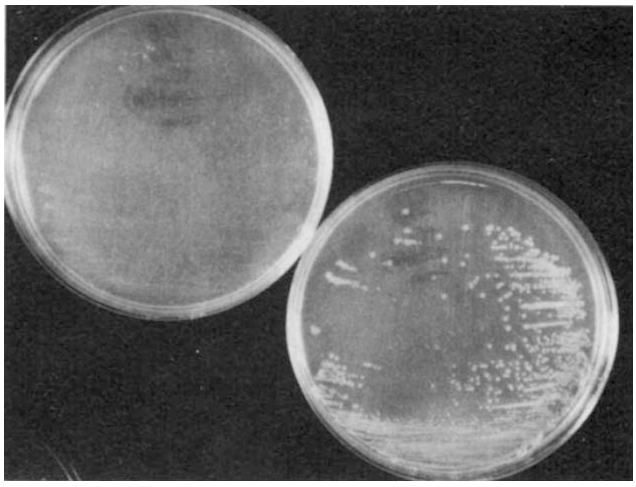

FIGURE 2. LB agar plates containing 10 μg/ml tetracycline, streaked with strain OM86 (DH5α/pOM98, pOM99) and incubated overnight at 37°C. The plate on the left contains arabinose, the plate on the right does not.

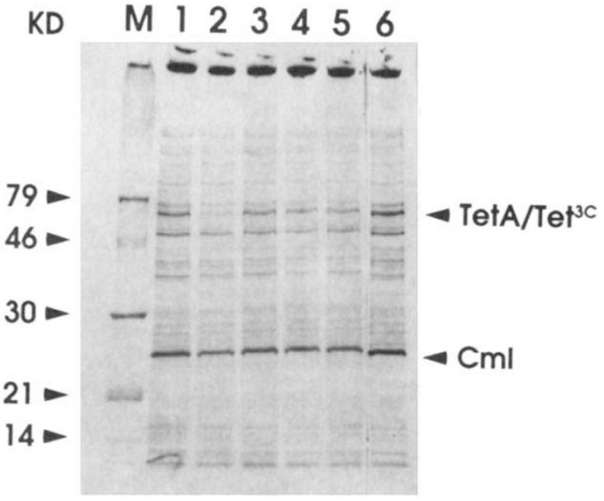

FIGURE 3. SDS-PAGE analysis of ^{35}S-methionine-labeled plasmid encoded proteins. To determine the fate of the tetracycline resistance protein under various growth conditions, cells were labeled for 30 min. with 250 μCi/ml of ^{35}S-methionine 1 hr. after the addition of arabinose. The labeled proteins were separated by SDS-PAGE and examined by autoradiography. Lanes 1–5, strain OM86 (DH5α/pOM98, pOM99); lane 6, isogenic strain with pACYC184 (*tetA*+) replacing pOM98. Lane 1, growth medium lacking arabinose; lanes 2–6, arabinose present. Lanes 3–5, inhibitors of protease 3C added to growth medium: lane 3, kalafungin (0.025 mg/ml), lane 4, radicinin (1.25 mg/ml), lane 5, citrinin hydrate (5 mg/ml). Abreviations: KD, kiloDaltons; M, denotes lane containing molecular weight standards; TetA/Tet^{3C}, position of wild-type and modified TetA protein bands; Cml, position of plasmid-coded chloramphenicol acetyltransferase protein band.

EVLFQGPVYR was ligated into the SalI site, resulting in the production of a TetA protein containing a protease 3C cleavage site (TetA^{3C}). The amino acid sequence inserted does not exactly correspond to any known rhinoviral cleavage site, but rather is one that has been shown to be efficiently cleaved *in vitro* using synthetic oligopeptides[13,14]. The modified pACYC184, designated pOM98, was introduced into the strain carrying the protease 3C expression plasmid pOM99. On plates containing 10 μg/ml tetracycline, overnight growth of this strain carrying the two plasmids was indistinguishable from that on plates lacking the antibiotic. However, when the growth medium was supplemented with arabinose to induce the synthesis of the protease 3C, growth was severely curtailed (Fig. 2). When pOM98 was replaced with pACYC184 in the same host, the addition of arabinose to the growth medium had no effect on growth (see below), indicating that inhibition of growth in the presence of arabinose was dependent upon the presence of the protease 3C target sequence within the TetA protein.

To verify that the protease 3C derivative actually cleaved the modified TetA protein within the bacterial cell, ^{35}S-methionine-labeled cultures were prepared in both the presence and absence of arabinose. Whole-cell protein extracts were subjected to SDS-PAGE and autoradiography (Fig. 3). Growth in the absence of arabinose (lane 1) produced two prominent bands, corresponding to the pOM98-coded Cml (chloramphenicol acetyltransferase) and TetA^{3C} proteins. When arabinose was included in the growth medium, the TetA^{3C} band was no longer observed (lane 2), while the Cml band remained. The resulting TetA^{3C} cleavage products were not visible, probably due to the rapid degradation of nonfunctional proteins often seen in *E. coli*[15]. When the protease 3C gene was absent from the expression vector, arabinose induction failed to cause the disappearance of the band. The expression strain lacking pOM98 or pACYC184 fails to produce the bands marked "Cml" and "TetA/Tet^{3C}" in Figure 3 (data not shown).

A high throughput screen for inhibitors. To enable large numbers of cultures to be handled and processed efficiently, cells were grown in 96 well microtiter plates, enabling the semiautomation of manipulations and full automation of data collection.

Although protease induction clearly sensitizes cells to tetracycline during overnight growth on agar plates, it was found that the difference in growth rate between induced and noninduced broth cultures was too small to be differentiated by monitoring the change in culture optical density over the shorter time periods (60–90 min.) desirable in a high-throughput assay (Fig. 4). However, since growth under the conditions used quickly becomes oxygen-limited, growth rate was determined by measuring the change in fluorescence emission of an oxygen-quenched ruthenium complex[16] applied to the floor of the microtiter wells. As oxygen in the medium is depleted by growing cells, fluorescence increases to a maximum level determined by the concentration of the indicator. The addition of arabinose caused protease induction and reduced cellular oxygen uptake due to the intracellular accumulation of inhibitory levels of tetracycline. Since the microtiter trays are incubated unsealed, a reduction in oxygen uptake allows air to diffuse into the growth medium and quench the fluorescence probe causing the gradual decrease in fluorescence (closed triangles, Fig. 4). The growth (respiration) of uninduced strain OM86 (DH5α/pOM98, pOM99) caused an increase in fluorescence by rapidly depleting available oxygen during the first hour of growth (open squares, Fig. 5). In the presence of arabinose, however, the growth rate was decreased, reflected by a much slower depletion of oxygen from the culture medium (closed symbols, Fig. 5). Under these conditions, the protease 3C synthesized is apparently able to cleave the tetracycline resistance protein, resulting in inhibition of growth by the antibiotic. Addition of the target oligopeptide (EVLFQGPVY) to the growth medium reversed the growth inhibition, presumably by competing with the target protein for proteolysis (triangles, Fig. 5). A control peptide of similar size (eight residues) had no effect on growth (data not shown). Oligopeptide added at 0 and 90 minutes produced a burst of growth followed by a decline during subsequent 30 minute periods. Thus the peptide can readily compete with the TetA^{3C} protein allowing growth in tetracycline-containing medium. As the intracellular pool of the target peptide diminishes, however,

hydrolysis of TetA^{3C} increases and the cells again become sensitive to tetracycline.

It has been found that peptides larger than about six amino acids are generally not efficiently taken up by wild-type *E. coli*[17]. However, our data strongly suggest that the nine amino acid target peptide is entering the cells. The size limitation on uptake is believed to be a function of outer membrane pore size since the hydrodynamic volume of the peptide, rather than the number of amino-acyl residues, appears to be the critical factor[17]. It is possible that the particular sequence of the target peptide allows compact folding or that very little needs to enter the cell to be effective. Alternatively, the TetA protein may be assisting in the entry of the peptide. Tetracycline resistance is known to be associated with increased uptake of aminoglycosides, compounds of greater molecular weight than the target peptide[18].

Novel inhibitors of protease 3C were identified using the fluorescence-based assay. More than 20,000 natural product extracts or purified compounds were examined using the high-throughput format described above. Two active compounds, shown in Figure 6, were recovered from microbial extracts. The phytotoxin radicinin[19] [1] was purified from a microbial extract identified using this assay, as was citrinin hydrate [2], a novel compound similar to the microbial toxin citrinin[20]. A protease 3C inhibitor was also identified from the group of pure compounds screened. Kalafungin [3], a polyketide antibiotic[21,22], was selected for testing due to its resemblance to thysanone, a natural product previously shown to be an inhibitor of protease 3C[23]. These three compounds were also found to inhibit the activity of purified protease 3C in an *in vitro* assay based on the hydrolysis of the target peptide (manuscript in preparation). The IC_{50} for these three substances were: radicinin, 500 μM; citrinin hydrate, 280 μM; kalafungin, 3.3 μM. A detailed description of these compounds will be published elsewhere.

We also tested these compounds, as well as TPCK (N-tosyl-L-phenylalanine chloromethylketone, a nonspecific thiol protease inhibitor[24]) and the target peptide by spotting solutions on LB plates containing both arabinose and tetracycline that had been seeded with strain OM86 (DH5α/pOM98, pOM99). All five compounds exhibited activity, seen as zones of growth surrounding each spot following overnight incubation (data not shown). Kalafungin, radicinin, and citrinin hydrate, all produced large zones of protection, probably due at least in part to their hydrophilic character and corresponding diffusibility. The target peptide, a larger hydrophobic molecule with limited diffusibility, produced a smaller zone.

Protease inhibitors reduce intracellular cleavage of the TetA^{3C} protein. To verify that exposure to these compounds has a direct effect upon the intracellular levels of TetA^{3C}, autoradiographs were prepared as before, using arabinose-induced cultures grown in the presence of three of the inhibitors. In Figure 3, lanes 3, 4 and 5 represent cells treated with kalafungin, radicinin and citrinin hydrate respectively. All show various levels of uncleaved TetA^{3C} protein, indicating substantial protection from 3C cleavage.

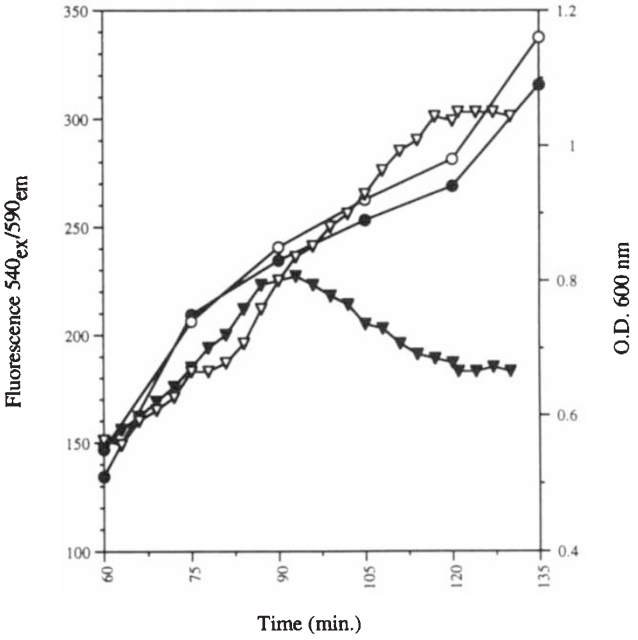

FIGURE 4. Comparison of cell growth determinations by optical density ($\lambda = 600$ nm) and by depletion of dissolved oxygen using fluorescence emission from a ruthenium complex ($540_{ex}/590_{em}$) as a function of time following the addition of arabinose to the growth medium. Triangular symbols: fluorescence measurements; round symbols: optical density measurements. Open symbols: arabinose absent; closed symbols: arabinose present. Growth conditions were as described in Experimental Protocol.

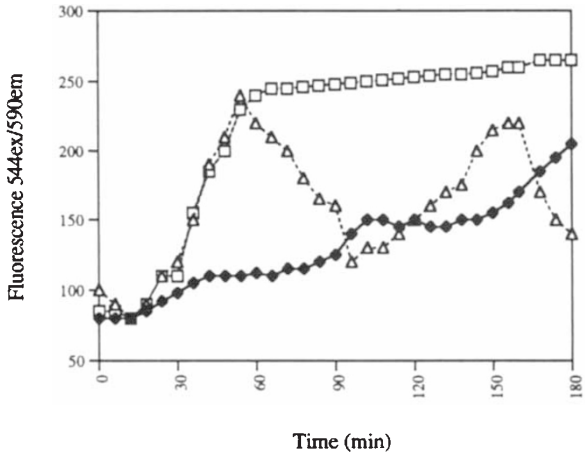

FIGURE 5. Measurement of cell-growth and the effect of target peptide on the inhibition of protease 3C. Cell growth was monitored for 3 hrs. as described in Experimental Protocol. Oligonucleotide concentration was 100 μmolar. Cell growth in the absence of arabinose (squares), and when sensitized to tetracycline in the presence of arabinose (diamonds) is shown. The addition of the target peptide at 0 and 90 min. (triangles) after arabinose induction produced a significant increase in the tetracycline tolerance of sensitive cells.

Discussion

In searching for enzyme-specific inhibitors, the initial screening is often done using an *in vitro* assay comprised of the purified enzyme and substrate, in this case the viral protease and a synthetic peptide target; the more laborious *in vivo* (infected cell) assay is usually reserved for verifying activities identified using the *in vitro* assay. *In vitro* assays may have limitations. First, a sufficient supply of soluble, active, purified enzyme must be available. Second, nonspecific inhibition of the protease can become a problem, especially in complex mixtures such as natural product extracts. Finally, large or hydrophilic molecules that appear as active in an *in vitro* screen often cannot traverse a cell membrane and are, therefore, not therapeutically useful. The screen described here is an attempt to overcome these limitations by using a bacterial cell-based design.

The strategy of this screen centers upon the rescue of a growing culture from the lethal activity of protease 3C against a (conditionally) essential bacterial protein. This strategy was first described by Block and Grafstrom, who constructed a prototype

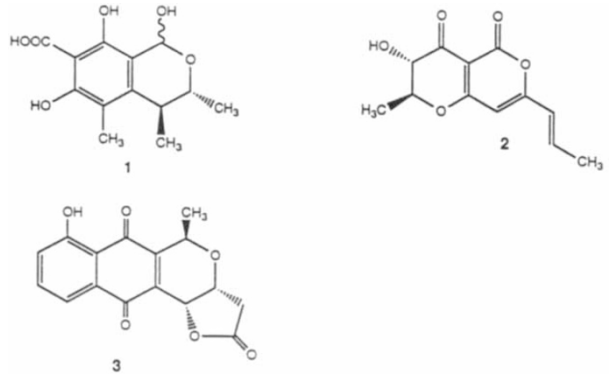

FIGURE 6. Chemical structures of citrinin hydrate [1], radicinin [2], and kalafungin [3].

screen based on the protease of HIV-1 but did not use it to actually screen compounds[12]. We found the prototype screen to be unworkable in practice, mainly due to the high toxicity of HIV-1 protease to the host cell (unpublished results). The screen presented here uses a viral protease with a much more restricted specificity and contains modifications to improve clonal stability and throughput.

The assay is based upon strain OM86 (DH5α/pOM98, pOM99) which expresses HRV-1b protease 3C under control of the *araBAD* promoter, and constitutively expresses a modified TetA protein containing a protease 3C cleavage site ($TetA^{3C}$). The addition of arabinose to the growth medium induces expression of the 3C protease, which results in the inactivation of cellular $TetA^{3C}$ protein, rendering the cells sensitive to tetracycline. Adding a protease inhibitor to the growth medium will reestablish tetracycline resistance and permit growth if (1) the inhibitor has sufficient activity against protease 3C that $TetA^{3C}$ cleavage will be blocked, (2) a sufficient amount can traverse the cytoplasmic membrane, and (3) it is not itself toxic to the cell. Cells growing in the presence of tetracycline therefore die following the addition of arabinose, unless rescued by the presence of an inhibitor of the 3C protease. In addition to the obvious need for a protease inhibitor to have a high level of activity against its target, it must also possess many other characteristics to be therapeutically useful. Chief among these are the ability to reach the target molecule within the cell and a lack of cellular toxicity. Since bacterial and human cells share many features, we reason that any candidate compound that is either unable to traverse a cell membrane, or shows initial toxicity, should be eliminated in a first screen. Although porin mutants of *E. coli* are available wherein the cell membrane presents less of a barrier than in wild-type cells, such strains were less favored, along with the naturally more permeable Gram positive species, due to an excessive rate of positive sample presentation (data not shown).

The *araB* operator/promoter was chosen to control 3C protease expression due to its very low basal level of transcription in the noninduced state[25]. We have found that this can be an important consideration as overexpression of some viral proteases is extremely toxic to bacterial cells (unpublished observations), and even sublethal baseline expression of such proteins could lead to clonal instability.

Experimental Protocol

Bacterial strain, plasmids and media. All work employed *E. coli* strain DH5α [F⁻ *ϕ80dlacZΔM15 endA1 recA1 hsdR17*(r_k^- m_k^+) *supE44 thi⁻1* λ⁻ *gyrA96 relA1 Δ(lacZYA-argF)U169*] (Bethesda Research Labs, Gaithersberg, MD). In all instances cells were grown using Luria-Bertani (LB) broth or plates[26], except for preparing samples for SDS-PAGE analysis, in which case growth was in "superbroth" (SB)[27]. Plasmids pKB130 and pACYC184 have been described previously[10,11].

Molecular biology. Unless stated otherwise all procedures were as in ref. 28. The HRV-1b 2B-3C region was cloned by reverse transcription and PCR amplification of RNA purified from a viral pellet. Sequence integrity of the cloned PCR product was verified by comparison with the published sequence[29]. The exact region amplified and cloned corresponds to base numbers 5116 to 5713 inclusive of the HRV strain 1B genome (Genbank accession number D00239). The engineered protease 3C target site within the Tet^{3C} protein was designed to have the highest possible cleavage efficiency. Using data from HRV type 14 protease 3C *in vitro* cleavage assays of synthetic peptides[13,14], the nine amino acid sequence of a single substitution variant of the type 14 2C-3A cleavage site was selected to define the cut site within the $TetA^{3C}$ protein. The $tetA^{3C}$ gene was produced by the insertion of a 30 nucleotide double-stranded synthetic oligonucleotide within the unique SalI site of the plasmid pACYC184. Small in-frame insertions at this site have been shown to be tolerated by the TetA protein with minimal loss of activity[12].

Shake-flask expression analysis. Cultures were grown to an OD_{600} of 1.0, at which time protease 3C production was induced by the addition of arabinose to a final concentration of 0.2%. Cell samples were collected at 0, 4 and 16 hrs. post-induction.

SDS-PAGE analysis. Prepoured Daiichi 10 × 10 cm 10–20% gradient gels (Enprotech, Natick, MA) were used exclusively, and run under manufacturer's suggested conditions. Expression was quantified by staining with ISS Pro-Blue (Enprotech, Natick, MA) followed by laser scanning densitometry using an LKB (now Pharmacia) Ultroscan XL (Albuquerque, NM).

Amino-terminal amino acid sequence analysis. Whole-cell protein extracts from arabinose-induced cultures were separated by SDS-PAGE and electroblotted onto PVDF membrane. The protein bands were visualized with coomassie blue R250 dye, and the protease band was closely excised from the membrane. The coomassie dye was removed by extraction with 0.1% triethylamine in methanol. The sample was analyzed on an Applied Biosystems Model 476 automatic sequencer (Foster City, CA).

Peptide synthesis. Oligopeptides were synthesized using Merrifield chemistry[30] on an Applied Biosystems (Foster City, CA) Model 430A synthesizer, purified by reverse phase HPLC on a Vydac (Hesperia, CA) C-18 column, and characterized by mass spectroscopy and amino acid analysis.

Fluorescence-based assay. High-throughput microtiter-based measurement of protease activity was done using strain OM86 (DH5α/pOM98, pOM99) grown to an OD_{600} of 0.6 in 75% (v/v) Luria broth (LB)[26] containing Ap (50 μg/ml) and Cm (10 μg/ml). Fifty microliters of these cells were added to 150 μl of fresh medium containing arabinose (Ara, 0.25%) and tetracycline (Tet, 2.5 μg/mL) at time zero. Cells were incubated in microtiter trays coated with the oxygen responsive indicator 4,7-diphenyl-1,10-phenanthroline ruthenium (II) chloride[16,31]. The oxygen-quenched fluorescence probe was synthesized from phenanthroline and ruthenium chloride by heating under reflux for 48 hrs. The reaction mixture was separated by C-18 silica gel column chromatography and material corresponding to 4,7-diphenyl-1,10-phenanthroline ruthenium chloride was identified by fluorescence and characterized by nuclear magnetic resonance. The ruthenium complex was dried and adsorbed on 40 μm silica gel to produce a dark yellow powder. The silica-bound material was suspended in a xylene-based clear silicone (Dow Corning, Midland, MI). The suspension was dispensed in 10 μl aliquots in each well of a 96-well microtiter tray and dried in a hood to remove xylene. Probe-coated trays were stored at room temperature until use. The drying process siliconized the probe to the bottom of the microtiter tray to form a watertight film allowing exchange of oxygen without aqueous solubility. Although the change in fluorescence caused by oxygen depletion during bacterial growth was reversible, probe-coated trays were discarded after a single use. The assay was carried out at 37°C in the plate reader, without shaking, in unsealed trays. Samples were tested at approximately 0.1 μg/well (0.5 μg/ml) and positives were obtained at a rate of 0.1%. These positive samples were then subjected to a plate assay designed to detect sugars such as fucose which act as antinducers of the *araB* promoter[32]. No other types of false positive sample were seen, i.e. all other positive samples tested were found to inhibit the purified enzyme. Growth was monitored continuously by measuring fluorescence emission (544ex/590em) with a Titertek Fluoroskan II microtiter fluorescence spectrophotometer (ICN, Huntsville, AL). Test samples were selected for further characterization if they supported growth in the presence of tetracycline.

Agar plate spot assay. An agar plate assay was developed to confirm the activity of samples which tested positive in the microtiter fluorescent assay. Early exponentially growing cells of OM86 were spread as a lawn on LB plates containing Ap, Cm, Ara and Tet at concentrations used in the microtiter assay. Samples to be tested for protease inhibition were spotted as 5 μl aliquots, directly on the agar surface, and the presence of a zone of growth in the vicinity of the sample was indicative of protease inhibition. A DMSO solution of TPCK, a non-specific thiol protease inhibitor[24], was used as control and spotted at double the volume of the test samples.

Protein labeling and autoradiography. Cells grown in a microtiter tray as described for the fluorescence-based assay were labeled with 250 μCi/ml of ^{35}S-methionine (1 mCi/mmolc) for 30 min. after a 60 min. preincubation without label. Cells harvested at 90 min. were washed twice with phosphate buffered saline, lysed in sample buffer and the proteins separated on a 10–20% gradient SDS-PAGE gel. The gel was dried and autoradiographed for 6–18 hrs.

Acknowledgments

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