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# A Novel Screening System to Characterize and Engineer Quorum Quenching Lactonases

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## ABSTRACT

*N*-acyl L-homoserine lactones are signaling molecules used by numerous bacteria in quorum sensing. Some bacteria encode lactonases, which can inactivate these signals. Lactonases were reported to inhibit quorum sensing-dependent phenotypes, including virulence and biofilm. As bacterial signaling is dependent on the type of molecule used, lactonases with high substrate specificity are desirable for selectively targeting species in communities. Lactonases characterized from nature show limited diversity in substrate preference, making their engineering appealing but complicated by the lack of convenient assays for evaluating lactonase activity. We present a medium-throughput lactonase screening system compatible with lysates that couples the ring opening of *N*-acyl L-homocysteine thiolactones with 5,5-dithio-bis-(2-nitrobenzoic acid) to generate a chromogenic signal. We show that this system is applicable to lactonases from diverse protein families and demonstrate its utility by screening mutant libraries of GcL lactonase from *Parageobacillus caldoxylosilyticus*. Kinetic characterization corroborated the screening results with thiolactonase and homoserine lactonase activity levels. This system identified GcL variants with altered specificity: up to 1900-fold lower activity for long-chain *N*-acyl L-homoserine lactone substrates and ~38-fold increase in preference for short-chain substrates. Overall, this new system substantially improves the evaluation of lactonase activity and will facilitate the identification and engineering of quorum quenching enzymes.

## 1 | Introduction

Quorum sensing (QS) is a bacterial communication system that uses the environmental concentration of autoinducer molecules to signal cell population density, and was first documented with the induction of bioluminescence through luciferase activity in *Vibrio fischerii* (Nealson 1977; Nealson, Platt, and Hastings 1970) and is controlled by synthase-receptor systems (Fuqua, Winans, and Greenberg 1994). As autoinducers accumulate, they trigger changes in gene expression profiles in the population. Some behaviors under QS regulation include biofilm formation and maturation (Labbate et al. 2004; Parsek and Greenberg 2005), antibiotic production (Bainton et al. 1992), and induction of virulence (Erickson et al. 2002; Nadal Jimenez et al. 2012). These behaviors contribute to infection and disease and make their control both economically and clinically important, for example in persistent antibiotic-resistant bacterial infections (Costerton, Stewart, and Greenberg 1999; Mehrad et al. 2015) such as those associated with cystic fibrosis (Bjarnsholt et al. 2010), marine aquaculture and aquatic biofouling (Dobretsov, Teplitski, and Paul 2009; Fitridge et al. 2012), and in agriculture (Pirhonen et al. 1993).

The first two authors contributed equally to this article.

Different types of autoinducers were identified, suggesting that different chemical languages are used by bacteria. Some of the main autoinducers are autoinducer peptides (AIP), *N*-acyl L-homoserine lactones (AHLs or autoinducer-I) and autoinducer-2 (AI-2). AIPs are small oligopeptides that can be cyclic or linear used in Gram-positive bacteria such as *Streptococcus pneumoniae* and *Staphylococcus aureus*. AI-2 are furanone compounds that occur broadly in both Gram-positive and Gram-negative bacteria (Pereira, Thompson, and Xavier 2013). AHLs vary in the length of the acyl chains and are used in most Gram-negative bacteria (Miller and Bassler 2001; Williams et al. 2007).

Interfering with QS is an enticing approach to control bacterial behaviors. Quorum quenching (QQ) involves the disruption of QS circuits, through the inhibition of signal synthesis or detection. QS inhibitors are molecules that interfere with signal reception or generation and reduce QS-related behaviors (Ahmed et al. 2019). Enzymatic QQ consists of the degradation of signaling molecules by enzymes. Enzymatic QQ was reported to result in reduction of biofilm and virulence in cultures (Chow et al. 2014; Vogel et al. 2022), and when directly applied to tissues and surfaces or immobilized, such as when orally administered to zebrafish and rats (Cao et al. 2012; Hraiech et al. 2014; Utari et al. 2018), immobilized on polyurethane (Grover et al. 2016; Guendouze et al. 2017), or on silicon catheters (Ivanova et al. 2015; Vogel et al. 2020). Additionally, recent studies suggest that QQ has impacts on microbiome dynamics, which include altering the transcriptome, proteome, and bacterial community composition in wastewater treatment and bioreactor settings (H.-W. Kim et al. 2013; Jo et al. 2016; de Celis et al. 2021).

AHL lactonases are key representatives of QQ enzymes. Lactonases hydrolyze the lactone ring of AHLs (Dong et al. 2001; Momb et al. 2008). Lactonases include representatives of four main protein families: metallo-β-lactamase like lactonases (MLLs) (Bergonzi, Schwab, and Elias 2016; Tang et al. 2015; Thomas et al. 2005), paraoxonases (Draganov et al. 2005; Elias and Tawfik 2012; Khersonsky and Tawfik 2005), phosphotriesterase-like lactonases (PLLs) (Elias et al. 2008; Hiblot et al. 2012, 2015), and α/β-fold hydrolases (Mei et al. 2010). MLLs tend to have higher catalytic efficiencies against a broad range of AHL substrates, in the $10^3$–$10^6\,M^{-1}s^{-1}$ range (Bergonzi et al. 2019; Momb et al. 2008; L.-H. Wang et al. 2004), while PLLs typically prefer AHLs with acyl chains greater than 8 carbons in length and have catalytic efficiencies in the $10^2$–$10^5\,M^{-1}\,s^{-1}$ range (Hiblot et al. 2012, 2015). Because high catalytic rates and high stability are desirable properties for QQ enzymes to be able to quickly and effectively remove QS signals in a sustained manner, there have been efforts to engineer these enzymes to increase their activity (Chow, Wu, and Yew 2009; J. Wang et al. 2019). Additionally, altering substrate specificity is especially of interest for targeting specific bacteria and has been explored to reduce virulence of, for example, *Burkholderia* (Koch et al. 2014) or as a means of avoiding attenuation of beneficial bacteria (Kyeong, Kim, and Kim 2015). Additionally, recent reports suggest that the substrate specificity profile of the QQ enzyme determines the effect it has on behavior in *Pseudomonas aeruginosa* (Mahan et al. 2020; Rémy et al. 2020).

The engineering of QQ enzymes such as lactonases is complicated by the lack of straightforward assays to evaluate enzymatic activity, which then limits the practicality of screening mutant libraries. Direct methods to record end-point kinetics for lactonase activity include gas chromatography (Charlton et al. 2000), thin-layer chromatography (Shaw et al. 1997), and high-performance liquid chromatography and mass spectrometry (Dong et al. 2001; L.-H. Wang et al. 2004). However, these methods are low-throughput and labor intensive. A time-course kinetic assay for lactone hydrolysis based on a pH indicator to report the release of a proton has been useful for biochemical characterization (Khersonsky and Tawfik 2005; Momb et al. 2008; Thomas et al. 2005), but its requirement of a controlled environment and weak buffer makes it impractical for screening. Indirect methods that indicate AHL degradation are also useful. For example, phenotypic screens such as the pigment production by *Chromobacterium violaceum* (McClean et al. 1997) and *P. aeruginosa* (Billot et al. 2022), as well as biosensor systems based on AHL receptors coupled with reporter GFP or luciferase genes (Andersen et al. 2001; Winson et al. 1998) can be used to evaluate mutant libraries (J.-H. Kim et al. 2010; Last et al. 2016; Torres et al. 2017). However, these reporter systems typically have a low dynamic range, are also complicated by high background and noise, and the evaluation of enzyme activity and substrate specificity can be challenging.

Here we report an easy and versatile time-course enzymatic assay that can be used to measure lactonase activity to the extent of kinetic characterization. This system allows for rapid screening of cell lysates, setting it apart from HPLC approaches which typically requires purified protein samples and is lower throughput. Moreover, this screening system produces quantitative measurements, allowing for the identification of the best variants in a single step, a task that is complicated with QS fluorescence-based assays due to their limited dynamic range. We took advantage of the ability of some lactonases to degrade *N*-acyl homocysteine thiolactones (HTLs) (Momb et al. 2006, 2008) and show that it can be used with lactonases from all of the different currently known protein families that they fall into. We worked with the lactonase GcL from *Parageobacillus caldoxylosilyticus* (Bergonzi, Schwab, and Elias 2016) and established conditions for kinetic determination and screening of mutant libraries using this HTL-based screening system. We show that this assay allows for the identification of new mutants with altered kinetic properties for HTLs and found that these results translate into altered kinetics for AHLs by testing select AHLs relevant to bacterial physiology. These include *N*-butyryl L-homoserine lactone (C4-HSL) used by *Aeromonas* species (Swift et al. 1997), *Pseudomonas aeruginosa* (Winson et al. 1995), and *Serratia liquefaciens* (Eberl et al. 1996); *N*-octanoyl L-homoserine lactone (C8-HSL) used by *Klebsiella pneumoniae* (Yin et al. 2012) and *Burkholderia cepacia* (Lewenza et al. 1999); *N*-decanoyl L-homoserine lactone (C10-HSL) used by *Acinetobacter baumanii* (Niu et al. 2008); and 3-oxo-dodecanoyl L-homoserine lactone (3OC12-HSL) which is also used by *P. aeruginosa* (Winson et al. 1995).

## 2 | Methods

### 2.1 | Materials

*N*-acyl homocysteine thiolactones (*N*-acetyl D,L-HTL [C2-HTL]; *N*-butanoyl L-HTL [C4-HTL)], and *N*-octanoyl D,L-HTL [C8-

HTL]) were custom synthesized by Enamine Ltd (Monmouth Junction, NJ, USA) or purchased from Millipore Sigma (Burlington, MA, USA). *N*-acyl L-homoserine lactones were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Cresol purple, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), and cobalt chloride were purchased from Alfa Aesar (now Thermo Fisher Scientific [Waltham, MA, USA]). Media components used were from BD Difco (Thermo Fisher Scientific) and VWR (Radnor, PA, USA). Ampicillin, dimethyl sulfoxide (DMSO), glycerol, and DNase I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloramphenicol, kanamycin sulfate, and phenylmethylsulfonyl fluoride (PMSF) were purchased from VWR. Buffer components were purchased from Thermo Fisher Scientific.

## 2.2 | Site Saturation Mutagenesis

Site saturation plasmid libraries of select GcL residues were ordered from Genscript Biotech Corporation (Piscataway, NJ, USA) in pET-22b(+). Chemically competent *Escherichia coli* DH5α (Invitrogen [Thermo Fisher Scientific]) was transformed with the pooled plasmid libraries through heat shock, then plated on LB agar containing 100 μg/mL ampicillin. Individual colonies were resuspended in phosphate-buffered saline and sequenced through direct colony sequencing (ACGT Inc. [Wheeling, IL, USA]). After sequencing and identification, individual cultures of *E. coli* DH5α carrying each single amino acid substitution plasmid were grown in LB media containing 100 μg/mL ampicillin and the plasmids were extracted and purified using the QIAprep Spin Miniprep kit (Qiagen [Hilden, Germany]). Purified plasmids were sequenced through Sanger sequencing at the University of Minnesota Genomics Center (Saint Paul, MN, USA) then used to transform *E. coli* BL21(DE3) Star (Invitrogen) containing plasmid pGro7 with L-arabinose inducible chaperones groES-groEL (TaKaRa [San Jose, CA, USA]) through heat shock.

## 2.3 | Protein Expression for Screening

*E. coli* BL21(DE3) Star cells containing pGro7 and GcL insert-containing pET-22b were grown from glycerol stocks in 500 μL of LB media containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37°C overnight to generate starter cultures. 40 μL of this starter culture was used to inoculate 1.5 mL of ZYP media containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol in 96-deep well plates. The plates were shaken at 500 rpm at 37°C for 4 h, then L-arabinose was added to a final concentration of 0.2% for chaperone induction, and $CoCl_2$ was added to a final concentration of 2 mM. The plates were cooled and allowed to shake at 18°C for 18–20 h. Cells were then harvested in the 96-deep well plate by centrifugation at 4000 rpm and the supernatant was removed. Cell pellets were resuspended in 300 μL lysis buffer containing Bugbuster Protein Extraction Reagent (Sigma-Aldrich), 1 μg/mL lysozyme (Research Products International [Mount Prospect, IL, USA]), 1 mM PMSF, and 50 ng/mL DNase, and incubated at room temperature for 2 h with shaking at 500 rpm. The cell lysate was then clarified by centrifugation in the same plate at 4000 rpm for 1 h.

## 2.4 | Protein Purification

GcL variants were grown and purified as previously described (Bergonzi et al. 2019). AaL was grown and purified as previously described (Bergonzi et al. 2018) but with the post-induction temperature set at 16°C. SsoPox W263I was grown and purified as previously described (Hiblot et al. 2013). Genes encoding lactonase AiiA from *Bacillus thuringiensis* serovar kurstaki (accession number AF478059.1) and lactonase JydB from *Rhodococcus sp*. BH4 (accession number ARE36482.1) were codon-optimized for expression in *E. coli* and cloned with an N-terminal His-tag and TEV cleavage site into pET29b(+) between the NdeI and XhoI restriction sites by Twist Bioscience (San Francisco, CA, USA). AiiA and JydB expression vectors were individually co-transformed with pGro7 into *E. coli* BL21(DE3) Star cells, then grown and purified according to the protocol described for GcL, except with the post-induction temperature at 16°C. For JydB, $CoCl_2$ was excluded from all steps of the production and purification process.

## 2.5 | Thiolactone Hydrolysis Assay on Bacterial Lysates

Thiolactonase activity was measured in 96-well plates in 200 μL reactions containing 30 μL cell lysate, 2 mM DTNB, 1 mM HTL substrate, and an activity buffer consisting of 50 mM HEPES pH 8.0, 150 mM NaCl, 0.2 mM $CoCl_2$. The absorbance increase over time at 412 nm was measured using a Biotek Synergy HT microplate reader (Marshall Scientific [Hampton, NH, USA]). The data was trimmed to determine the initial rate of reaction, and the mean initial rate of reaction for each mutant was divided by the mean initial rate of reaction for the wild-type (WT) enzyme to obtain a normalized activity value for each mutant. The ratio of the normalized activity between each substrate was then calculated to determine shifts in substrate preference for each mutant. Variants with low activity, that is, with less than 5% activity for all substrates relative to WT were excluded from the calculations as these mutants are nearly inactive.

## 2.6 | Thiolactone Hydrolysis Assay on Purified Proteins

Thiolactonase activity was measured in 96-well microplates in 200 μL reactions containing purified recombinant enzymes at their indicated concentrations, 1 mM or 2 mM DTNB, HTL substrates at their indicated concentrations, and activity buffer. $CoCl_2$ was excluded from the activity buffer for JydB. Five to eight substrate concentrations ranging from 1 μM to 2 mM were used. Reactions were performed in triplicate along with a blank containing the buffer and substrate. The absorbance increase at 412 nm was measured using a BioTek Synergy HT or HTX microplate reader over time. The data was trimmed and the respective blank was subtracted to determine the initial rate of reaction. The mean of the initial rate of the reaction was plotted against substrate concentration and fit to the Michaelis–Menten equation using GraphPad Prism to determine kinetic parameters.

### 2.7 | pH Indicator Based AHL Lactonase Assay

Lactonase activity was measured in 96-well plates, in 200 μL reactions consisting of a lactonase buffer containing 2.5 mM bicine pH 8.3, 150 mM NaCl, 0.2 mM $CoCl_2$, 0.25 mM cresol purple as a pH indicator, and enzyme at 2.5–25 μg/mL. For longer chain, more hydrophobic substrates, DMSO was added to 5% v/v. The change in absorbance at 577 nm was measured using a Biotek Synergy HT or HTX microplate reader (Marshall Scientific). Five to eight substrate concentrations were used, ranging from 1 μM to 2 mM. The reaction was performed in triplicate or quadruplicate, along with a buffer-only blank, and the mean of the initial rate of the reaction was fit to the Michaelis–Menten equation using GraphPad Prism to obtain the catalytic parameters.

## 3 | Results and Discussion

### 3.1 | Multiple *N*-Acyl L-Homoserine Lactonases Hydrolyze *N*-Acyl L-Homocysteine Thiolactones

We aimed to engineer lactonases with altered substrate preferences and were looking for a system to screen mutant libraries quickly and efficiently. It was previously reported that the lactonase AiiA showed significant levels of activity against C6-HTL ($k_{cat}/K_M = 3.0 \times 10^4\ M^{-1}\ s^{-1}$), as measured using the phenol red pH-indicator method (Momb et al. 2008). *N*-acyl L-homoserine lactones (AHLs) and *N*-acyl L-homocysteine thiolactones (HTLs) differ only by one atom, the O/S substitution in the lactone ring (Figure 1A,B). This extreme structural similarity causes *N*-acyl L-homocysteine thiolactones to be agonists of *N*-acyl L-homoserine lactones for AHL receptors (Boursier, Combs, and Blackwell 2019). Here, we used GcL to establish a kinetic assay to monitor degradation of HTLs. Upon hydrolysis, HTLs release a free thiol that can subsequently react with the Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid) [DTNB]) (Figure 1C). Hydrolysis can therefore be monitored by measuring the increase in absorbance at 412 nm and a color change of the reaction mixture from clear to yellow (Ellman 1959; Ellman et al. 1961) (Figure 2A). The enzymatic assay is compatible with pHs at which the DTNB reagent remains stable (up to and including pH 6–10.5) with the best dynamic range in the pH 8–10 range. At higher pHs, the background of the reaction also increases as the DTNB spontaneously degrades (Figure 2B).

We determined the kinetic parameters for a diverse set of quorum quenching lactonases from different protein families against three different HTLs: *N*-acetyl D/L-HTL (C2-HTL), *N*-butanoyl L-HTL (C4-HTL), and *N*-octanoyl D/L-HTL (C8-HTL) (Figure 2C–E, Table 1). Specifically, we evaluated three representatives from the MLLs: AaL from *Alicyclobacillus acidoterrestris* (Bergonzi et al. 2018); AiiA from *Bacillus thuringiensis* (Thomas et al. 2005), and GcL; one representative from the PLLs: SsoPox W263I (Hiblot et al. 2013), and one α/β hydrolase: JydB (Ryu et al. 2020). Interestingly, the results show that all the tested lactonases can degrade HTLs to varying degrees, highlighting the value of this substrate as a convenient substrate proxy for activity against AHLs.

The catalytic efficiency values for HTL hydrolysis are generally lower than those observed against AHLs, possibly due to the lower polarization of the C-S bond (compared to C–O) of the thiolactone ring. For example, the catalytic efficiency of GcL WT against C4-HTL ($k_{cat}/K_M = 1.7 \times 10^2\ M^{-1}\ s^{-1}$) is ~700× lower than its activity against C4-HSL ($k_{cat}/K_M = 8.3 \times 10^4\ M^{-1}\ s^{-1}$) (Bergonzi, Schwab, and Elias 2016), and AaL has a ~1200× lower catalytic efficiency against C4-HTL compared to C4-HSL (Table 2). An exception to this is AiiA, which has a higher catalytic efficiency against C2- and C8-HTL, preferring them by 13× and 47× more than the equivalent AHL (Table 2).

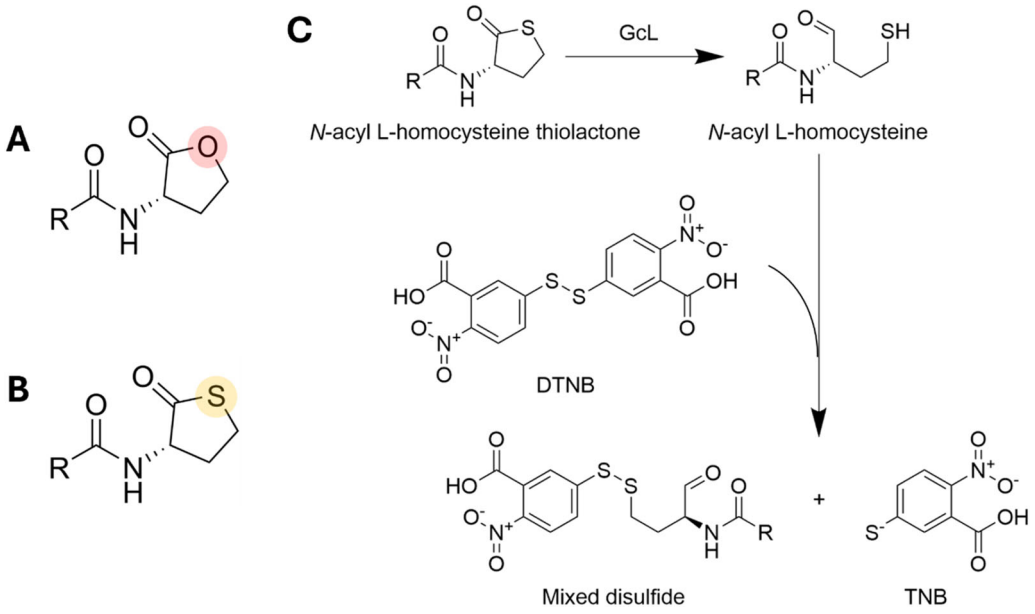


**FIGURE 1** | *N*-acyl L-homocysteine thiolactones hydrolysis assay. Chemical structures of (A) *N*-acyl L-homoserine lactones and (B) *N*-acyl L-homocysteine thiolactones. (C) Reaction scheme of the colorimetric assay system to measure lactonolysis of *N*-acyl L-homocysteine thiolactone using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB).

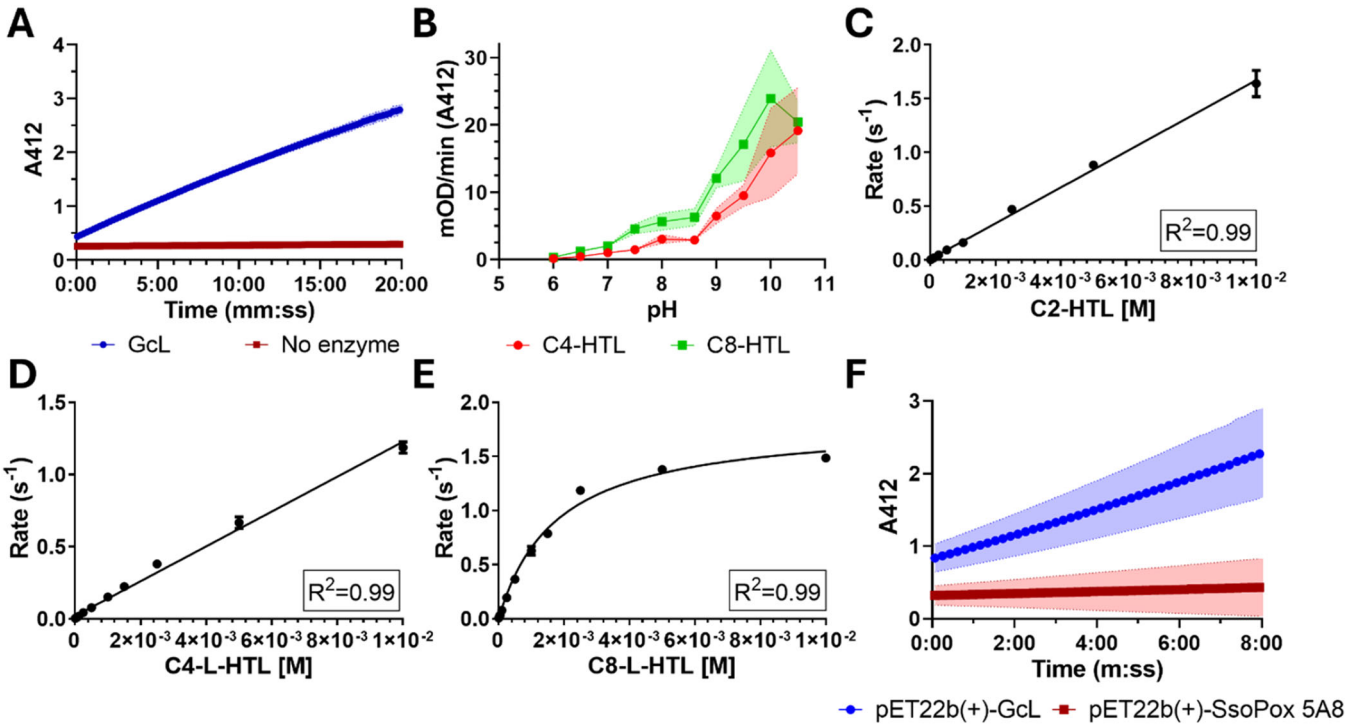


**FIGURE 2** | Kinetics of *N*-acyl L-homocysteine thiolactone hydrolysis. (A) Real-time increase in absorbance at 412 nm on hydrolysis of *N*-acetyl homocysteine thiolactone at pH 8.0. (B) pH profile of the assay system with GcL and *N*-butyryl L-homocysteine thiolactone (red) or *N*-octanoyl L-homocysteine thiolactone (green). Shaded areas represent standard deviation ($n = 3$). (C–E) Kinetic curves for GcL against homocysteine thiolactone substrates: (C) *N*-acetyl D-/L-homocysteine thiolactone, (D) *N*-butyryl L-homocysteine thiolactone, (E) *N*-octanoyl L-homocysteine thiolactone. Corresponding Michaelis–Menten curves for other lactonases can be found in the Supporting Information S1: Figures S1–S4. For substrates which did not fit the Michaelis–Menten equation, a linear regression was generated to determine catalytic efficiency. (F) *N*-acetyl homocysteine thiolactonase activity in cell lysates overexpressing the lactonase GcL as reported by DTNB and recorded at 412 nm with lysates expressing active lactonase (GcL, blue), inactive lactonase (SsoPox 5A8, red). Shaded regions represent standard deviation ($n = 8$).

**TABLE 1** | Biochemical kinetic parameters of lactonases used in this study against homocysteine thiolactones (HTLs).

| Enzyme | HTL | $k_{cat}$ ($s^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($s^{-1} M^{-1}$) |
|---|---|---|---|---|
| GcL WT | C2 | nd | nd | $(1.66 \pm 0.02) \times 10^2$ |
| | C4 | nd | nd | $(1.21 \pm 0.02) \times 10^2$ |
| | C8 | $1.82 \pm 0.04$ | $1777.00 \pm 112.90$ | $(1.03 \pm 0.07) \times 10^3$ |
| AaL | C2 | nd | nd | $(1.24 \pm 0.01) \times 10^3$ |
| | C4 | nd | nd | $(1.08 \pm 0.04) \times 10^3$ |
| | C8 | nd | nd | $(4.02 \pm 0.11) \times 10^3$ |
| AiiA | C2 | $33.56 \pm 7.47$ | $4358.00 \pm 1271.00$ | $(7.70 \pm 2.83) \times 10^3$ |
| | C4 | $176.30 \pm 17.13$ | $1831.00 \pm 301.50$ | $(9.63 \pm 1.84) \times 10^4$ |
| | C8 | $260.30 \pm 14.95$ | $522.60 \pm 67.20$ | $(4.98 \pm 0.70) \times 10^5$ |
| SsoPox W263I | C2 | na | na | na |
| | C4 | na | na | na |
| | C8 | $7.39\ (\pm 0.01) \times 10^{-3}$ | $11.60 \pm 1.26$ | $(6.37 \pm 0.70) \times 10^2$ |
| JydB | C2 | $17.45 \pm 0.47$ | $37.82 \pm 4.65$ | $(4.61 \pm 0.58) \times 10^5$ |
| | C4 | $2.71 \pm 0.05$ | $40.43 \pm 3.76$ | $(6.70 \pm 0.64) \times 10^4$ |
| | C8 | $0.62 \pm 0.02$ | $69.63 \pm 10.42$ | $(8.94 \pm 1.37) \times 10^3$ |

*Note:* Kinetic curves are shown in Figure 2 and Figures S1, S2.
Abbreviations: C2, acetyl-D/L-homocysteine thiolactone; C4, butyryl L-homocysteine thiolactone; C8, octanoyl D/L-homocysteine thiolactone; na, no detectable activity; nd, not determined: saturation could not be reached.

**TABLE 2** | Biochemical kinetic parameters of lactonases used in this study against *N*-acyl L-homoserine lactones (HSLs).

| Enzyme | HSL | $k_{cat}$ ($s^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) | HSL/HTL ratio |
|---|---|---|---|---|---|
| GcL WT | C4[a] | $19.06 \pm 1.51$ | $229 \pm 57$ | $(8.3 \pm 2.2) \times 10^4$ | 687 |
| | C6[a] | $8.95 \pm 0.48$ | $7.97 \pm 1.89$ | $(1.1 \pm 0.3) \times 10^6$ | — |
| | C8[a] | $1.29 \pm 0.04$ | $3.12 \pm 0.75$ | $(4.1 \pm 1.0) \times 10^5$ | 400 |
| | C10[a] | $5.48 \pm 0.37$ | $1.45 \pm 0.47$ | $(3.8 \pm 1.3) \times 10^6$ | — |
| | 3OC12[b] | $1.45 \pm 0.05$ | $0.85 \pm 0.24$ | $(1.7 \pm 0.49) \times 10^6$ | — |
| AaL[c] | C4 | $13.54 \pm 0.91$ | $10.5 \pm 0.3$ | $(1.29 \pm 0.37) \times 10^6$ | 1193 |
| | C6 | $13.97 \pm 0.43$ | $82.7 \pm 11.0$ | $(1.69 \pm 0.23) \times 10^5$ | — |
| | C10 | $5.13 \pm 0.35$ | $49.6 \pm 1.4$ | $(1.03 \pm 0.30) \times 10^5$ | — |
| | 3OC12 | $5.03 \pm 0.25$ | $14.0 \pm 3.4$ | $(3.60 \pm 0.88) \times 10^5$ | — |
| AiiA | C4[d] | 37.63 | 5110 | $7.36 \times 10^3$ | 0.08 |
| | C6[e] | $510 \pm 10$ | $360 \pm 40$ | $1.4 \times 10^6$ | 14.54 |
| | C8[d] | 27.53 | 2610 | $1.05 \times 10^4$ | 0.021 |
| SsoPox W263I | C4[f] | nr | nr | $(4.61 \pm 1.22) \times 10$ | — |
| | C6[f] | nr | nr | $(4.58 \pm 0.48) \times 10^2$ | — |
| | 3OC12[g] | $1.80 \pm 0.05$ | $17.8 \pm 4.9$ | $(1.01 \pm 0.28) \times 10^5$ | — |
| JydB | C4[h] | $300 \pm 17.32$ | $0.16 \pm 0.031$ | $(1.88 \pm 0.31) \times 10^6$ | 4.08 |
| | C6[e] | $666 \pm 50.92$ | $15.28 \pm 2.41$ | $(4.36 \pm 0.11) \times 10^4$ | — |
| | 3OC6[e] | $347 \pm 24.73$ | $0.24 \pm 0.07$ | $(1.45 \pm 0.39) \times 10^6$ | — |
| | C8[b] | $1.88 \pm 0.11$ | $11.79 \pm 4.10$ | $(1.59 \pm 0.56) \times 10^5$ | 17.79 |

[a]Data from Bergonzi et al. (2019).
[b]This study (Supporting Information S1: Figures S5–S6).
[c]Data from Bergonzi et al. (2017).
[d]Data from L.-H. Wang et al. (2004).
[e]Data from Momb et al. 2008).
[f]Data from Billot et al. (2022).
[g]Data from Hiblot et al. (2013).
[h]Data from Ryu et al. (2020).
Abbreviations: C4, *N*-butyryl L-homoserine lactone; C6, *N*-hexanoyl L-homoserine lactone; C8, *N*-octanoyl L-homoserine lactone; C10, *N*-decanoyl L-homoserine lactone; 3OC12, 3-oxo-dodecanoyl l-homoserine lactone; nr, not reported.

AiiA is also the most active lactonase against the tested HTLs, with a catalytic efficiency value against C8-HTL of $4.9 \times 10^5\,s^{-1}\,M^{-1}$, only ~2.8× less than its catalytic efficiency against the AHL counterpart *N*-hexanoyl L-homoserine lactone (C6-HSL) at $1.4 \times 10^6\,M^{-1}\,s^{-1}$ (Momb et al. 2008). AiiA prefers longer chain HTLs, where the catalytic efficiency of hydrolysis of C8-HTL is ~5.2× higher than that of C4-HTL ($9.6 \times 10^4\,M^{-1}\,s^{-1}$), which of itself is ~12.5× more efficient than hydrolysis of the C2-HTL ($7.7 \times 10^3\,M^{-1}\,s^{-1}$). Similarly, the lactonase JydB also prefers AHL substrates over HTL, but to a much smaller degree than the other tested lactonases: its catalytic efficiency is only four times higher against C4-HSL than C4-HTL (Table 2). Interestingly, JydB prefers shorter chains for both AHL and HTL substrates (Tables 1 and 2). Both GcL and AaL show preferences for longer HTL substrates ( ~fourfold for AaL; ~eightfold for GcL in favor of C8-HTL relative to C4-HTL), consistent with their slight preference for longer AHLs: GcL has a ~ fivefold preference for C8-HSL over C4-HSL (Bergonzi et al. 2019). The tested SsoPox mutant (W263I) shows no measurable activity against shorter HTL substrates and very low activity with C8-HSL. This behavior is consistent with the preference of SsoPox W263I for longer AHL substrates. Indeed, it is ~2200-times more active against 3OC12-HSL than C4-HSL (Billot et al. 2022; Hiblot et al. 2013) (Table 2).

Overall, while being generally less active with HTLs than with AHLs, the tested lactonases are capable of degrading HTLs to varying degrees and, importantly, show similar acyl chain length substrate preferences as with AHLs. These results suggest that this system could potentially be extended to more enzymes, including other lactonases such as zearalenone hydrolase (M. Wang et al. 2024).

### 3.2 | Utilizing *N*-Acyl L-Homocysteine Thiolactones as a Chromogenic Substrate for Medium-Throughput Screening

The finding that a broad range of lactonases can hydrolyze HTLs provides the opportunity to adapt the thiolactonase assay for medium-throughput screening. Specifically, we used bacterial lysates expressing GcL lactonase and established conditions to measure thiolactonase activity as a proxy for AHL lactonase activity in crude lysates. The assay has a satisfactory dynamic range, showing a high signal with lactonase-containing lysates and very little background with control lysates expressing an inactive form of the lactonase SsoPox (variant 5A8 [Hiblot et al. 2013]) (Figure 2F) or the empty expression pET22b(+) vector (Supporting Information S1: Figure S7).

This makes the HTL assay suitable for medium-throughput screening in 96-well plates with cell lysates. We chose to demonstrate the utility of this system by using it to screen site saturation libraries of the lactonase GcL to identify amino acid substitutions that influence substrate preference. Residues that line the binding cleft of GcL were previously identified by structural analysis of the crystal structures of GcL in apo form and bound to AHLs (Bergonzi et al. 2019). The active site of GcL was divided into three regions: residues that interact with the lactone ring moiety (M22, F48, L121, and Y223); residues that form a hydrophobic patch interacting with the acyl chain of AHL substrates (W26, M86, F87, G156, A157, I237) and residues located at the outer edge of the active site (S82, T83, E155) (Figure 3). The design approach and all the identified mutants, including their complete structural characterization will be described in detail elsewhere. Here, we describe the new screening procedure that can be applied to a wide range of AHL lactonases. Specifically, we describe the screening of the site saturation libraries for residues W26, M86, F87, and G156, and how we assessed their activities against C2-, C4-, and C8-HTL (Figures 4 and 5).

### 3.3 | Site Saturation of Bulky Aromatic Residues Results in Reduced Catalytic Activity and Variants With Increased Preference for Longer-Chain Substrates

The GcL crystal structures reveal that F87 interacts very closely with the acyl chain (carbon atom C2), while W26 interacts with atoms C4 and C8 of the acyl chain (Bergonzi et al. 2019) (Figure 3). The screening of the W26 saturation library shows dramatic loss of activity against all tested substrates for most variants, showing that this position is not permissive to substitutions (Figure 4). This is not surprising because in addition to its substrate binding role, W26 is also a core, hydrophobic residue and mutation of such residues can be highly destabilizing (Tokuriki et al. 2007). Moreover, this residue is also located near the enzyme dimerization interface. Conservative substitutions, such as those to large hydrophobic residues like W26F, W26I, and W26V, retained similar activity levels than WT. Few of the substitutions to W26 resulted in meaningful shifts in substrate preference. W26K and W26R each showed, respectively, a ~ 9-fold and ~2.5-fold shift in favor of short-chain HTLs (C2- and C4-HTL, respectively) vs. C8-HTL (Figure 4). A similar, albeit lower shift is observed for polar residues, such as W26N and W26S. This suggests that shorter-chain HTLs are preferred when residues at position W26 are hydrophilic, consistent with the proposed role of this residue in binding the aliphatic acyl chain of the substrate in the WT enzyme.

Substitution of residue F87 resulted in a number of variants with greatly decreased activity, particularly the substitutions for charged and polar residues such as F87D, E, N, Q, & R (Figure 4). Interestingly, numerous mutants showed a large reduction in their activity towards short-chain HTLs. Indeed, 10 out of 15 variants showed a > 2.5-fold shift in favor of C8-HTL over C2 and C4-HTL. This suggests that F87 plays a more crucial role in the accommodation of short-chain HTLs than long-chain HTLs, or that mutating this position creates an unfavorable environment for longer-chain substrates. There are two notable exceptions to this pattern, the bulky aromatic mutants F87W and F87Y. Both substitutions shift the enzyme preference in favor of C2 and C4 HTL by more than 2.5-fold. Given the position of the residue deep within the binding cleft, substitution from phenylalanine to

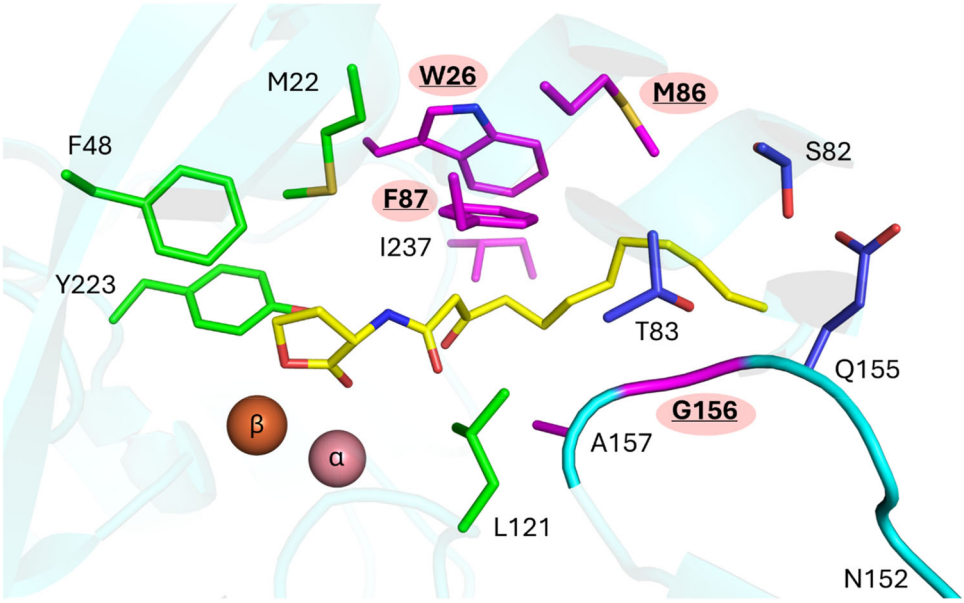


**FIGURE 3** | Substrate binding cleft of the GcL lactonase enzyme. Structure of GcL (PDB: 6N9R) bound to 3OC12-HSL (yellow sticks) showing the active site metals (spheres; α: cobalt cation, β: iron cation) and the side chains of the amino acid residues lining the substrate binding cleft (sticks). Residues in the vicinity of the lactone ring are shown in green; residues interacting with the acyl chain are in magenta, and residues lining the cleft exposed to the solvent are in indigo. Residues mutated in this study are bolded, underlined, and highlighted in red circles. The flexible loop section between N152 and A157 that carries G156 is highlighted as an opaque cyan cartoon. The surrounding protein environment is shown as transparent cyan cartoon.

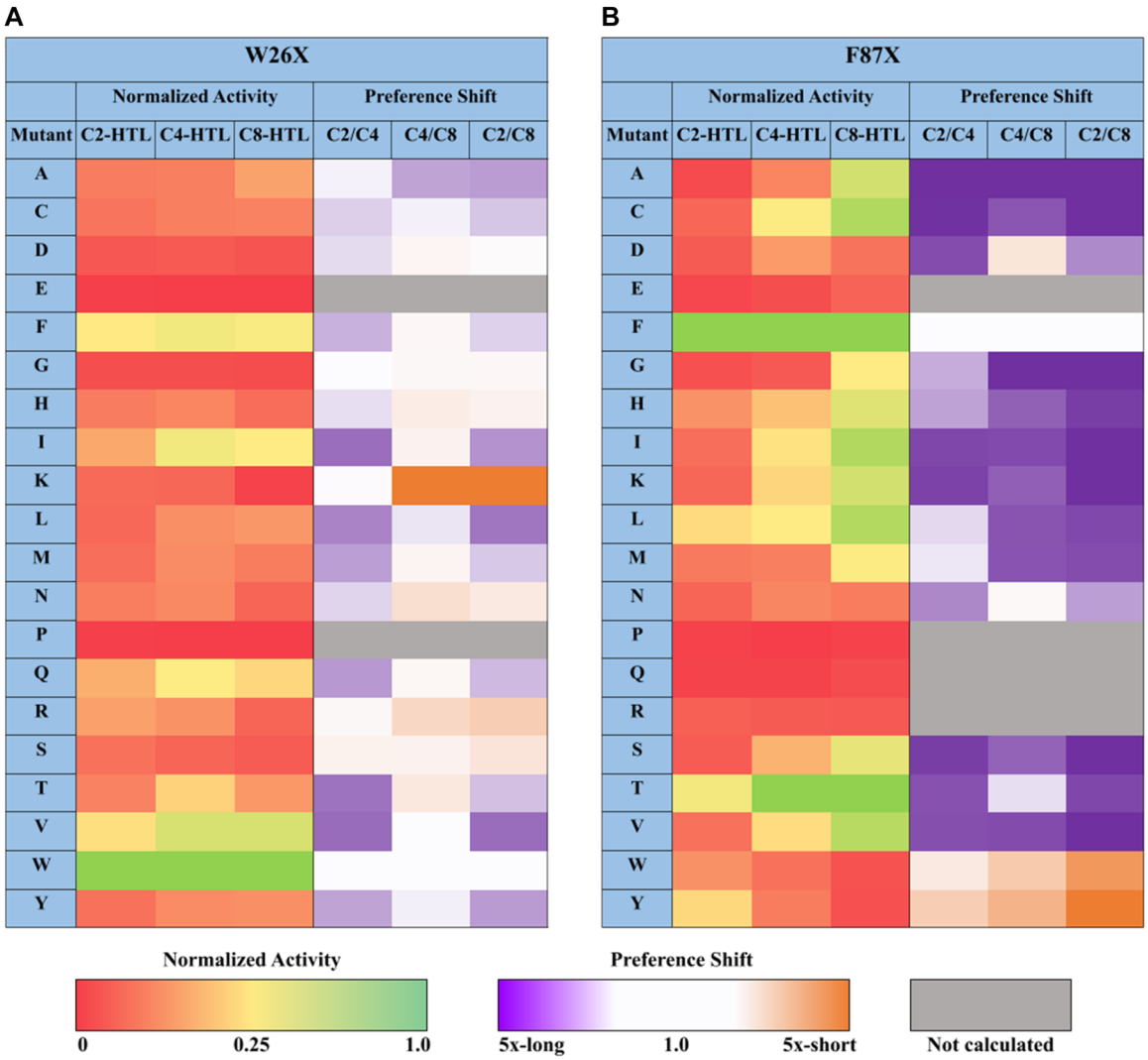


**FIGURE 4** | Heat maps showing normalized activity and substrate preference of GcL W26 (A) & F87 (B) saturation libraries. The color gradient of red–yellow–green reflects the normalized activity of each variant relative to the WT, with red representing the lowest activity and green representing WT-like activity. Each row shows a different variant and each column represents a HTL substrate with a different length acyl-chain. The color gradient of purple–white–orange shows changes in substrate preference relative to WT by calculating activity ratios between the different substrates. Purple indicates a shift in favor of longer-chain HTLs, orange indicates a shift in favor of shorter-chain HTLs, and white shows WT-like substrate preference. Variants with extremely low activity (< 5% relative to WT) were omitted from substrate preference calculations and are shown in gray.

tryptophan or to tyrosine may sterically occlude longer-chain HTLs from the active site.

### 3.4 | Residues on the Outer Part of the Hydrophobic Patch Are Permissive to Substitutions

The residues M86 and G156 are located at the edge of the hydrophobic patch in the GcL active site, before the active site residues become surface exposed to the surface (Figure 3). In the structure of GcL in complex with 3OC12-HSL, the two residues sit opposite each other, on either side of the substrate acyl chain. Substitution of M86 could disrupt interactions with the substrate acyl chain and alter the hydrophobic character of the binding cleft. The α-carbon of G156 also interacts closely with the acyl chain. Substitution of this residue may generate new interactions or sterically block access to the active site.

Screening results show that both M86 and G156 are somewhat tolerant to substitution. Four M86 substitutions and 11 G156 substitutions maintained over 25% activity relative to WT for all substrates, and only one variant in each library had low activity (< 5%) for all substrates (Figure 5). This likely reflects the relatively weak interactions between these residues and the HTL chain. Most M86 substitutions showed a slight, < 1.5-fold increase in preference for short-chain HTL over long chain. Stronger effects (> 2.5-fold) are seen in the polar/charged variants M86D, E, N, and Q. This suggests that disruption of the hydrophobic nature of the binding cleft may favor short-chain HTLs over long chains, as seen for W26.

Conversely, most variants of the G156 library showed a slight increase in preference for long-chain HTLs. This is potentially due to the creation of new interactions with the acyl chain at this position. Despite this pattern, few mutants of G156 showed

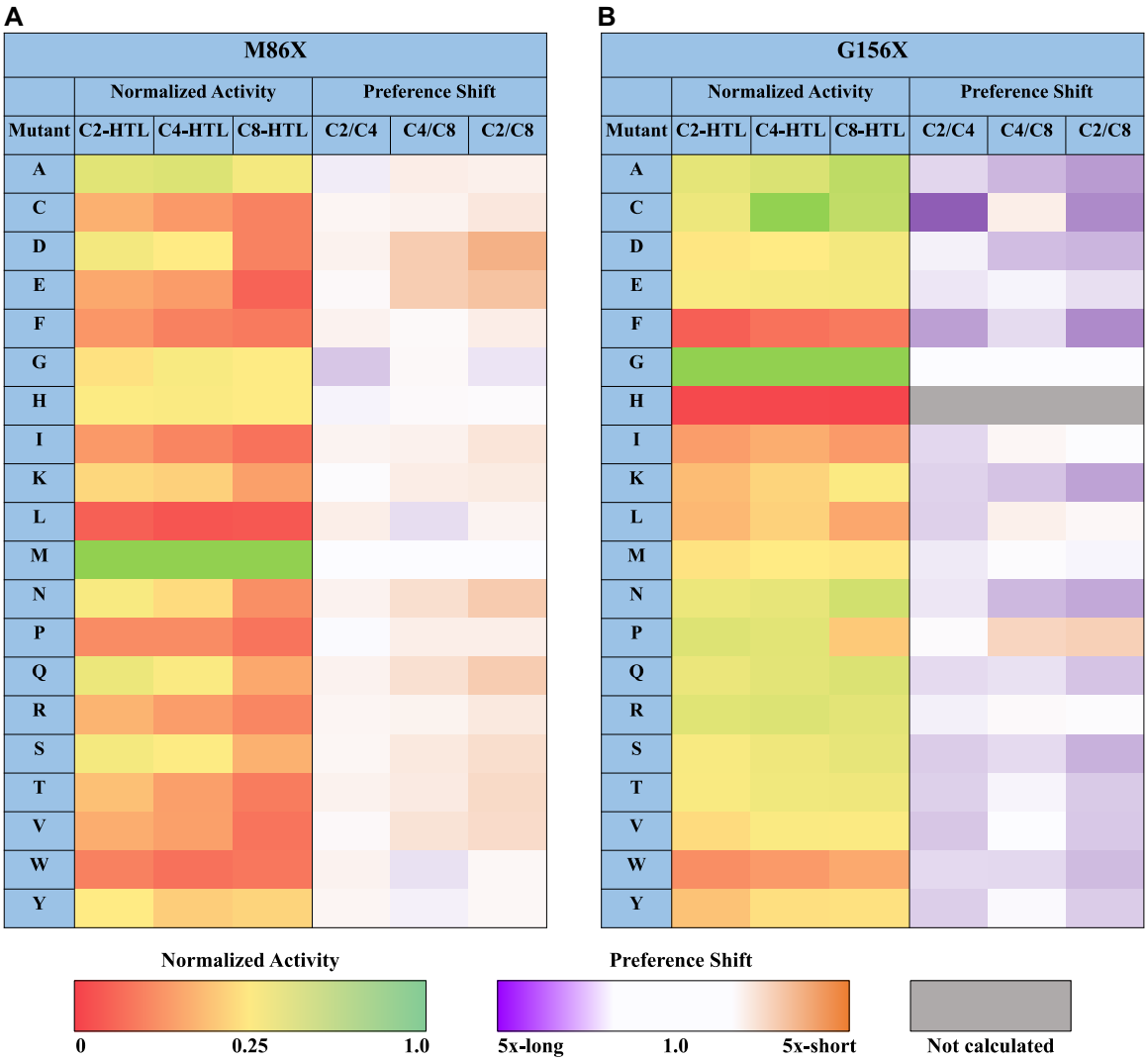


**FIGURE 5** | Heat maps representing changes in normalized activity level and substrate preferences for GcL M86 (A) and GcL G156 saturation libraries (B). The red-green scale represents activity relative to the WT (green) while the purple-orange scale represents preference changes for long (purple) or short (orange) HTL substrates based on activity ratios between substrates. Gray boxes denote values not calculated due to low activity (< 5% of the WT).

remarkable shifts in substrate preference. Because G156 lies on a flexible loop, from N152 to A157 (Figure 3) toward the exterior of the binding cleft, it may be a key residue that modulates the conformation of this loop, which we observed in the recently solved structure of G156P that revealed an altered loop conformation (Corbella et al. 2024). The variant G156P showed a roughly twofold increase in preference for C2- and C4-HTL over C8-HTL.

### 3.5 | Thiolactonase Activity Levels Correlate With Homoserine Lactonase Activity Levels

Screening of the GcL active site mutational libraries produced at least one key variant from each library that had substantially altered substrate specificity, preferring shorter chain HTL substrates. We further characterized these variants with appreciable specificity shifts (W26K, M86D, F87W, G156P) for kinetic determination against HTL (Table 3) and AHL (Table 4) substrates. As described above (Table 1), the catalytic efficiencies against the HTL substrates were about 10-fold less than those for AHL substrates and were improved for short-chain HTLs compared to the WT GcL (Tables 3 and 4). This establishes a good agreement between the results from the screening using HTL substrates and the kinetic parameters measured against AHL substrates using purified protein.

The variants identified here show substantially increased preference for short-chain substrates, as exemplified by their activity ratios (ratio value greater than 1; Tables 3 and 4). The largest change was seen in the M86D variant: its catalytic efficiency was increased by 2.6- and 2.5-fold against C2 and C4-HTL and decreased by 4.7-fold against C8-HTL (Table 3). Consequently, the M86D variant shows a 1.95-fold preference for C2-HTL over C8-HTL; a ~12-fold specificity change compared to the WT GcL. The next best variant, W26K, has a 1.78-fold preference for C2-HTL over C8-HTL, representing an ~11-fold change compared to WT GcL. The changes in preference for C4-HTL compared to C8-HTL were similar. The largest improvement seen was in M86D, which prefers C4-HTL over

**TABLE 3** | Kinetic parameters of GcL WT and select variants against homocysteine thiolactones.

| Enzyme | HTL | $k_{cat}$ ($s^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) | Fold change | C2/C4 | C4/C8 | C2/C8 |
|---|---|---|---|---|---|---|---|---|
| GcL WT | C2 | nd | nd | $(1.66 \pm 0.02) \times 10^2$ | — | 1.37 | 0.12 | 0.16 |
| | C4 | nd | nd | $(1.21 \pm 0.02) \times 10^2$ | — | | | |
| | C8 | $1.82 \pm 0.04$ | $1777.00 \pm 112.90$ | $(1.03 \pm 0.07) \times 10^3$ | — | | | |
| W26K | C2 | nd | nd | $(3.71 \pm 0.04) \times 10^2$ | 2.2↑ | 1.41 | 1.26 | 1.78 |
| | C4 | nd | nd | $(2.63 \pm 0.06) \times 10^2$ | 2.2↑ | | | |
| | C8 | nd | nd | $(2.08 \pm 0.00) \times 10^2$ | 5.0↓ | | | |
| M86D | C2 | nd | nd | $(4.25 \pm 0.10) \times 10^2$ | 2.6↑ | 1.40 | 1.39 | 1.95 |
| | C4 | nd | nd | $(3.03 \pm 0.12) \times 10^2$ | 2.5↑ | | | |
| | C8 | nd | nd | $(2.18 \pm 0.09) \times 10^2$ | 4.7↓ | | | |
| G156P | C2 | nd | nd | $(6.49 \pm 0.10) \times 10^2$ | 3.9↑ | 1.14 | 1.00 | 1.14 |
| | C4 | nd | nd | $(5.68 \pm 0.07) \times 10^2$ | 3.2↑ | | | |
| | C8 | nd | nd | $(5.70 \pm 0.01) \times 10^2$ | 1.6↓ | | | |
| F87Y | C2 | nd | nd | $(5.77 \pm 0.27) \times 10^2$ | 3.5↑ | 1.49 | 0.59 | 0.88 |
| | C4 | nd | nd | $(3.87 \pm 0.04) \times 10^2$ | 3.2↑ | | | |
| | C8 | $0.70 \pm 0.06$ | $1072.00 \pm 118.60$ | $(6.57 \pm 0.90) \times 10^2$ | 1.6↓ | | | |

*Note:* Rate determination curves can be found in Figures S8–S11.
Abbreviations: C2, acetyl-D/L-homocysteine thiolactone; C4, butyryl L-homocysteine thiolactone; C8, octanoyl D/L-homocysteine thiolactone; nd, not determined; saturation could not be reached.

C8-HTL by 1.39-times, an 11.6-fold specificity shift compared to the WT GcL.

The W26K, M86D, G156P, and F87W variants were further characterized against AHL substrates that are natively used by different bacteria and their activities compared to the GcL WT (Table 4). Consistent with the screening results, the variants are generally less active than WT GcL against AHLs. Importantly, the comparison of the kinetic parameters against HTLs and AHLs reveal that the substrate preference shifts of the variants generally translated from the HTLs to AHLs. For most variants with specificity changes, their increased preference for shorter-chain AHL substrates is due to a substantially greater reduction in their catalytic efficiencies against longer-chain substrates such as C8-, C10-, and 3OC12-HSL (~190–1900×), as compared to the shorter chain substrates (C4-HSL, ~3–50× fold range; Table 4).

All of the variants identified from the screening show higher preferences for C4-HSL than WT GcL. Variant G156P only exhibits a 1.5× improvement in C4/3OC12-HSL activity ratio compared to the WT, but other GcL variants showed improvements to the C4/3OC12-HSL activity ratios by over 10-fold. For example, W26K shows a 26.5-fold change in the C4/3OC12-HSL activity ratio. Similarly, the catalytic efficiency ratio values for C4/C10-HSL were also largely altered (~19–28-fold, as compared to WT, except for variant G156P). The biggest improvements were observed for the C4/C8-HSL ratio values: though G156P only had a modest increase, the remaining variants all now hydrolyze C4-HSL more efficiently than C8-HSL. This translates to a ~35–38-fold shift in substrate preference, as compared to the WT.

These changes in substrate preference are significant in light of the few previous engineering efforts for these enzymes. A significant engineering effort on the MLL AiiA yielded single amino acid substitution variants with ~1.03–14.7-fold increases in C4-HSL preference over C10-HSL, while our study, using the new HTL-based screening system, generated single amino acid substation variants with ~1.5–34.8-fold increases in this ratio (Supporting Information S1: Table S1). The previous study was able to generate a triple mutant variant (AiiA F64W/V69W/A206F [Kyeong, Kim, and Kim 2015]) with 101-fold change in preference for C4/C10-HSL (Supporting Information S1: Table S1), suggesting that a similar combinatorial mutagenesis strategy may also be effective for increasing C4-HSL preference of GcL.

Overall, this data shows that the results of the HTL screen are broadly transferable to AHL substrates and illustrate the utility of this system for identifying lactonase variants with improved/altered kinetic properties.

## 4 | Conclusion

The molecular engineering of quorum quenching enzymes such as lactonases is complicated by a lack of enzymatic assays that are applicable for screening enzyme libraries. Here, we describe the use of *N*-acyl L-homocysteine thiolactones as a proxy for *N*-acyl L-homoserine lactones and their use in screening cell lysates of engineered lactonase libraries for increased lactonase activity. With a rapid colorimetric assay that has low background noise, amino acid substitution libraries can be screened quickly and effectively to identify variants with altered kinetic properties such as substrate specificity. Applying this system to the lactonase GcL, variants with altered substrate specificities were identified during screening with HTLs substrates and the determination of their kinetic parameters against HTLs and

**TABLE 4** | Kinetic parameters of GcL WT and select variants against *N*-acyl L-homoserine lactones.

| Enzyme | HSL | $k_{cat}$ ($s^{-1}$) | Fold Δ | $K_M$ (μM) | Fold Δ | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) | Fold Δ | C4/C8 | C4/C10 | C4/3OC12 |
|---|---|---|---|---|---|---|---|---|---|---|
| GcL WT | C4 | $19.5 \pm 2.6$ | — | $581 \pm 157$ | — | $(3.4 \pm 1.0) \times 10^4$ | — | 0.04 | 0.03 | 0.02 |
| | C8 | $2.60 \pm 0.10$ | — | $2.93 \pm 0.77$ | — | $(8.9 \pm 2.4) \times 10^5$ | — | | | |
| | C10 | $1.85 \pm 0.10$ | — | $1.41 \pm 0.40$ | — | $(1.3 \pm 0.4) \times 10^6$ | — | | | |
| | 3OC12 | $1.45 \pm 0.10$ | — | $0.85 \pm 0.24$ | — | $(1.7 \pm 0.5) \times 10^6$ | — | | | |
| W26K | C4 | nd | — | nd | — | $6.4\ (\pm 0.3) \times 10^2$ | 53↓ | 1.39 | 0.58 | 0.53 |
| | C8 | nd | — | nd | — | $4.6\ (\pm 0.3) \times 10^2$ | 1935↓ | | | |
| | C10 | $0.09 \pm 0.01$ | 21↓ | $83.8 \pm 13.3$ | 60↑ | $1.1\ (\pm 0.2) \times 10^3$ | 1182↓ | | | |
| | 3OC12 | $0.20 \pm 0.01$ | 7.3↓ | $172 \pm 48$ | 202↑ | $1.2\ (\pm 0.3) \times 10^3$ | 1417↓ | | | |
| M86D | C4 | nd | — | nd | — | $(1.5 \pm 0.1) \times 10^3$ | 23↓ | 1.52 | 0.58 | 0.20 |
| | C8 | nd | — | nd | — | $(9.9 \pm 0.4) \times 10^2$ | 899↓ | | | |
| | C10 | $0.57 \pm 0.05$ | 3.2↓ | $218 \pm 68$ | 155↑ | $(2.6 \pm 0.8) \times 10^3$ | 500↓ | | | |
| | 3OC12 | $1.37 \pm 0.12$ | 1.1↓ | $186 \pm 74$ | 219↑ | $(7.4 \pm 3.0) \times 10^3$ | 230↓ | | | |
| G156P | C4[a] | $8.72 \pm 0.69$ | 2.2↓ | $771 \pm 140$ | 1.3↑ | $(1.1 \pm 0.2) \times 10^4$ | 3↓ | 0.06 | 0.02 | 0.04 |
| | C8[a] | $1.44 \pm 0.09$ | 1.8↓ | $8.22 \pm 2.92$ | 2.8↑ | $(1.8 \pm 0.6) \times 10^5$ | 5↓ | | | |
| | C10[a] | $0.73 \pm 0.02$ | 2.5↓ | $1.31 \pm 0.34$ | 1.1↓ | $(5.6 \pm 1.5) \times 10^5$ | 2↓ | | | |
| | 3OC12 | $0.70 \pm 0.02$ | 2.1↓ | $2.26 \pm 0.55$ | 2.7↑ | $(3.1 \pm 0.8) \times 10^5$ | 6↓ | | | |
| F87Y | C4 | nd | — | nd | — | $(2.4 \pm 0.1) \times 10^3$ | 14↓ | 1.50 | 0.83 | 0.27 |
| | C8 | nd | — | nd | — | $(1.6 \pm 0.1) \times 10^3$ | 556↓ | | | |
| | C10 | $0.23 \pm 0.01$ | 8.0↓ | $78.4 \pm 8.8$ | 56↑ | $(2.9 \pm 0.3) \times 10^3$ | 448↓ | | | |
| | 3OC12 | $2.2 \pm 0.12$ | 1.5↑ | $246 \pm 39$ | 289↑ | $(8.9 \pm 1.5) \times 10^3$ | 191↓ | | | |

*Note:* Rate determination curves can be found in Supporting Information S1: Figures S5, S12–S15.
Abbreviations: 3OC6, 3-oxo-hexanoyl l-homoserine lactone; C4, *N*-butyryl L-homoserine lactone; C6, *N*-hexanoyl L-homoserine lactone; C8, *N*-octanoyl L-homoserine lactone; C10, *N*-decanoyl L-homoserine lactone; nd, not determined due to limitations in substrate solubility.
[a]data from Corbella et al. (2024).

AHLs shows that the screening results mostly translated to corresponding changes in preferences for AHLs. Analysis of the change in activity of some of the best mutants suggests that the hydrophobic nature of the binding cleft is key for the accommodation of long acyl chain substrates and will inform the future design and engineering of lactonase active sites. We also show that many lactonase representatives are capable of hydrolyzing HTLs, demonstrating the versatility of this system and the possibility of its wide range application to AHL lactonases at large.

## Author Contributions

**Kitty Sompiyachoke:** data curation, formal analysis, investigation, visualization, original draft preparation, writing–review and editing. **Joseph Bravo:** formal analysis, methodology, investigation, visualization, writing–original draft preparation, writing–review and editing. **Rakesh Sikdar:** formal analysis, investigation, visualization, writing–review and editing. **Jowan Abdullah:** investigation, writing–review and editing. **Mikael H. Elias:** conceptualization, methodology, writing–original draft preparation, writing–review and editing, supervision, project administration, funding acquisition.

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## Conflicts of Interest

M.H.E. is an inventor of patents WO2014167140A1, WO2015014971A1, WO2020185861A4, WO2020187861A1, FR3093894B1, FR3132715A1. M.H.E. is a cofounder, a former CEO and an equity holder of Gene & Green TK, a company that holds the license to WO2014167140A1 and contributed to WO2020187861A1, FR3093894B1, FR3132715A1. These interests have been reviewed and managed by the University of Minnesota in accordance with its Conflict-of-Interest policies. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data Availability Statement

Data for this study were deposited to the Open Science Framework: https://osf.io/h62rz/?view_only=dc9850ded25648ba87a63ad694e8df72.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.