BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 2551–2562 2551
DOI: 10.1021/bi9020764

Parallel Multiplicative Target Screening against Divergent Bacterial Replicases: Identification of Specific Inhibitors with Broad Spectrum Potential[†]

H. Garry Dallmann,[‡] Oliver J. Fackelmayer,[‡] Guy Tomer,[‡] Joe Chen,[‡] Anna Wiktor-Becker,[‡] Tracey Ferrara,[‡] Casey Pope,[‡] Marcos T. Oliveira,[∥] Peter M. J. Burgers,[§] Laurie S. Kaguni,[∥] and Charles S. McHenry*[,‡]

[‡]*Department of Chemistry and Biochemistry, University of Colorado, Campus Box 215, Boulder, Colorado 80309,* [§]*Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110, and* [∥]*Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824-1319*

Received December 3, 2009; Revised Manuscript Received February 8, 2010

ABSTRACT: Typically, biochemical screens that employ pure macromolecular components focus on single targets or a small number of interacting components. Researches rely on whole cell screens for more complex systems. Bacterial DNA replicases contain multiple subunits that change interactions with each stage of a complex reaction. Thus, the actual number of targets is a multiple of the proteins involved. It is estimated that the overall replication reaction includes up to 100 essential targets, many suitable for discovery of antibacterial inhibitors. We have developed an assay, using purified protein components, in which inhibitors of any of the essential targets can be detected through a common readout. Use of purified components allows each protein to be set within the linear range where the readout is proportional to the extent of inhibition of the target. By performing assays against replicases from model Gram-negative and Gram-positive bacteria in parallel, we show that it is possible to distinguish compounds that inhibit only a single bacterial replicase from those that exhibit broad spectrum potential.

Typically, in vitro high-throughput screening (HTS)[1] assays target single proteins or protein pairs. This approach has enabled significant success. To exploit all of the targets available in complex pathways or molecular machines, researchers often resort to cellular screens, to ensure the availability of all relevant targets. Using these approaches, novel targets have been revealed that have led to the discovery of new interactions, validating the power of forward chemical genetics (*1*). However, cellular screens have the drawback of missing compounds that cannot achieve suitable intracellular concentrations because of low permeability, unfavorable metabolism, or efflux. These issues could be overcome if all of the machinery involved in a complex process could be reconstituted in vitro, enabling a biochemical screen. This approach would permit identification of inhibitors that could subsequently be optimized for potency and permeability in parallel with other favorable pharmacological properties.

DNA replication is an essential process for the proliferation of all pathogens and offers a largely unexplored target for the development of novel antibacterials. Therapeutically useful inhibitors have been developed that inhibit processes upstream (nucleotide precursor biosynthesis) (*2*) and downstream (DNA gyrase) (*3*) of DNA replication. Most of the subunits of the bacterial DNA replication apparatus are essential, suggesting that their inhibition should lead to blockage of cell proliferation or death (*4*). This has been validated by a class of compounds, 6-anilinouracils, targeted to the polymerase subunit of the Gram-positive replicase, Pol C. These compounds not only are potent biochemical inhibitors but also specifically block DNA replication in Gram-positive bacteria (*5*). While screens targeting individual replicase subunits have been described (*6*−*9*), complete bacterial replicases have not been explored by chemical genetic approaches.

Cellular replicases are tripartite multiprotein assemblies (for reviews, see refs (*10*−*13*)). They contain specific DNA polymerases that function as the catalytic elongation component and that derive enormous processivity from interaction with a braceletlike "sliding clamp" processivity factor that encircles DNA, tethering the replicative polymerase to it. The sliding clamp is loaded onto DNA by an ATP-powered "clamp loader". The clamp loader serves additional roles, including communication with the replicative helicases and linking the leading and lagging strand polymerases, at least in *Escherichia coli* (*14*, *15*).

The 10 subunits of the *E. coli* DNA Pol III holoenzyme interact to form a remarkably complex protein machine (*10*, *14*, *16*−*18*). Protein interactions change at the various steps of the replicative reaction. Counting all of the individual protein components and their interactions with other subunits and substrates, we estimate upward of 100 essential targets that are potentially useful for development of antibacterial agents (see Results and Discussion for enumeration). Given the impracticality of running 100 specific screening assays and preferring to avoid the problems inherent in whole cell screens, we established a biochemical HTS in which inhibition of any of the essential targets could be detected through a common end point. This would permit screening for all targets within the replicase in a single well of a microtiter plate, a goal never achieved for such a complex target in a biochemical screen using purified

[†]This work was supported by National Institutes of Health Grants AI064382 and AI076287.

*To whom correspondence should be addressed. E-mail: Charles.McHenry@colorado.edu. Telephone: (303) 735-0071. Fax: (303) 735-1347.

[1]Abbreviations: HTS, high-throughput screening; SSB, single-stranded DNA binding protein; Pol, polymerase; Pol III*, DNA polymerase III* (a subcomplex of all subunits of the *Escherichia coli* Pol III holoenzyme except β_2); MIC, minimal inhibitory concentration; MMS, macromolecular synthesis; IDD, intercalator dye displacement; SAR, structure−activity relationship.

Published on Web 02/25/2010

components. Using conversion of single-stranded DNA binding protein (SSB)-coated single-stranded DNA to a duplex, detected by binding of the fluorescent dye, PicoGreen, we developed robust screens for model Gram-negative and Gram-positive bacterial replicases. By screening a small trial 20000-compound library against these related targets in parallel, we were able to distinguish compounds that inhibited the replicase of a single species from those compounds that exhibited broad spectrum potential. Counterscreens against nonorthologous enzymes with related activities revealed those compounds that are most likely to be target-specific.

MATERIALS AND METHODS

Chemicals and Reagents. Screening compounds were purchased from TimTec (Newark, DE) (10000 compounds, Diversity Set collection) and Chembridge (San Diego, CA) (10000 compounds, DIVERset collection). All nucleotide triphosphates, PicoGreen double-stranded (ds) DNA detection reagent, and fluorescein digalactoside were purchased from Invitrogen (Carlsbad, CA). T4 and T7 DNA polymerases and apyrase were purchased from New England Biolabs (Ipswich, MA). The *E. coli* RNA polymerase core ($\alpha\beta\beta'\omega$) was purchased from Epicenter Biotechnologies (Madison, WI). β-Galactosidase was purchased from Worthington Biochemical Corp. (Lakewood, NJ).

Buffers. Buffer Q consists of 50 mM HEPES (pH 7.0), 20 mM NaCl, 10% glycerol, 5 mM DTT, and 1 mM EDTA. Buffer Q2 consists of 40 mM HEPES (pH 7.5), 50 mM NaCl, 10% glycerol, 0.5 mM DTT, and 0.5 mM EDTA. Buffer B consists of 50 mM Tris-HCl (pH 7.5), 10% glycerol, 1.0 M ammonium sulfate, 0.5 mM EDTA, and 5 mM DTT. Buffer QS consists of 50 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM DTT, 0.1 M NaCl, and 0.5 mM EDTA. Buffer HA consists of 25 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM DTT, 150 mM NaCl, and 0.5 mM EDTA. Buffer S consists of 25 mM HEPES (pH 7.5), 10% glycerol, 0.2 M NaCl, 0.5 mM EDTA, and 5 mM DTT. Buffer QE consists of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 5 mM DTT, and 0.5 mM EDTA. Buffer H consists of 50 mM imidazole (pH 7.0), 10% glycerol, and 5 mM DTT. Buffer S consists of 50 mM Tris-HCl (pH 7.5), 10% glycerol, 10 mM NaCl, 0.1 mM EDTA, and 5 mM DTT. Buffer I consists of 50 mM imidazole (pH 6.0), 20% glycerol, 20 mM NaCl, and 5 mM DTT. Buffer H2 consists of 50 mM Tris-HCl (pH 7.5), 400 mM NaCl, 20% glycerol, 0.5 mM EDTA, and 0.25 mM DTT.

Cloning and Expression of Bacillus subtilis DNA Replication Proteins. All *B. subtilis* DNA Pol III holoenzyme subunit genes were amplified by PCR of the relevant coding region from *B. subtilis* genomic DNA (ATCC) and the resulting amplified segments inserted into expression plasmid pA1-CB (*19*, *20*) immediately behind the IPTG-inducible $P_{A1/04/03}$ promoter/operator using appropriate restriction enzymes. This resulted in the construction of expression plasmids pA1-BS-polC, pA1-BS-dnaX, pA1-BS-holBA, pA1-BS-holB, pA1-BS-dnaN, and pA1-BS-ssb1, expressing *B. subtilis* DNA Pol III subunits PolC, τ, δ, δ', β, and SSB, respectively. All individual *B. subtilis* replicase subunits were heterologously expressed as native sequences, without affinity tags in *E. coli* strain DH5-α. Due to extremely low levels of expression of the *B. subtilis* δ subunit in an initial expression construct containing only the *holA* gene, it was cloned into the polylinker region immediately downstream of the *holB* gene in pA1-BS-holB (resulting in expression plasmid pA1-BS-holBA). The level of expression of δ (*holA*) was significantly increased to levels above that of δ' (*holB*), which allowed purification of the single isolated δ subunit.

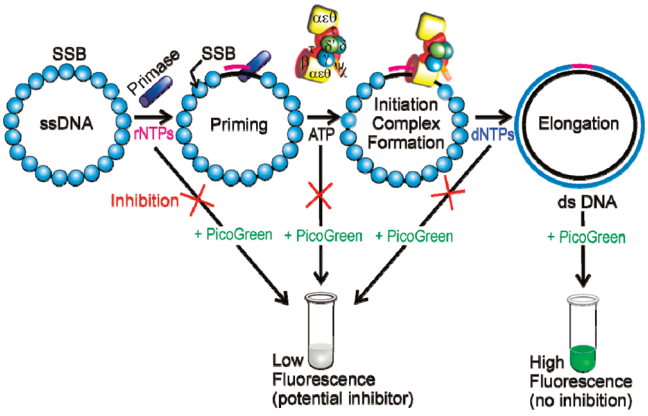


FIGURE 1: Homogenous, PicoGreen fluorescence-based assay for high-throughput screening of bacterial replicases. In all replicase screening assays, the conversion of single-stranded (ss) phage DNA to the duplex form was monitored by an increase in fluorescence upon binding the ds DNA-specific dye, PicoGreen. This permitted detection of inhibitors that act at any of the reaction steps, including priming on the SSB−ss DNA nucleoprotein filament, ATP-dependent initiation complex formation by the Pol III holoenzyme, and subsequent elongation in the presence of dNTPs. Assays of the *B. subtilis* replicase were similar, except a primer was provided by annealing of a synthetic oligonucleotide.

Cell lysates were prepared by procedures identical to those used for *E. coli* replicase component purifications. For all purifications, enzyme activity was monitored using DNA Pol III holoenzyme reconstitution assays measuring the incorporation of [^{3}H]TTP into DNA synthesized on either $M13_{Gori}$ templates (dnaG primase-dependent *E. coli* system) or oligonucleotide-primed $M13_{Gori}$ templates (*B. subtilis* system), in a filter binding assay essentially as outlined in Figure 1 and ref *21*.

Purification of Bacterial DNA Replication Proteins. *E. coli* Pol III*, β, SSB, and DnaG were expressed and purified using published methods (*22*−*24*).

B. subtilis PolC was expressed in *E. coli*, precipitated from a cell lysate with a 40−55% ammonium sulfate cut, and purified by chromatography over Q-Sepharose (buffer QE, gradient from 0.05 to 0.6 M NaCl), hydroxylapatite (buffer HE, gradient from 0.05 to 0.4 M potassium phosphate), BioRex70 (buffer I, gradient from 0.075 to 0.4 M NaCl), and Sephacryl S-300 gel filtration in buffer S. A total of 25 mg of purified PolC (specific activity of 1.0×10^7 units/mg) was obtained from 600 g of cells.

B. subtilis τ was precipitated from cell lysate with a 35−45% saturated ammonium sulfate cut and purified by chromatography over Q-Sepharose (buffer QE, gradient from 0.1 to 0.4 M NaCl), hydroxylapatite (buffer HE, gradient from 0 to 0.25 M potassium phosphate), and Sephacryl S-400 gel filtration in buffer S. A total of 160 mg was obtained from 280 g of cells (specific activity of 3.3×10^5 units/mg).

B. subtilis δ was precipitated from extracts with a 35−50% saturated ammonium sulfate cut and purified by chromatography over Q-Sepharose (buffer QS, gradient from 0.1 to 0.35 M NaCl), hydroxylapatite (buffer H, gradient from 0 to 0.2 M potassium phosphate), and Sephacryl S-100 gel filtration in buffer S. A total of 22 mg was obtained from 100 g of cells (specific activity of 1.6×10^7 units/mg).

B. subtilis δ' was precipitated from extracts with a 40−55% saturated ammonium sulfate cut and purified by chromatography over Q-Sepharose (buffer Q, gradient from 0.02 to

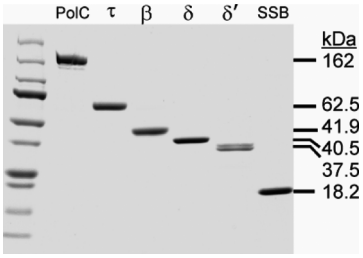


FIGURE 2: Purified *B. subtilis* DNA replication proteins. Purified *B. subtilis* minimal replicase components PolC, τ, β_2, δ, δ', and SSB were analyzed on a 4 to 20% gradient SDS−polyacrylamide gel. The first lane contained protein molecular mass standards. Other lanes contained 2 μg samples of each *B. subtilis* replicase protein as indicated. Molecular masses of each replicase protein are listed on the right.

0.18 M NaCl) and Butyl Sepharose (buffer B, gradient from 0.8 M ammonium sulfate and 10% glycerol to 0 M ammonium sulfate and 30% glycerol). A total of 44 mg was obtained from 400 g of cells (specific activity of 1.3×10^7 units/mg). Although two distinct bands are present in the purified preparation of δ' when it is analyzed by SDS−PAGE (Figure 2), both bands contain the δ' subunit. This doublet is also observed in purified *E. coli* δ' (*25*, *26*) and *Streptococcus pyogenes* δ' (*27*). The basis for this electrophoretic behavior of δ' is not known.

B. subtilis β was precipitated from extracts with a 65−75% saturated ammonium sulfate cut and purified by chromatography over Q-Sepharose (buffer Q2, gradient from 0.05 to 0.5 M NaCl) and heparin Sepharose (buffer Q2, gradient from 0.05 to 0.25 M NaCl). A total of 25 mg was obtained from 400 g of cells (specific activity of 8.0×10^7 units/mg).

B. subtilis SSB was precipitated from extracts with a 0 to 40% ammonium sulfate cut followed by backwashing the resulting pellet with 36% ammonium sulfate. The pellet was then resuspended in buffer QS and purified by chromatography over Blue Sepharose FF (*24*). This column was successively washed with 0.1, 0.4, 1, and 2 M NaCl in buffer QS before the final elution in 4 M NaCl. SSB was further purified by hydroxylapatite (buffer H2, gradient from 0 to 0.1 M potassium phosphate). A total of 110 mg was purified from 900 g of cells.

A polyacrylamide gel showing the final purified *B. subtilis* replicase subunits and SSB is shown in Figure 2.

High-Throughput Screening Assays. HTS assays of the *E. coli* replicase were performed using final protein concentrations and conditions listed in the legend of Figure 3 using Biomek FX liquid handling robotics (Beckman Coulter Ltd.) equipped with a 96-well pipettor head and a plate stacker unit. An enzyme mixture containing all of the protein components for the assay in a buffer was optimized for maximal enzyme stability and activity [50 mM HEPES (pH 7.5), 5% glycerol, 0.02% Pluronic F-68, 80 μM TCEP, 150 mM potassium glutamate, and 7 mM magnesium acetate]. During HTS, the enzyme mixture was kept on a chilled reservoir (4 °C), to ensure stability over a 6 h screening period. Substrate mix (nucleotides and $M13_{Gori}$ ss circular DNA template) and test compounds were kept at room temperature. The HTS assay was performed by the addition of 1.5 μL of compound (500 μM in DMSO, final concentration of 30 μM) into 18.5 μL of enzyme mix in black 384-well assay plates (Greiner Bio-One, Monroe, NC) and mixed by repeated aspiration. The compounds and proteins were incubated at room temperature for 10 min, after which 5 μL of substrate mix was added to initiate DNA synthesis (see the legend of Figure 3 for

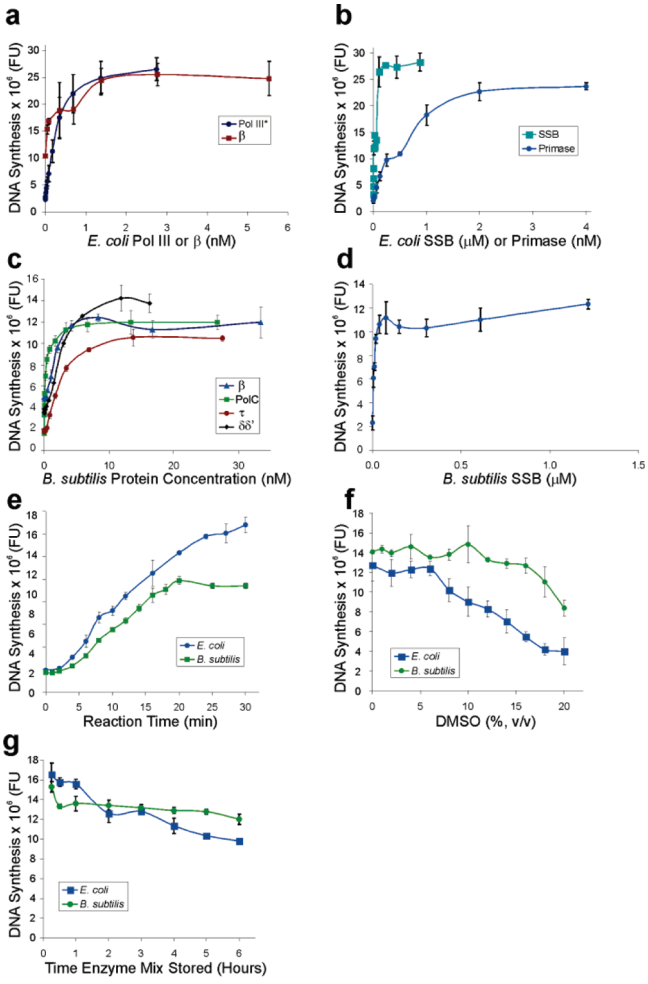


FIGURE 3: Optimization of *E. coli* and *B. subtilis* replicase high-throughput screening assays. (a) Titration of *E. coli* Pol III holoenzyme components in the presence of saturating levels of the other assay components. Screening assays (volume of 25 μL) were conducted using 0.35 nM Pol III* and 1.4 nM β_2. Substrate concentrations used in all *E. coli* HTS assays were as follows: 5 μM dATP, dGTP, dCTP, and dTTP, 200 μM CTP, GTP, and UTP, 50 μM ATP, and 40 pmol of ss $M13_{Gori}$ (as the nucleotide). (b) Titration of *E. coli* SSB and DnaG primase in the presence of saturating levels of the other *E. coli* assay components. Screening assays were conducted using 154 nM SSB_4 and 2 nM DnaG. (c) Titration of *B. subtilis* replicase components in the presence of saturating levels of the other assay components. Screening assays (volume of 25 μL) were conducted using 1.7 nM PolC, 2.8 nM β_2, 12 nM τ, and 6 nM δ'. Substrate concentrations used in all *B. subtilis* HTS assays were as follows: 7 μM dATP, dGTP, dCTP, and dTTP, 15 μM ATP, and 40 pmol of oligonucleotide-primed ss $M13_{Gori}$ (as the nucleotide). (d) Titration of *B. subtilis* SSB in the presence of saturating levels of the other assay components. Screening assays were conducted using 156 nM SSB_4. (e) Determination of time course of optimized, reconstituted replicase reactions. All assay components for each experiment were used at the concentrations specified for panels a−d, including 5% DMSO. All screening assays were performed for the indicated times before reactions were quenched with a PicoGreen dye/EDTA mixture as described in Materials and Methods. (f) Determination of the sensitivity of the HTS assay to DMSO. All assays were performed as specified for panels a−d. *E. coli* screening assays were conducted at a maximum of 8% (v/v) DMSO. *B. subtilis* screening assays were conducted at a maximum of 15% (v/v) DMSO. (g) Determination of the stability of assay components at room temperature (22 °C). An enzyme premix containing protein components (at concentrations specified for panels a−d) diluted in the optimized HTS assay buffer was mixed with 8% (*E. coli*) and 15% (*B. subtilis*) DMSO and incubated for the indicated times at room temperature before the addition of substrates. Reactions were conducted for 20 min before the addition of PicoGreen dye reagent and EDTA as described in Materials and Methods.

details). After incubation for 20 min at room temperature (22 ± 1 °C), 75 μL of PicoGreen dye reagent [containing 0.1 μL of reagent stock, as supplied by Invitrogen, in 10 mM Tris-HCl (pH 7.5) and 10 mM EDTA] was added to stop the reaction. Assay plate fluorescence intensity was read using an excitation wavelength of 485 nm and an emission wavelength of 535 nm on an EnVision plate reader (Perkin-Elmer, Waltham, MA). Compounds were not prescreened for fluorescence at 535 nm, and this could have caused a small number of false negative readings.

The *B. subtilis* HTS assay was performed essentially as described for *E. coli* except that the *B. subtilis* DnaG primase was not included in the HTS. Instead, a synthetic DNA primer (5′-AGGCTGGCTGACCTTCATCAAGAGTAATCT) was annealed to ss circular $M13_{Gori}$ DNA in an equimolar ratio in a quantity sufficient for all assays and kept frozen until it was needed. The assay buffer for the *B. subtilis* screen was optimized for maximal enzyme stability and activity and contained 40 mM HEPES (pH 7.5), 0.5% polyethylene glycol, 0.02% Pluronic F-68, 20 μM TCEP, 200 mM potassium glutamate, 3 μM zinc sulfate, 12.5 mM manganese chloride, and 12.5 mM magnesium acetate. The *B. subtilis* HTS assay used 0.5 μL of compound (500 μM in DMSO, final concentration of 10 μM) added to 19.5 μL of enzyme mix. All subsequent steps were identical to those of the *E. coli* HTS assay described above. We also included the known inhibitor of *B. subtilis* PolC, HB-EMAU [provided as a kind gift from N. Brown and G. Wright, University of Massachusetts Medical Center, Worcester, MA (*5*)] as a positive control inhibitor in all *B. subtilis* replicase screening and IC_{50} assays (compound 13, Table S1 of the Supporting Information).

Primary Screening of Bacterial Replicases. Screening compounds from TimTec and Chembridge were tested in each screening system in duplicate (Figure 4). Each 384-well screening plate contained DMSO (volume equivalent to the volume of compound added in each screen as a negative control) and EDTA (final concentration of 20 mM as a positive control) in the two outer columns of every assay plate. Data from these controls were used to normalize compound activity calculations and determine assay signal-to-background ratios and Z and Z' factors (*28*). Compounds exhibiting ≤50% of the normalized DMSO control activity in both screens were scored as primary hits and were reordered from suppliers or sampled from internal stores for retesting to confirm inhibitory activity and potency.

IC_{50} Measurements. IC_{50} values were determined using protocols identical to the HTS and enzyme specificity assays described above and in the Supporting Information, except that assays were conducted in 96-well plates using ten 2-fold serial dilutions of test compounds dissolved in DMSO at starting concentrations of 5 mM. This resulted in dilution series ranging from 300 to 0.29 μM for all assays. IC_{50} values were calculated by nonlinear least-squares curve fitting of the fluorescence data to the equation $Y = Y_{min} + (Y_{max} - Y_{min})/[1 + (IC_{50}/x)^h]$, where Y is the normalized percent activity of the reaction, x is the inhibitor concentration, and h is the Hill coefficient.

Secondary Screening in Specificity Assays. Test compound-intercalator dye displacement (IDD) assays (*29*) were performed by modifications of published procedures (*30*). Double-stranded calf thymus DNA (5 ng) was mixed with 0.1 μL of PicoGreen reagent in 23.5 μL of the *E. coli* HTS assay buffer and allowed to bind for 10 min; 2-fold dilutions of test compounds (1.5 μL) were added in a final volume of 25 μL. Fluorescence intensity and IC_{50} values were determined as described above. *E. coli* β-galactosidase assays were performed by published

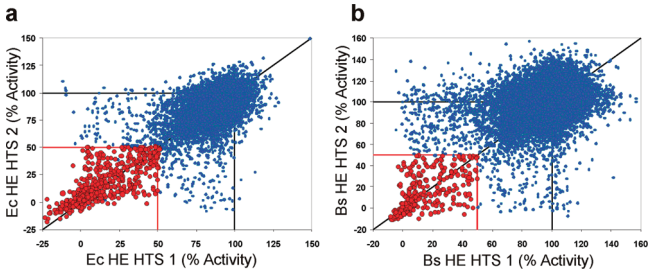


FIGURE 4: Duplicate screening plots of the bacterial DNA Pol III holoenzyme HTS. Compound activity (expressed as a percentage of the normalized DMSO control activity) for two screening runs is plotted. The black diagonal line represents the theoretical position of equivalent activity for the 20000 compounds in each duplicate screen. The red lines outline the hit definition zone in which compounds scoring ≤50% of the activity of the uninhibited DMSO controls are considered primary hits (red points). (A) *E. coli* DNA Pol III HTS. Average assay signal-to-background ratios were 9.4 ± 2.2 and 9.8 ± 1.8 for screens 1 and 2, respectively. Average Z' factors were 0.73 ± 0.14 and 0.76 ± 0.16, respectively. (B) *B. subtilis* DNA Pol III HTS. Average assay signal-to-background ratios were 11.7 ± 1.0 and 10.3 ± 0.7 for screens 1 and 2, respectively. Average Z' factors were 0.71 ± 0.10 and 0.74 ± 0.10, respectively.

procedures (*31*) using fluorescein digalactoside as a fluorogenic substrate (fluorescence intensity was read using an excitation wavelength of 485 nm and an emission wavelength of 535 nm). Apyrase ATPase activity was assayed using 0.5 mM ATP at 22 °C for 10 min using a colorimetric assay employing ammonium molybdate and malachite green dye to detect inorganic phosphate as described in ref *32*.

E. coli RNA polymerase core transcription was assayed essentially as described in ref *33*. T4 DNA polymerase was assayed in a gap-filling DNA polymerase assay essentially as described in ref *34*. Both of these assays used DNaseI-activated calf thymus DNA as templates and measured the incorporation of either [^{3}H]UTP (*E. coli* RNA polymerase core) or [^{3}H]TTP (T4 DNA polymerase) into their respective RNA or DNA products. RNA and DNA synthesis was assessed via scintillation counting after the addition of poly-L-lysine-coated polyvinyl acetate scintillation proximity assay beads (Invitrogen) in 0.3 M sodium citrate (pH 3.0) (*35*).

Saccharomyces cerevisiae Pol δ holoenzyme (*36*) and human mitochondrial DNA polymerase (*37*) were assayed as described previously. These assays and the T7 DNA polymerase assay used the oligonucleotide-primed $M13_{Gori}$ template described for the *B. subtilis* replicase assay and PicoGreen as described in Materials and Methods. The yeast Pol δ holoenzyme assay contained 25 ng of Pol δ, 2 ng of PCNA, 1 ng of RFC, and 0.6 μg of RPA in a total volume of 25 μL. Assays were performed at 22 °C for 40 min. The human mitochondrial polymerase assay contained 4 ng of Pol γ α subunit, 10 ng of Pol γ β subunit, and 1 μg of human mitochondrial SSB in a total volume of 25 μL.

Antimicrobial Susceptibility Testing. The reference strains for antimicrobial susceptibility testing, *E. coli* ATCC 25922 and *B. subtilis* ATCC 6633, were used for all experiments and were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Antimicrobial susceptibility testing was done using Mueller-Hinton broth according to the Clinical Laboratory Standards Institute (*38*). Microtiter plates containing serial dilutions of each test compound (beginning at a final concentration of 512 μg/mL) were inoculated with each organism (10^5 colony forming units/mL) in a final volume of 100 μL. Assay plates were incubated for 20 h at 35 °C in ambient air.

Minimum inhibitory concentrations (MICs) were scored as the lowest compound concentrations at which no bacterial growth was visible. The antibiotics tetracycline, rifampicin, and novobiocin were used as reference standards (*39*).

Macromolecular Synthesis Assays. Macromolecular synthesis (MMS) assays were adapted from ref *40*. *E. coli* strain ATCC 25922 and *B. subtilis* strain ATCC 6633 were grown overnight in L Broth at 37 °C before inoculation into M9 minimal medium (10 mL) and grown to an absorbance (600 nm) of 0.15. Radiolabeled precursors of three MMS pathways (DNA synthesis, 10 μCi/mL of [*methyl*-^{3}H]thydmidine; RNA synthesis, 3 μCi/mL of [5-^{3}H]uridine; protein synthesis, 1 μCi/mL of L-[2,5-^{3}H]histidine) were then added and incubated at 37 °C to allow incorporation of radiolabeled precursors in the presence of test compounds or control inhibitors. Compounds were added to 25 μL of cells at concentrations 4, 2, 1, 0.5, and 0.25 times the MIC in a final volume of 50 μL. Novobiocin (and HB-EMAU for *B. subtilis*), rifampicin, and tetracycline were used as controls for inhibition of DNA, RNA, and protein synthesis, respectively. For *B. subtilis* MMS assays, radiolabeled cells were incubated with compounds for 40 min before lysis. For *E. coli* MMS assays, cells were incubated for 30 min for DNA and RNA synthesis MMS assays and for 10 min for the protein synthesis MMS assay before lysis. Labeled cells were lysed using the detergent lysis reagent, Y-PER (Pierce Chemical), which lyses both Gram-negative and Gram-positive bacteria. After 100 μL of Y-PER reagent had been added and the cells had been mixed by repeated pipetting, they were lysed by incubation at 37 °C for 20 min with shaking. Macromolecules were precipitated by the addition of TCA to a final concentration of 10% (v/v) and incubation on ice for 1 h prior to filtration on 96-well Whatman GF/C filter plates. Filter plates were washed eight times with 10% cold TCA (200 μL/well) and once with 100% ethanol. Incorporation of radioactivity into DNA, RNA, and protein was detected by scintillation counting. Data were expressed as the percentage of macromolecule precursor incorporated, normalized to DMSO controls.

RESULTS

DNA replication is essential for the propagation of living cells. In spite of the widespread use of drugs that target processes upstream and downstream of DNA replication, no clinically useful compounds have been developed that target the central DNA replication apparatus. We describe an approach in which purified, reconstituted bacterial replicases are assayed by a screen that simultaneously detects inhibitors of the numerous component targets. Parallel screens of replication systems from Gram-negative and Gram-positive organisms and comprehensive counterscreening, including eukaryotic replicases, allow the biochemical assessment of the spectrum of potential antibacterial agents as well as potential toxins. The strategies outlined here should prove general and useful in initiating reverse chemical genetic approaches to any complex biological process.

Development of HTS Assays for Detect Inhibitors of Bacterial DNA Replicases. The origin of the ss circular DNA of bacteriophage G4 cloned into M13 provides a convenient template for the assay of the *E. coli* Pol III holoenzyme, SSB, and DnaG primase (*41*, *42*). The G4 origin possesses an ss DNA element that allows primase to bypass normal DnaB helicase-dependent primase recruitment during chromosomal replication, allowing direct primer synthesis (*43*).

We adapted a fluorescent PicoGreen assay (*44*) to monitor conversion of ss templates to a duplex and optimized the assay in a format suitable for HTS. PicoGreen binds preferentially to ds DNA and becomes fluorescent (Figure 1), providing a homogeneous and sensitive indicator of compounds interfering with the synthesis of ds DNA. Given the complexity of this multicomponent system, the *E. coli* replicase HTS assay was extensively optimized and was dependent upon all of the purified protein components (Figure 3a,b). Each protein component was assayed at limiting concentrations that would respond linearly to binding of an inhibitor. Nucleotide substrate concentrations were also near-limiting (data not shown), permitting the detection of inhibitors of dNTP binding to the polymerase active site, rNTP binding to the primase active site, or dATP or ATP binding to the ATPase site within the DnaX complex.

We next extended our capability to screen for inhibitors of a Gram-positive replicase. This would permit identification of inhibitors of both Gram-negative and Gram-positive DNA replication and, thus, would have the potential of being developed into a detection system for broad spectrum antibacterials or chemical biological tools. A minimal five-subunit replicase has been demonstrated from *S. pyogenes* (*27*). For the development of basic chemical biological approaches, we chose to use a replicase from a related model organism, *B. subtilis*, so that we could exploit biochemical and genetic tools and knowledge available for this organism in downstream studies. We expressed the *B. subtilis* PolC, the β_2 processivity factor, the three subunits of the DnaX complex (τ, δ, and δ'), and SSB and purified them in sufficient quantity for HTS (Figure 2).

The *B. subtilis* replicase HTS assay was formulated using a strategy similar to that used for *E. coli*. The identical DNA template was used in both assays. Since the *B. subtilis* DnaG primase cannot synthesize primers directly on the G4 origin (unlike the *E. coli* primase) without the participation of additional proteins (*45*, *46*), we annealed an oligonucleotide primer complementary to the $M13_{Gori}$ ss DNA template. Titrations of all protein components and HTS assay optimization studies were performed as described for the *E. coli* replicase (Figure 3c,d).

Reaction kinetics were measured to determine the longest time for which a linear response was observed, ~20 min for both *E. coli* and *B. subtilis* (Figure 3e). As our screening compounds are dissolved in DMSO, we tested the DMSO tolerance of the assay to determine compound screening concentration limits (Figure 3f). More than 80% activity was retained at 8 and 15% DMSO for the Gram-negative and Gram-positive screens, respectively. Since screening would be conducted over the course of a day, we tested the stability of the enzyme in assay buffer and determined that approximately 20% the activity was lost after 4 h at room temperature (Figure 3g). Consequently, the *E. coli* protein component mixes used during screening were kept in a chilled reservoir (at 4 °C) for no longer than 6 h to ensure maximum enzyme activity.

HTS of both Gram-Negative and Gram-Positive Replicases against a Compound Library. Having established robust, reliable replicase-targeted biochemical HTS systems, we tested a trial library comprised of 20000 commercially obtained compounds in duplicate screens against both the Gram-negative and Gram-positive replicases (Figure 4). Compounds that limited enzyme activities to $\leq$50% of uninhibited DMSO controls in both duplicates (red circles in Figure 4) were considered primary hits and selected for follow-up studies. Statistics from the screens yielded Z' factor averages of >0.7, which was extremely

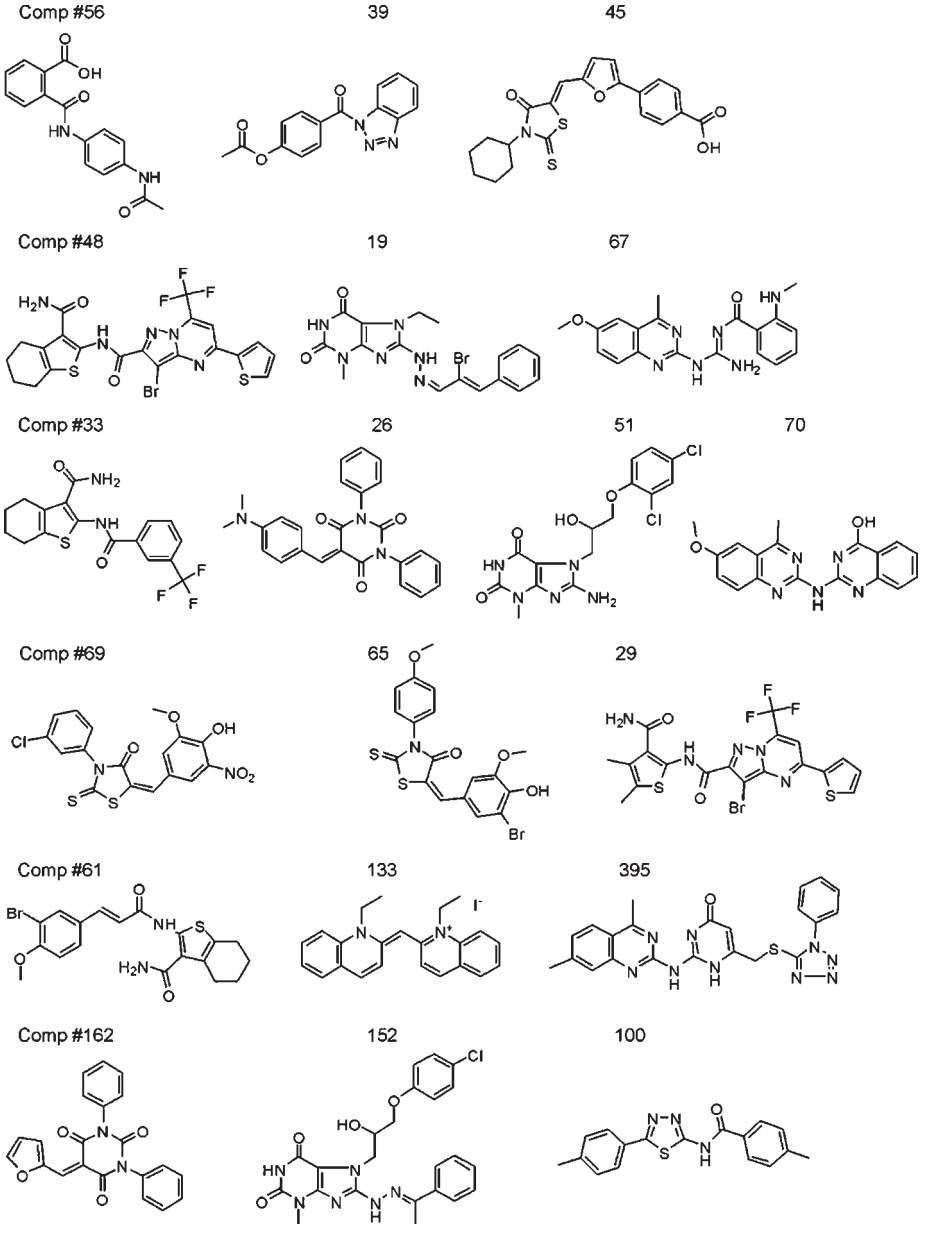


FIGURE 5: Representative inhibitors identified through HTS against bacterial replicases. Structures of selected inhibitors of the *B. subtilis* and *E. coli* replicases are shown. Activity profiles of each compound in all replicase screens and specificity assays are listed in Table 1 and in Table S1 of the Supporting Information.

encouraging given the complexity of both systems. Typically, Z factors between 0.5 and 1 indicate a very robust assay (*28*). A total of 831 and 290 compounds were identified as primary hits in the Gram-negative and Gram-positive screens, respectively (Figure 4).

Initial hit confirmation was done using a combination of compound titrations against the primary screen and a DNA intercalator dye displacement (IDD) assay (*29*). Preliminary studies indicated that the most common sources of artifactual hits were compounds that affected the PicoGreen assay readout either by binding to the DNA template or product, subsequently blocking polymerase activity, by blocking PicoGreen dye binding to ds DNA, or, more trivially, by interfering with the fluorescence of the PicoGreen−ds DNA complex due to absorption. The IDD assay measures the ability of a test compound to interfere with binding of the ds DNA-selective dye PicoGreen to ds calf thymus DNA. For example, compounds such as 133 (Figure 5 and Table 1) yielded comparable IC_{50} values in both bacterial replicase assays and the IDD assay; 251 and 143 of the primary hits resulting from the *E. coli* and *B. subtilis* screens, respectively, titrated with IC_{50} values of ≤ 100 μM in the primary screen and were inactive (with IC_{50} values > 5-fold greater than the replicase IC_{50}) in the IDD assay, strongly suggesting that these were specific inhibitors. These compounds were chosen for further analysis against a panel of specificity assays (Table S1 of the Supporting Information).

Development of Assays To Eliminate Nonspecific Inhibitors. Since our screening systems comprised a large range of potential enzymatic, protein−protein, and protein−nucleic acid targets against which hits could act, we counterscreened active compounds against a diverse panel of specificity assays to distinguish specific inhibitors of the bacterial Pol III holoenzymes from nonspecific inhibitors of unrelated DNA polymerases (bacteriophage T7 and T4 DNA polymerases, *S. cerevisiae* DNA Pol δ holoenzyme, and human mitochondrial DNA polymerase), an unrelated RNA polymerase (*E. coli* RNA

Table 1: Representative Inhibitors Identified through HTS against Bacterial Replicases

	DNA replication IC_{50} (μM)[b]		specificity assay IC_{50} (μM)								
compound[a]	Eco	Bsu	DNA binding	T7	T4	Pol δ	Pol γ	Apy	RNAP	β-Gal	specificity[c]
56	5.6	86.0	> 300	110	300	> 300	> 300	> 300	> 300	> 300	*E. coli*
39	2.7	62.9	> 300	> 300	> 300	> 300	> 300	> 300	> 300	> 300	*E. coli*
45	13.4	192	300	> 300	> 300	> 300	> 300	> 300	> 300	> 300	*E. coli*
48	60.6	> 300	> 300	> 300	> 300	> 300	> 300	> 300	> 300	> 300	*E. coli*
19	> 300	8.7	> 300	> 300	> 300	300	> 300	> 300	> 300	> 300	*B. subtilis*
67	> 300	1.3	> 300	28.0	> 300	> 300	> 300	> 300	> 300	> 300	*B. subtilis*
33	260	35.0	> 300	> 300	> 300	180	> 300	> 300	> 300	> 300	*B. subtilis*
26	> 300	20.5	> 300	> 300	> 300	> 300	> 300	> 300	> 300	> 300	*B. subtilis*
51	28.6	22.3	> 300	250	> 300	300	> 300	250	> 300	> 300	broad
70	1.3	3.2	> 300	10.2	> 300	> 300	> 300	> 300	> 300	> 300	broad
69	0.9	0.9	55.4	11.3	14.6	4.5	> 300	8.1	129	> 300	broad
65	6.0	3.0	33.0	31.8	57.2	36.6	> 300	300	> 300	> 300	broad
29	25.2	25.2	175	> 300	> 300	147	> 300	> 300	> 300	> 300	broad
61	46.2	9.4	> 300	68.1	127	> 300	> 300	> 300	> 300	> 300	broad
133	8.2	22.3	19.5	14.2	99.4	12.0	> 300	198	94.5	15.8	nonspecific
395	36.9	2.8	> 600	12.6	> 600	> 600	> 600	> 600	> 600	> 600	T7 Pol/*B. subtilis*
162	20.4	34.0	> 300	200	2.8	> 300	> 300	> 300	> 300	> 300	T4
152	12.4	10.5	> 300	> 300	> 300	25.0	> 300	> 300	> 300	> 300	Pol δ/broad
100	22.7	18.8	> 300	> 300	> 300	> 300	> 300	0.8	> 300	> 300	apyrase

[a]Compound refers to compounds shown in Figure 5 and Table S1 of the Supporting Information. [b]IC_{50} values for both bacterial replicase assays (Eco, *E. coli* replicase; Bsu, *B. subtilis* replicase) and all specificity assays (DNA binding, IDD assay; T7, T7 DNA polymerase; T4, T4 DNA polymerase; Pol δ, *S. cerevisiae* DNA Pol δ; Pol γ, human mitochondrial DNA polymerase; Apy, apyrase ATPase; RNAP, *E. coli* RNA polymerase core; β-Gal, β-galactosidase) are shown. Bacterial replicase assays were conducted as described in Materials and Methods. IC_{50} values containing a greater than sign indicate that the compound did not inhibit the assay lower than the 50% activity level at the highest compound concentration tested. [c]A threshold of 5-fold was used to score the specificity of a given compound for a particular assay or bacterial replicase target. Specificity assays were conducted as described in Materials and Methods.

polymerase core), an unrelated ATPase (apyrase), and a mechanistically unrelated enzyme (*E. coli* β-galactosidase).

The IC_{50} values of confirmed inhibitors were determined and compared with IC_{50} values determined in specificity assays. A threshold of 5-fold was used to score the specificity of a given compound for a particular assay or bacterial replicase target. A compound such as 133 (Figure 5 and Table 1) was scored as nonspecific with respect to most specificity assays, since the IC_{50} values all fell within 5-fold of one of the bacterial holoenzyme target IC_{50} values. Compound 26 (Figure 5 and Table 1) was considered specific for the *B. subtilis* replicase, because the IC_{50} (20.5 μM) was more than 5-fold more potent compared to the *E. coli* replicase assay IC_{50} (> 300 μM), or that of any specificity assay. A complete listing of the annotated activities of all 394 compounds tested is provided in Table S1 of the Supporting Information. Figure 5 and Table 1 illustrate the structures and activity profiles of selected compounds that will be presented as examples of the classes of inhibitors we have identified.

The panel of specificity assays was selected not only to identify problematic compounds such as protein reactive and aggregation-based inhibitors (*47*) but also to detect mechanistically nonspecific compounds (i.e., general polymerase, ATPase, or RNA polymerase inhibitors) and, as such, proved to be critical to a thorough evaluation of the replicase inhibitors identified here. Of all compounds tested, relatively few were found to be active against β-galactosidase, *E. coli* RNA polymerase, or human mitochondrial DNA polymerase (16, 19, and 18 compounds, respectively). Most of these compounds were generally nonspecific, inhibited many other targets, and were eliminated from further consideration.

DNA Pol IIIs, involved in chromosomal replication, are a special subclass of type X polymerases and have a unique fold in their active site that distinguishes them from type B polymerases, which replicate eukaryotic chromosomes, or type A polymerases, which are responsible for mitochondrial replication (*48*−*51*). To eliminate compounds that generally inhibited DNA polymerases, we performed counterscreens against two type A polymerases (bacteriophage T7 and human mitochondrial DNA polymerase) and two type B polymerases (bacteriophage T4 and *S. cerevisiae* DNA Pol δ holoenzyme). The bacteriophage T7 DNA polymerase assay was by far the most efficient specificity filter, identifying a total of 96 compounds that inhibited T7 DNA polymerase (and an additional 50 that inhibited T7 and the three other polymerase specificity targets). Compounds such as 395 exhibited comparable levels of inhibition in the T7 assay and the bacterial replicase screens. T7 DNA polymerase is a highly processive polymerase (*52*) and may be particularly sensitive to compounds that interact with the ss DNA template, blocking processive replication by blocking progression of the polymerase. In contrast to T7 DNA polymerase, the human mitochondrial DNA polymerase (*53*) was inhibited by only 18 compounds, all of which were also active against T7. This enzyme was included because it is a known predictor of mitochondrial toxicity. DNA replication inhibitors (notably anti-reverse transcriptase inhibitors) have displayed toxicity directed at mitochondrial DNA polymerase (*53*, *54*) and have even led to the clinical failure of drugs such as the anti-hepatitis B agent Fialuridine (*55*). The majority of the compounds identified here have proven to be inactive against this specificity filter.

Only five compounds (e.g., compound 162) were found to significantly inhibit T4 DNA polymerase. The *S. cerevisiae* DNA Pol δ holoenzyme (*56*) specificity filter was included to permit detection of compounds that result from inhibition of a eukaryotic enzyme highly similar to the human replicase. Such compounds, of course, would have toxic potential from the perspective of antibacterial therapeutic agents but could also be among the most useful as chemical biological tools, as they may target central mechanisms common to diverse life forms.

We identified 15 compounds (e.g., compound 152) that fall into this class of agents that do not inhibit the simpler DNA polymerases or other specificity targets tested but do inhibit both a bacterial and the eukaryotic replicase.

Because a major enzymatic target in both replicase screens is the processivity clamp loader ATPase, a eukaryotic E-type ATPase, apyrase (*57*), was included to identify compounds that could target ATPases nonspecifically. A total of 14 compounds inhibited apyrase (e.g., compound 100) at IC_{50} values comparable to that of either bacterial replicase, as well as 48 others that inhibit both apyrase and T7 DNA polymerase. While the majority of these compounds do not appear to inhibit any other specificity target (notably the *S. cerevisiae* DNA pol δ, which contains an AAA^+ ATPase clamp loader, as in the bacterial replicases), it will be of interest to determine if selected compounds in this set do inhibit bacterial clamp loader ATPases in future work and if structural analogues of these compounds that confer greater specificity toward the bacterial replicases could be identified.

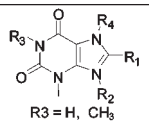
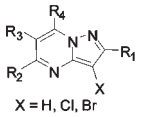
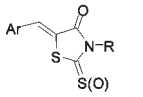
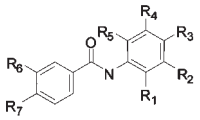
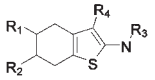
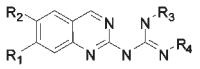
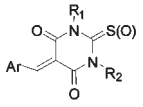
Identification of Specific Bacterial Replicase Inhibitors. Most encouragingly, a total of 74 compounds were determined to exhibit a significant degree of specificity toward one or both bacterial replicases. According to our specificity threshold, a 5-fold difference in IC_{50} values between replicases and specificity assays, 18 compounds were specific for the *E. coli* replicase (examples are compounds 56, 39, 45, and 48), 28 compounds were specific for the *B. subtilis* replicase (e.g., compounds 19, 67, 33, and 26), and 28 compounds were found to inhibit both replicases (e.g., compounds 51, 70, 69, 65, 29, and 61), revealing a potential to be developed into broad spectrum antibacterial agents. Our specificity threshold is based on relative numbers. We acknowledge that some compounds, such as 3 (Table S1 of the Supporting Information), were designated Gram-positive-specific but still yield a credible IC_{50} against the *E. coli* replicase of 25 μM.

We anticipate that these highly specific replicase inhibitors will act by a variety of mechanisms against numerous protein subtargets within the replicase assays. Although we have identified compounds that appear to be specific to Gram-negative replicases, this conclusion cannot be firmly established until the actual targets are identified. The primase activity in the Gram-positive replicase systems was not included, and templates were primed by annealing an exogenous primer. Thus, a broad spectrum primase inhibitor could appear to be Gram-negative-specific in our assays, as constituted.

Identification of Compound Structural Series. We examined all 394 compounds identified from the bacterial replicase screens using a chemical structure clustering algorithm (ChemTK, Sage Informatics LLC, Santa Fe, NM) to identify structurally similar inhibitors. We identified seven distinct chemical series (A−G, Table 2), all with representatives among the specific bacterial replicase inhibitors listed in Table 1 (and further described in Table S1 of the Supporting Information).

The largest series identified (series A, 46 compounds) contained a purine-2,6-dione core and included nine compounds predominantly displaying broad spectrum replicase-specific inhibition and also *B. subtilis* replicase specificity, as shown with compounds 51 and 19. Other members of this series showed nonspecific activities primarily against apyrase, T7 DNA polymerase, and *S. cerevisiae* DNA Pol δ, possibly reflecting the nucleoside-like core structure.

Table 2: Inhibitor Series Identified in Replicase Screens

Series	Core Structure [a]	Comp # [b]	Number of Compounds Identified [c]	Replicase-Specific Compounds [d]
A	 R3 = H, CH_3	19,51 152	46	9
B	 X = H, Cl, Br	29,48	7	3
C		45,65 69	28	6
D		56	13	1
E		33,61	6	3
F		67,70 395	20	8
G		26,162	10	1

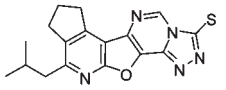
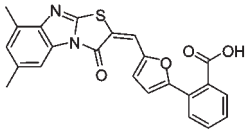
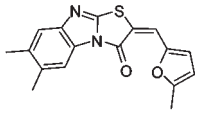
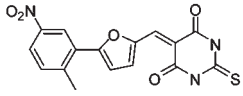
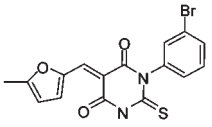
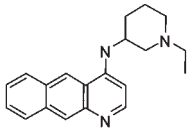
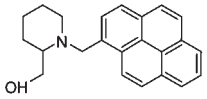
[a] Ar, aromatic substitution. [b] Comp # refers to all compounds shown in Figure 5 and Table 1. [c] Compounds that were hits in the HTS and titrated with an IC_{50} of ≤100 μM. [d] Compounds that titrated with an IC_{50} ≤5-fold greater than the most inhibited specificity assay.

The next-largest series (series C, 28 compounds) contained 5-arylmethylenethiazolidine-2,4-diones or -2-thioxo-4-ones. Many of these compounds showed broad spectrum activity (e.g., compounds 65 and 69), and a few were specific for the *E. coli* targets (e.g., compound 45). Other members of the series displayed nonspecific activities predominantly toward other DNA polymerases.

An additional series (series F, 20 compounds) contained variously substituted 2-quinazolinylguanidines. The eight bacterial replicase-specific members of this series (e.g., compounds 67 and 71) predominantly targeted the *B. subtilis* replicase. Other nonspecific members of the series predominantly inhibited T7 DNA polymerase (compound 395) and the other DNA polymerases in the specificity panel. This series has been previously identified as possessing antimicrobial activity (*58*) and, later, as a polymerase inhibitor (*59*).

The remaining series identified (series B, compounds 29 and 48; series D, compound 56; series E, compounds 33 and 61; series G, compounds 26 and 162) all contain compounds displaying broad spectrum and organism-specific replicase inhibition. The data obtained from these series of compounds provide information that can be used to derive preliminary structure−activity relationships (SAR) that will prove to be useful during the pursuit of more defined SARs and the design of library expansion strategies. Likewise, the 44 remaining bacterial replicase-specific compounds that do not fall into obvious inhibitor series can also provide starting points for testing structurally related

Table 3: Inhibitors of Cellular DNA Replication

Comp #	Structure	Antimicrobial Activity MIC (μg/mL)[a] *E. coli*	*B. subtilis*	MMS Assay Specificity[b]
72		>512	64	*B subtilis* DNA Replication
4		512	64	*B subtilis* DNA Replication
153		512	128	*B subtilis* DNA Replication
161		>512	128	*B subtilis* DNA Replication
285		>512	256	*B subtilis* DNA Replication
309		16	16	*E coli* DNA Replication
345		128	16	*E coli* DNA Replication

[a]MIC values of > 512 μg/mL indicate no growth inhibition at the highest compound concentration tested. [b]Data for MMS assay of these compounds are shown in Figure S1 of the Supporting Information.

compounds to establish whether a common structural series can be identified.

Identification of Compounds with Target-Specific Biological Activity. To determine which compounds, initially identified using our multiplicative target screen, exhibited antimicrobial activity and retained target specificity in a whole cell context, we took a two-pronged approach. First, we screened all compounds with promising biochemical profiles for antimicrobial activity. Minimal inhibitory concentrations (MICs) were determined. Then, all compounds that exhibited a MIC of ≤256 μg/mL were screened using macromolecular synthesis assays in which incorporation of precursors for proteins, RNA, and DNA was quantified. Among 196 compounds with biochemical replicase specificity (or activity against few other specificity targets) screened, 33 compounds exhibited MICs of ≤256 μg/mL. Among these, seven preferentially inhibited DNA replication and provide candidates for the development of more potent and specific probes that can be used to block bacterial DNA replication (Table 3). All of the compounds identified show biochemical activity against both Gram-positive and Gram-negative replicases (Table S1 of the Supporting Information). We were unable to explore the specificity for the *E. coli* replicase for five compounds because of high MICs (Table 3). Two compounds (309 and 345) that exhibited low MICs against both *E. coli* and *B. subtilis* exhibited replicase specificity against only *E. coli*, not *B subtilis*. In all cases, increasing the potency and specificity with attention to cell permeability issues promises to yield specific biological probes.

DISCUSSION

Typically, screens of compound libraries are performed on single targets. While this has led to the efficient identification of inhibitors of an enzymatic activity or a targeted interaction, it misses inhibitors of interactions absent from the relatively simple screening assay. To target more complex interactions, we generally performed whole cell screens. In a particularly elegant early example, Mitchison and colleagues, using a screen for mitotic arrest in tissue culture cells, identified monastrol, an inhibitor of the kinesin Eg5 that binds to a site remote from the ATP or microtubule binding sites (*60*).

An alternative approach to the whole cell screens would be to include all of the components of a multiprotein machine in a fully reconstituted biochemical system. Indeed, progress has been reported in the development of more complex biochemical assays; for example, a fatty acid biosynthesis pathway screen in which a precursor is converted to product through several intermediates and independent enzyme-catalyzed reactions has been described (*61*). Screens have also been performed against multisubunit enzymes, permitting identification of individual subunits and their interactions (*62*, *63*). In this report, we exploited the most complex biochemical system reassembled from purified components for screening purposes to date. We reconstituted and screened the entire apparatus for both Gram-positive and Gram-negative bacterial replicases. For the *E. coli* assay, this includes the 10 subunits of the Pol III holoenzyme with SSB and DnaG primase.

We estimate that this assay contains upward of 100 targets, including all of the essential subunits and their interactions and essential conformers. These include an α−ε interaction, required for shuttling of the primer terminus between these two essential subunits (*64*). Polymerization itself contains at least six kinetic steps and essential changing enzyme conformations (*65*). Additionally, the replicative elongation reaction includes additional steps involved in partitioning of the primer terminus between the polymerase and exonucleolytic proofreading active sites (*66*). Each stage of the replication pathway provides a target that can be trapped by a small molecule, arresting the overall reaction. Indeed, the Benkovic lab has demonstrated that gp59 arrests the bacteriophage T4 replicase by trapping a conformation that blocks both polymerase and exonuclease activity (*67*).

In addition, α interacts with the ss DNA template, the duplex immediately behind the template, the primer of the preceding Okazaki fragment, and the essential β and τ subunits (*16*, *19*, *68*, *69*). The subunit interactions change during the formation of the initiation complex and also in the dissociative reactions requisite for cycling during Okazaki fragment synthesis on the lagging strand of the replication fork (*18*, *69*). The DnaX complex contains essential intracomplex interactions: τ−τ, τ−γ, γ−δ′, δ′−δ, δ−τ, γ−ψ, and ψ−χ. This is just the static picture. Assembly of a processivity factor onto DNA requires at least six kinetic steps in which the intersubunit interactions change during a tightly regulated process that involves ATP binding and turnover, accompanied by ordered β and DNA binding and release (*70*). Blockage of any of these discrete kinetic steps by

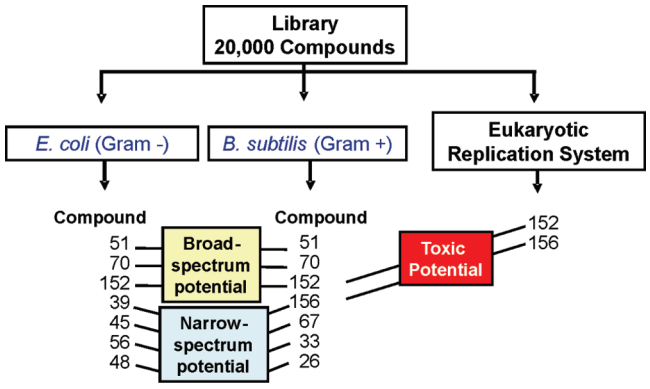


FIGURE 6: Use of parallel multiplicative target screening assays to identify specific bacterial DNA replication inhibitors with broad spectrum potential. The use of the *E. coli* replicase permits identification of compounds that have potential for general Gram-negative antibacterial activity. The same approach, utilizing the *B. subtilis* replicase, permits us to distinguish compounds with general Gram-positive activity. Compounds that inhibit both Gram-positive and Gram-negative replicases have the highest potential for being developed into broad spectrum agents. For therapeutic purposes, having a spectrum that is so broad that it includes the analogous human target is, of course, undesirable. Inclusion of a human target or a homologous eukaryotic target permits elimination of compounds inhibiting the eukaryotic target. Compound numbers correspond to those in Table 1 and Table S1 of the Supporting Information.

trapping the complex in a specific conformation should result in inhibition of the whole reaction.

SSB interacts with DNA and DnaG primase and regulates the transfer of nascent primers to the polymerase, a process that likely involves DnaG−χ−ψ−α or DnaG−α interactions (*71*). An additional SSB−χ interaction is important for the initiation of complex formation and stabilization of the polymerase on DNA in the presence of physiological salt levels (*72*, *73*). Blockage of any of these targets by a small molecule inhibitor would prevent conversion of the ss DNA template to the ds DNA product detected in our HTS assay. We refer to the exploitation of complex dynamic protein machines for screening purposes as multiplicative target screening to reflect the combinatorial nature of interacting components providing a quantity of targets that is a multiple of the number of individual components required.

We have extended multiplicative target screening to replication systems from two diverse organisms. Parallel analysis of a compound library in replication systems from diverse organisms provided a direct determination of which compounds have broad spectrum potential and which compounds are specific to one or more related organisms. Compounds that inhibit replicases from distinct Gram-negative and Gram-positive organisms would be expected to have broad spectrum potential. Compounds that inhibit replicases from only closely related organisms would be expected to have a narrower spectrum (Figure 6). This approach, which we refer to as parallel target screening, provides information useful for both reverse chemical genetic approaches and the development of therapeutics. For chemical genetics, knowing which small molecules are inhibitors of a specific enzyme in a variety of systems will improve their utility as a tool, both in serving to block a common process in multiple organisms and in serving as a probe to study conserved mechanistic features. For therapeutics, broad spectrum agents are most often pursued, but a need for targeted therapeutics exists (*74*), such as antimicrobial agents used to treat chronic diseases without interfering with normal flora.

Inclusion of counterscreens against targets that, if inhibited, could result in toxicity permitted early discrimination of compounds with an undesirable lack of specificity. In our studies, we used the *S. cerevisiae* Pol δ replicase as a surrogate for the closely related human replicase and the recombinant human mitochondrial replicase. Screening for compounds that interact with ds DNA and interfere with dye binding provided a preliminary indication that a compound might be acting by interacting with the substrate of the replicase rather than the protein itself. Inclusion of unrelated phage polymerases provided an additional means of detecting compounds that can block DNA synthesis by general or nonspecific mechanisms. The *E. coli* replication system contains a specialized RNA polymerase that makes primers for DNA replication. Inclusion of the unrelated *E. coli* RNA polymerase provided a means of eliminating general RNA polymerase inhibitors. In both the *E. coli* and *B. subtilis* systems, the β processivity factor is placed on DNA by the action of a AAA+ ATPase, the DnaX complex. We included an unrelated ATPase, apyrase, to eliminate simple ATP analogues or nonspecific ATPase inhibitors.

We have also demonstrated that our approach yields a subset of interesting compounds with biological activity, retaining the specificity indicated by the biochemical screen. The good yield (seven compounds with biological target specificity) from a small 20000-compound test library is an indication of the richness of the target explored by the biochemical multiplicative target screening approach. We expect that, via selection of a range of compounds showing high biochemical specificity, further optimization to increase potency will reveal additional useful biological probes as well as useful compounds for studying the structure and mechanism of replicases. However, a strength of the biochemical approach resides in the ability to identify compounds and their accompanying targets that would have been missed in whole cell screens because the compounds fail to achieve the high cellular concentrations required for the relatively weak inhibitors expected from first-stage screening efforts. Those additional compounds that demonstrate high biochemical specificity can be further optimized to increase both their potency and cellular permeability.

A key advantage of our multiplicative target screening strategy is that a very large number of targets can be screened simultaneously. These targets include subtle conformational changes and interactions that may not yet be understood. However, this powerful approach leaves the challenge of identifying the exact target, in terms of both the binding sites and the specific interactions and mechanistic/kinetic steps affected. We are developing an efficient deconvolution process whereby the target can be identified without all possibilities being tested. We anticipate this will involve broad classification of the general reaction stage (priming, initiation complex formation, and elongation) and iteratively narrowing our search until the specific target is identified. For example, inhibitors shown to block formation of the initiation complex specifically will be screened to determine whether ATP, DNA, or β_2 binding to the DnaX complex is blocked or if the ATPase activity of DnaX is repressed. Once these details are understood, the specific kinetic step affected and specific binding site can be pursued, exploiting the extant wealth of functional and structural information. This will not only permit direct identification of known interactions and kinetic steps but also reveal unknown features of our complex system, via the discovery of compounds that interfere with weak, essential interactions or block fast non-rate-limiting

kinetic steps that have eluded detection by other techniques. As our studies progress, we hope to gain a better understanding of the structure of target−inhibitor complexes and exploit this information to gain mechanistic insight and to make rational progress with promising inhibitors toward useful therapeutics and valuable biological and mechanistic probes.

NOTE ADDED AFTER ASAP PUBLICATION

After this paper was published ASAP February 25, 2010, the Supporting Information document was updated. The revised version was reposted March 1, 2010.

SUPPORTING INFORMATION AVAILABLE

A figure showing the results of the MMS assays reported in Table 3 and the complete screening, specificity, and microbiological data for the 394 compounds identified in the *E. coli* and *B. subtilis* HTS. This material is available free of charge via the Internet at http://pubs.acs.org.

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